STUDY OF THE ROLE OF THE ENDOGENOUS KISSPEPTIN TONE IN THE REGULATION OF HYPOTHALAMIC PITUITARY GONADAL AXIS

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TANZEEL HUMA

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A THESIS SUBMITTED FOR THE PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Dedication

I dedicate this thesis to my father "**Muhammad Anwar**". He was the one who always helped me throughout my studies and my research work. He always sacrificed his personal work and helped in my research. In arranging things, animals and everything I needed.

Declaration

I, Tanzeel Huma, hereby declare that all the content provided here is my original work. I have not presented any part of this work previously for any other degree.

Tanzeel Huma

In the Name of ALLAH,
The Most GRACIOUS,
the Most MERCIFUL

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May Allah bless all those who pray for me (Aameen)

TANZEEL HUMA

List of Abbreviations

ARC	Arcuate area
AVPV	Anteroventral periventricular nucleus
CNS	Central nervous system
DAG	Diacyl glycerol
GFAP	Glial fibrillary acidic protein
DMN	Dorsomedial nucleus
DTI-MR	Diffusion tensor imaging-magnetic resonance
ER	Estrogen receptor
FSH	Follicle stimulating hormone
GnIH	Gonadotropin inhibitory hormone
GnRH	Gonadotropin releasing hormone
GPR54	G-protein coupled receptor 54
HPG-Axis	Hypothalamic-pituitary gonadal axis
iHH	Idiopathic hypogonadotropic hypogonadism
IP_3	Inositol triphosphate
ir	Immunoreactivity
Kp	Kisspeptin
LD	Long days
LH	Luteinizing hormone

MAPK s	Mitogen activated protein kinases
MBH	Mediobasal hypothalamus
MBP	Myelin basic protein
MTT	Microculture tetrazolium assay
ME	Median eminence
NKB	Neurokinin B
NPY	Neuropeptide Y
OPCs	Oligodendrocyte progenitor cells
PIP_2	Phosphotidyl inositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
POA	Preoptic area
RFa	RF amide
RNC	Rossette neurnal cells
RFRPs	RF amide related peptides
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCN	Suprachiasmatic nucleus
SD	Short days
VMN	Ventromedial nucleus

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General Abstract

Reproductive activity in mammals is progressed by the neuroendocrine coordination between the hypothalamus pituitary and gonads constituting hypothalamic-pituitary-gonadal axis (HPG-axis). The discovery of kisspeptin and its receptor and fundamental role of kisspeptin in regulating reproduction diverted the scientific attention toward investigating its role in reproduction. Present series of studies was designed to investigate the role of endogenous kisspeptin in the regulation of HPG-axis by applying a variety of paradigms using adult male rhesus monkey as a model: 1. for checking the role of endogenous kisspeptin tone in the regulation of HPG- axis, we peripherally antagonized kisspeptin by using peptide 234 (kisspeptin receptor antagonist) 2. to assess if seasonal modulation of the HPG-axis is mediated by the kisspeptin signaling. We used assay, immunocytochemical and RT-qPCR approaches to report the effect of seasons on gene and protein correlation of kisspeptin signaling in the mediobasal hypothalamus (MBH) of adult male rhesus monkeys and finally 3. to determine whether kisspeptin can bring any change in the pluripotent stem cells, we determined the effect of kisspeptin-10 on rhesus monkey derived Lyon -ES cells differentiation and the proliferation.

In the first experiment five adult male monkeys were administered with either peptide-234 (P234) (38.8 µg/kg body weight) or vehicle (0.9% saline). Plasma testosterone and adiponectin levels, as a positive control, were measured using specific ELISA in blood samples collected sequentially at 15 min intervals during a 60 min pre and a 360 min post P234/vehicle injection period. In three monkeys, the experiment was repeated where the animals received a human kisspeptin-10 (50 µg) challenge 30 min after the P234 or vehicle treatment. Our results indicated that systemic administration of P234 did not alter the plasma testosterone levels, but slightly decreased ($p<0.01$) the plasma adiponectin concentration. Further, kisspeptin dependent testosterone and adiponectin release was significantly ($p<0.05$) decreased by P234 treatment.

For the second experiment, we hypothesized that modulation of expression of Kiss1, Kiss1r and GnRH underlies seasonal changes in reproduction. We examined

the expression of Kiss1, Kiss1r, GnRH, pDYN, KOR, NKB and NK3R mRNA levels using RT-qPCR and of Kiss1, Kiss1r and GnRH immunoreactivity by using immunocytochemisty in the MBH of adult male rhesus monkeys during breeding season (BS; January; n=3) and non-breeding season (NBS; July; n=3). The animals were maintained under free ranging conditions in the primate breeding colonies of Kunming Institute of Zoology (102.71º longitude, 25.03º latitude). Additionally, we measured CSF (four samples collected at 30 min interval during a period from 9:00-10:30, from the lumbar vertebrae; n=3) kisspeptin and peripheral testosterone levels (blood samples were collected at 20 minutes interval during a period from 1000-1600 hrs; n=4) in BS (November) and NBS (August) monkeys by using specific RIA's. We observed an increase ($p<0.01$; $p<0.0005$; $p<0.0001$, respectively) in the relative mRNA expression of Kiss1, Kiss1r and GnRH in the MBH of the adult male rhesus monkeys during the BS. The number of Kiss1 positive cells in the arcuate area (ARC) was also increased ($p<0.0001$) in BS. The Kiss1r positive and Kiss1r positive GnRH cell bodies were also increased ($p<0.01$) during the BS along with an increase in the number of contacts between Kiss1 and GnRH cell bodies ($p<0.01$). Kisspeptin levels in the CSF and the peripheral testosterone levels were also increased ($p<0.0001$) during the BS.

In the third experiment, rhesus monkey derived GFP Lyon-ES cells were treated with the human kisspeptin-10. We tested how different doses of kisspeptin-10 treatment would influence specific neuronal differentiation among Lyon ES cells. At the early rosette stage, we incubated rhesus macaque derived tau GFP-Lyon ES cells with 0.1, 1, 10 and 100 nM of kisspeptin-10 or ddH_2O as a control, for three days. Different parameters were then assessed at 24, 48 and 72 hr time points. We used tunnel assay for checking apoptotic activity, flow cytometry and cell count to check the effect on proliferation and later immunocytochemistry was done for glial fibrillary acidic protein (GFAP), nesting, β-tubulin III and GnRH expression. The result revealed that kisspeptin treatment affected the cell proliferation in a dose ($p<0.001$) and time ($p<0.0001$) dependent manner. The interaction between dose and time was also significant ($p<0.01$). Lyon-ES cells were also differentiated in to GnRH neuronal type cells in response to kisspeptin exposure. This observation suggests that kisspeptin is an important neurological and stimulatory factor for proliferation and

differentiation of the Lyon ES cells and specially in inducing GnRH expression. This observation indicates the role of kisspeptin in inducing the GnRH expression in the Lyon ES cells.

In summary, we observed that kisspeptin dependent testosterone release was decreased by systemic infusion of P234 which suggests that kisspeptinergic signaling plays key role in regulating the HPG-axis. During breeding season the kisspeptinergic signaling was increased, resulting in the up regulation of the HPG axis. A neurotropic role of kisspeptin was evident in Lyon ES experiment in which kisspeptin treatment induced the GnRH like neuronal expression and GnRH gene expression. This indicates a potential therapeutic use of kisspeptin in future to treat various reproductive disorders i.e., infertility, IHH.

Overall from this thesis, we can conclude that kisspeptinergic signaling is playing a key role in regulating the HPG-axis and is being modulated by the season. Additionally, kisspeptin has potential to cause GnRH expression in the pluripotent cells, which is a novel finding and can be used further in the future to cure reproductive disorders i.e., infertility, IHH.

Chapter 1: General Introduction

General Introduction

Reproductive activity in mammals is progressed by the endocrine linkage between the hypothalamus, pituitary and gonads. Gonadotropin releasing hormone (GnRH) plays an important role in the general pathway for the central regulation of reproduction and fertility. GnRH is secreted in a pulsatile pattern from the neurons resident in the arcuate area (ARC) nuclei of the hypothalamic origin (Clarke and Cummins, 1982; Levine et al., 1982).This pulsatile release of GnRH plays an essential role in the release of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary (Belchetz et al., 1978; Levine et al., 1982; Kaiser et al., 1997), which in turn leads to the steroid hormones production and ultimately gametogenesis by the gonads. This series of events represents strong interaction between brain, pituitary and gonads, and constitute the hypothalamic pituitary gonadal axis (HPG) which ensures a successful reproduction. The GnRH pulse pattern is regulated by the gonadal steroids feedback (Karsch et al., 1993). This feedback is largely mediated via steroid-sensitive afferents (Wintermantel et al., 2006; Mayer et al., 2010). Recent discovery of the hypothalamic kisspeptinergic system has totally changed prevailing view about the regulation of GnRH system. Kisspeptin neurons act one step upstream to the GnRH neurons (Smith et al., 2005; Oakley et al., 2009: Hameed et al., 2011).

The nomenclature for kisspeptin and its receptor was officially classified by the Committee on receptor nomenclature and drug classification named as “International Union of Basic and Clinical Pharmacology”. The human kisspeptin gene was designated as KISS1 while non human gene was designated as Kiss1. The human kisspeptin receptor was designated as KISS1R while non human as Kiss1r (Gottsch et al., 2009). This nomenclature would be followed throughout this dissertation.

The current thesis focuses on the investigation of the role of kisspeptin signaling in regulating the HPG axis of higher primates taking male rhesus monkey as a model. Accordingly, this general introduction starts with a detailed description of the kisspeptin system history.

1.1. Discovery and Basic Biology of Kisspeptin and Kisspeptin Receptor

In 1996, kisspeptin gene (kiss1) was discovered as a metastasis-suppressor gene in melanoma tumor cells (Lee et al., 1996). Kiss1 gene is located at chromosome 1q32 (West et al., 1998), however, some elements at chromosome 6q16.3-q23 (Lee et. al., 1996; Miele et al., 1996; Goldberg et al., 2003; Mitchell et al., 2007) cause upstream regulation of Kiss1 expression (Murphy et al., 2005). Kisspeptin belongs to RF-amides super-family of peptides, which are stamped by Arg-Phe-NH2 (RF-NH2) terminal signature (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Ukena and Tsutsui, 2001; Dockery, 2004). Kisspeptin (154 aa precursor peptide) is cleaved to a 54 amino acid (aa) peptide called kisspeptin-54 or metastin (Ohtaki et al., 2001, Takino et al., 2003). The precursor kisspeptin is highly unstable sequence and is considered to be proteolytically cleaved by furin or prohormones convertases (Kotani et al., 2001). The shorter products formed after cleavage are termed kisspeptin-10, -13, -14, -16 according the number of aa contained. Metastin and other shorter fragments all are collectively called kisspeptins. Kisspeptins express similar affinity for the kisspeptin receptor, which indicates that the activation and the binding of the receptor is regulated through the C-terminal end of the peptide (West et al., 1998; Kotani et al., 2001; Ohtaki et al., 2001, Caraty et al., 2012).

In 2001, four independently working groups recognized that kisspeptin acted as a high-affinity peptide ligand for the orphan G protein-coupled receptor GPR54 (referred to as AXOR12) (Clements et al., 2001; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). First described in the rat in 1999, GPR54 has now been termed as“Kiss1r” because of its role as a Kiss1 receptor (reviewed in Oakley et al., 2009). Kiss1r gene is located on the short arm of chromosome 19 (19p13.3). Kiss1r is a 398 amino acid GPR with expected seven trans-membrane domains. In addition to the amino- and the carboxyl-terminus, all GPRs have three extracellular and three intracellular (or cytosolic) loops (Figure 1.1). This receptor has 40-45% homology with galanin receptors. Kiss1r has a short extracellular domain which has potential N-glycosylation sites and an intracellular domain having phosphorylation activity (Lee

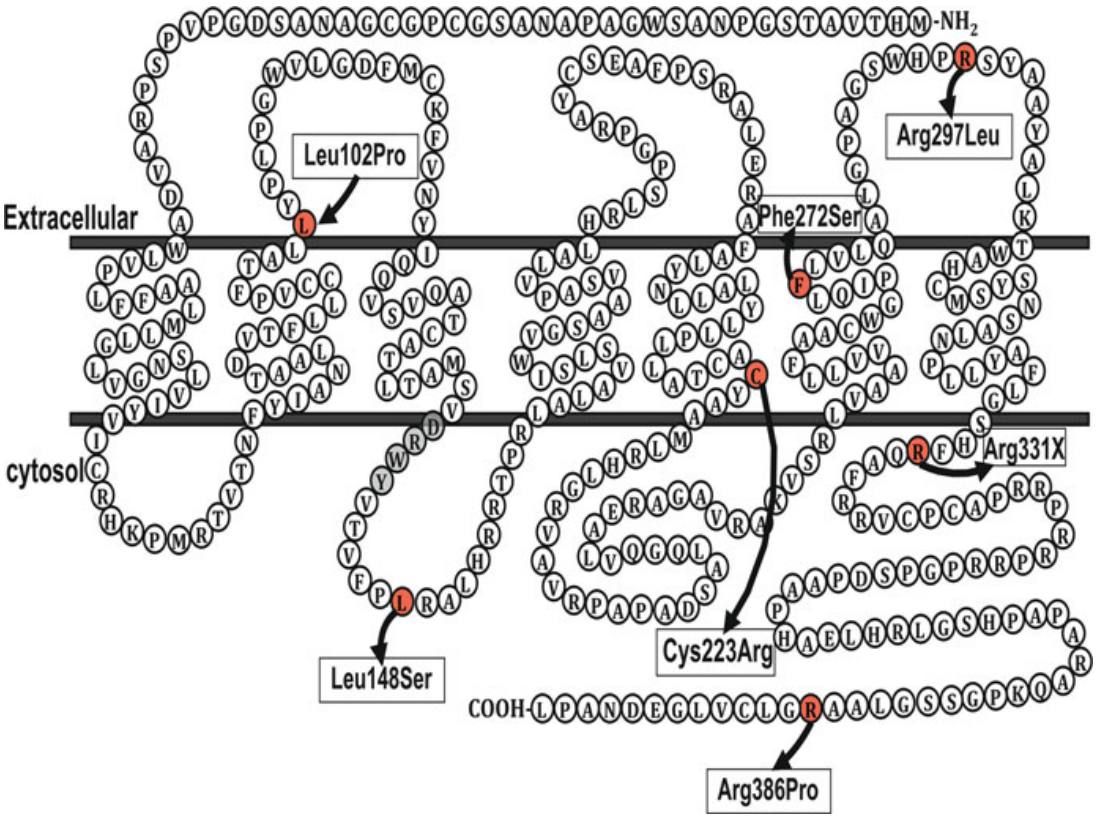

Figure 1.1: Predicted membrane topology of Kiss1r: Also indicating the reported mutations (Bianco and Kaiser, 2013).

et al., 1999). Kisspeptin interaction with Kiss1r leads to the activation of phospholipase C and cAMP transduction pathways, an amassing of inositol-(1,4,5)-triphosphate, responsible for Ca^{++} mobilization, arachidonic acid discharge and phosphorylation of ERK1/2 and p38 MAP kinases in CHO-K1 cell lines (Kotani et al., 2001) (Figure 1.2).

1.2. *Central Expression of Kisspeptin*

Kisspeptin expression has been found in several central as well as peripheral tissues. In mice, Kiss1 is expressed in the anteroventral periventricular nucleus (AVPV), periventricular nucleus (PeN), arcuate nucleus (ARC), medial amygdala anterodorsal preoptic nucleus, and bed nucleus of the stria terminalis (Gottsch et al., 2004; Clarkson and Herbison 2006). In rat, Kiss1 gene is expressed throughout central nervous system, including spinal cord, medulla and pons, hypothalamus, midbrain, ARC, paraventricular nuclei (PVN), dorsomedial hypothalamus (DMH), caudoventrolateral reticular nucleus, ventromedial hypothalamus (VMH), nucleus of solitary tract, lateral reticular nucleus, cerebral cortex, amygdala, nucleus accumbens, bed nucleus of stria terminalis, thalamus, diagonal band of broca (DBB), caudate putamen, septal nuclei, periaqueductal gray, lateral parabrachial nucleus zona incerta, raphe nuclei, spinal trigeminal tract locus coeruleus, medullary reticular nucleus, rostral ventrolateraland medial preoptic area (POA) (Dun et al., 2003; Irwig et al., 2004; Brailoiu et al., 2005; Adachi et al., 2007). Kiss1 expression is sexually differentiated in some areas of the rat brain. In sheep, Kiss1 expression is reported in ARC, DMH, medial POA, PVN, VMH, POA and caudal region of the paraventricular nucleus (Franceschini et al., 2006; Pompolo et al., 2006). Kiss1 mRNA is expressed in the ARC and AVPV (Revel et al., 2006b; Mason et al., 2007) and in preoptic periventricular zone of the hypothalamus in hamster and in DMH and median eminence (ME) in horse (Decourt et al., 2008; Magee et al., 2009). In rhesus monkey, Kiss1-neurons have been identified in the ARC/ME and medial basal hypothalamus (MBH) but not in the POA (including the AVPV) (Shibata et al., 2007; Ramaswamy et al., 2008).

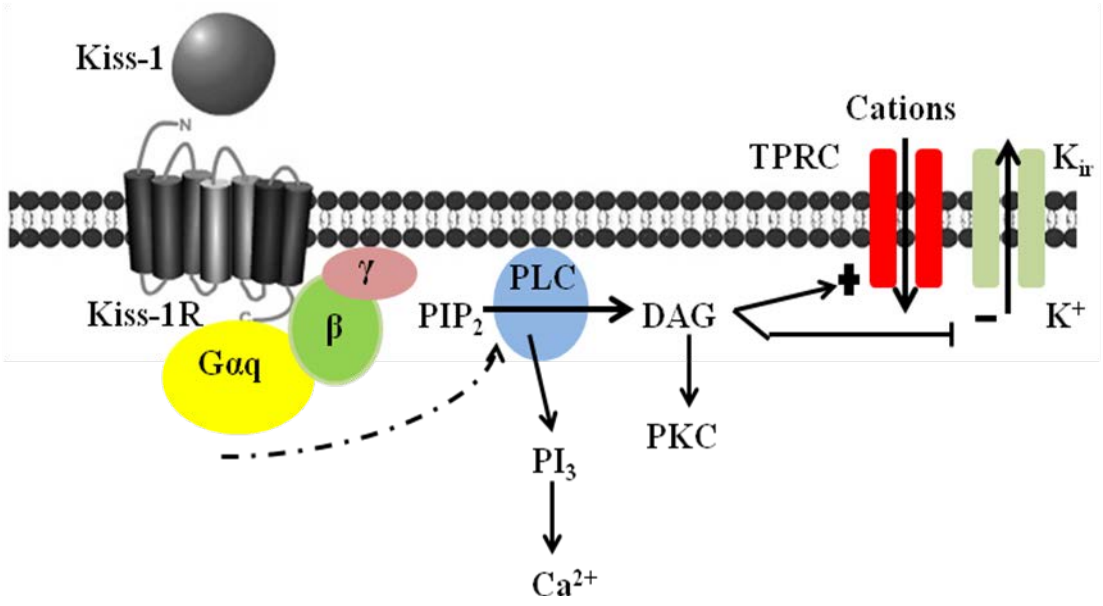

Figure 1.2: A proposed model for kisspeptin signaling.

Kisspeptin expression was noted in human hypothalamus and kisspeptin neurons were observed in the infundibular stalk, lamina terminalis, ventral PeN, POA, PVN, infundibular nucleus, tuberal subdivision of supraoptic nucleus, VMH, DMH, medial septum and stria terminalis (Hrabovszky et al., 2008; Hrabovszky et al., 2010).

Many non mammalian species also show Kiss1 mRNA expression in brain. Kiss1 mRNA is expressed in forebrain and hindbrain of frog (Lee et al., 2009). In many piscine species, such as medaka, gold fish, zebra fish and sea bass, Kiss1 mRNA is expressed in the brain (thalamus and hypothalamic periventricular regions) (Kanda et al., 2008; Li et al., 2009; Mechaly et al., 2009; Parhar et al., 2004).

1.2.1. Kisspeptin Cell Bodies and Fibers

In different species, kisspeptin cells have been reported in two locations i.e, diencephalon and POA. The largest population of these cells is observed in the hypothalamus (Lehman et al., 2010). In rodents, the hypothalamic kisspeptin cells are located rostral-caudal (Gottsch et al., 2004; Smith et al., 2005) while the POA cells are located in rostral periventricular of the third ventricle (RP3V) and clustered in AVPV which extends caudally in to PeN. This distribution in rodents is based on the studies in the females. Males have few kisspeptin cells in this region (Goodman and Lehman, 2012). In monkeys and sheep cell bodies in ARC are located in the middle and caudal levels (Goodman et al., 2007; Ramaswamy et al., 2008). In contrast to female rodents the second group of kisspeptin cell population in other female mammals (primates and ruminants) lacks well defined RP3V population. The kisspeptin cells in these mammals are scattered a little more in lateral POA. The cells in the RP3V of rodents and those in POA of the sheep, goat and primates are homologous but the functional role of each of the population may differ in different species (Goodman and Lehman, 2012). The horse is the only species in which the preoptic population has yet to be demonstrated despite the use of specific antibodies (Decourt et al., 2008; Magee et al., 2009). These rostal kisspeptin cell populations play role in estrogen induced pre-ovulatory LH surge in many species (Goodman and Lehman, 2012). Their absence in horse correlates with the evidence that this surge in

mares is due to withdrawal of steroid negative feedback, rather than the stimulatory action of the estrogen (Ginther et al., 2005).

In addition to ARC and POA, there are other hypothalamic areas which express the kisspeptin cells in mammalian brain. In monkeys, small number of kisspeptin cells, most likely the extension of ARC has been observed in the median eminence (ME) (Ramswamy et al., 2008). Kisspeptin cell bodies are found in infundibular stalk of humans (Hrabovszky et al., 2010). Kiss1 cells are 25 times more in adult females compared to males (Irwig et al., 2004; Smith et al., 2006; Adachi et al., 2007; Kauffman et al., 2007; Kalamatianos et al., 2008). In mice, guinea pigs, sheep and horses and small groups of kisspeptin cells are found in the DMH, but are absent in the rat or hamster (reviewed by Lehman et al., 2013).

A number of kisspeptin cell population resides outside the boundaries of hypothalamus. This included clusters of Kiss1 mRNA expressing cells in the medial nucleus of the amygdala, observed in both rats and mice (Kim et al., 2011)

Recent literature suggests that kisspeptin cell bodies may be present in different areas of brain outside the hypothalamus and limbic system. This observation is based on studies undertakenin the transgenic mice, in which Kiss1 derives Cre recombinase and another reporter gene (Cravo et al., 2011). Using transgenic mice, Cre (Kiss1) expression has been reported in the cortical areas, including insular cortex, piriform cortex, layer 5 and 6 of neo-cortex, in the lateral septum and nucleus of solitary track in brain stem. It is possible that Cre expression in some of these regions is present only during development and not in adulthood (Padilla et al., 2010). The purpose/ function of this expression in these regions is still needs to be explored.

In addition to kisspeptin cell bodies, the kisspeptin fibers have also been reported in different regions of brain. The kisspeptin fibers have been reported in ARC, RP3V, ME internal in the most dense form. Other regions of kisspeptin fibers include POA, ME external, PVN, VMH, LHA and lateral septum in the humans (Hrabovszky et al., 2010). In rhesus monkeys ARC and ME inter contain the dense fibers while the POA and ME external also contain some (Ramaswamy et al., 2008; Ramaswamy et al., 2010). Dense fibers in ARC and ME inter and low density fibers

in POA, RP3V, PVN, DMH, LHA, supra optic nucleus (SON), organum vasculosum of lamina terminalis (OVLT), bed nucleus of stria terminalis (BNST), medial and lateral septum, lateral POA, contralateral ARC, posterior hypothalamic area (PHA), medial amygdale, paraventricular thalamic nucleus, locus coeruleus and periaqueductal gray have been reported in mouse (Clarkson et al., 2009; Hanchate et al., 2012). In rat, kisspeptin fibers have been reported in the ARC, POA, RP3V ME, DMH, LHA, SON, BNST, OVLT, diagonal *band of Broca* (DBB), medial and lateral septum, contralateral ARC, posterior hypothalamus, medial tuberal nucleus, retro-chiasmatic area, supra chiasmatic nucleus and septohypothalamic area (Knoshita et al., 2005; Adachi et al., 2007; Desrozier et al., 2010; Krajewski et al., 2010; True et al., 2011; Xu et al., 2012). In hamster, kisspeptin fibers have been reported in ARC and RP3V (Mason et al., 2007). In guinea pigs the fibers have been observed in ARC, POA, RP3V, ME, VMH, DMH, BNST, OVLT, medial and lateral septum, DBB and ventral premammilary nucleus (Bosch et al., 2012). In sheep, the fibers have been reported in ARC, POA, ME, VMH, DMH and BNST (Franceschini et al., 2006; Goodman et al., 2007; Smith et al., 2008; Smith et al., 2009). Kisspeptin fibers have also been reported in ARC, ME and POA of horse (Decourt et al., 2008) and goat (Ohkura et al., 2010).

1.2.2. Kisspeptin Interaction with other Peptides/Neurotransmitters

In the last few years, kisspeptin neurons have been reported to be receptive either directly or indirectly to various regulatory cues like sex steroids, metabolic factors and circadian factors (Reviewed by Oakley et al., 2009). This has brought kisspeptin in spotlight to play a major role in regulation of GnRH. The heterogeneity among the kisspeptin cell population is providing increasing evidence of their interaction with other neuropeptides/neurotransmitters. For example, in ARC nucleus the kisspeptin neurons are expressed with other two neurotransmitters, i.e., neurokinin B (NKB) and dynorphin (DYN) (Figure 1.3). Both of these have been strongly implicated to play physiological role in controlling GnRH release (Goodman et al., 2004; Topaloglu et al., 2009). In several mammalian species, together with humans, this co-localization is greatly preserved (Goodman et al., 2007; Hrabovsky et al., 2008 Rance, 2009).

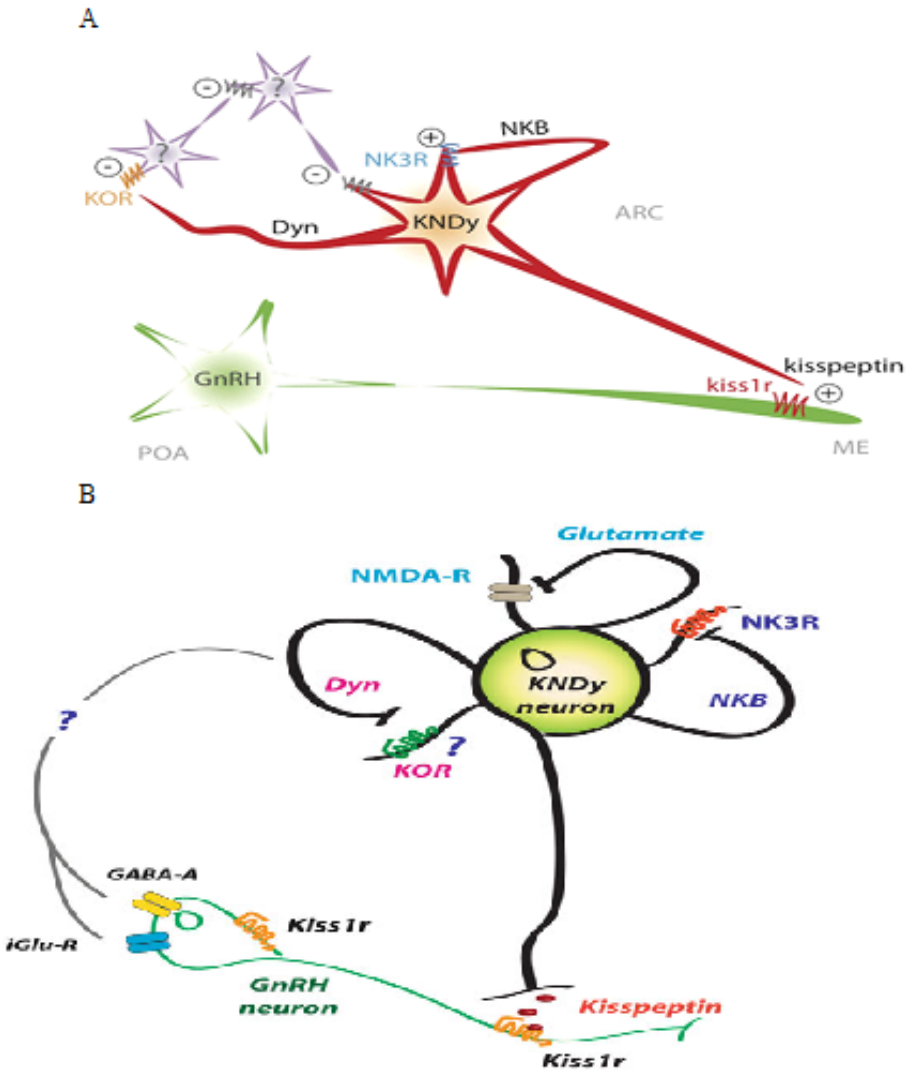

Figure 1.3: Schematic presentation of KNDy interaction (Navarro, 2012) (A). Proposed autoregulatory loop of KNDy neurons (Navarro, 2013) (B).

Dynorphin is an endogenous opioid peptide which plays a part in the progesterone-mediated negative feedback control of GnRH release (Goodman et al., 2004).

NKB is a member of the substance P-related tachykinin family (Krajewski et al., 2005). Tachykinin neurokinin 3 receptor (NK3R) is the receptor for NKB, and is expressed on GnRH neurons (Todman et al., 2005).The loss of function mutations in the gene encoding NKB (TAC3), or its receptor, result in the normosomic hypogonadotropic hypogonadism and also pubertal failure (Topaloglu et al., 2009). This phenotype is similar to rodents and human models of defective kisspeptin signaling (de Roux et al. 2003; Seminara et al. 2003; d'Anglemont de Tassigny et al., 2007; Topaloglu et al. 2009).

In the POA of sheep and goats the kisspeptin cells do not appear to be co-localized with the NKB or dynorphin. In mouse, the RP3V area contains both the peptide co-localized in the kisspeptin cells (Navarro et al., 2009). It is also found in both mouse and rat that a good population of RP3V cells appears to be in close proximity with immunoreactivity of tyrosine hydroxylase (TH) (Kauffman et al., 2007; Semaan et al., 2010; Clarkson and Herbison, 2011), which is a key enzyme involved in dopamine biosynthesis (Clarkson and Herbison, 2011). However, kisspeptin cells in the POA and ARC of the sheep are not in contact with TH (Merkley et al., 2012).

The kisspeptin population of both ARC and RP3V also contains the glutamate and GABA, well known classic transmitters as exposed by the co-localization of the markers. RP3V cells are predominantly GABAergic while the ARC cells contain major populations of the glutamate (Cravo et al., 2011). Some observations in sheep (Merkley et al., 2009) suggest that such difference in ARC and RP3V exists in other mammals as well.

1.3. Central Expression of Kisspeptin Receptor

In 1999, Lee and colleagues reported the presence of the Kiss1r in the rat brain and found expression in pons, thalamus, mid brain, hippocampus, amygdala, striatum and frontal cortex. Further detailed examination of rat forebrain revealed Kiss1r

expression in DBB, medial POA, medial septum, lateral POA, median preoptic nucleus, anterior hypothalamus and lateral hypothalamus (Irwig et al., 2004). Kiss1r expression was observed in PeN and ARC of pig (Ieda et al., 2014), in ARC, MBH and POA in rhesus monkey (Shahab et al., 2005; Shibata et al., 2007). In human KISS1R has been reported in cerebellum, cerebral cortex and brainstem (Muir et al., 2001).

Kiss1r is also expressed in a number of piscine species 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). Two distinct transcripts of the Kiss1r differentiated by approximately 80 bp in length and named Ss Kiss1r1 and Ss Kiss1r2 are expressed in Senegalese sole, medaka, goldfish, zebrafish and sea bass (Biran et al., 2008; Filby et al., 2008; Kanda et al., 2008; Li et al., 2009; Mechaly et al., 2009; Kitahashi et al., 2009). In many non- mammalian vertebrates, the Kiss1r has been reported; e.g., frog (hypothalamus, forebrain and hindbrain) and bullfrog (forebrain and hypothalamus) (Lee et al., 2009; Moon et al., 2009). Kiss1r expressed in the telencephalon brain (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).

1.4. *Peripheral Expression of Kisspeptin*

Peripherally, KISS1 is highly expressed in human placenta, pituitary, and pancreas, with lower levels evident in other tissue, such as stomach, small intestine, thymus, spleen, lung, testis, kidney, and fetal liver (Lee et al., 1999; Chen et al., 2011). Kisspeptin mRNA expression has been reported in human placenta, testis, pancreas, liver and small intestine (Kotani et al., 2001; Muir et al., 2001).

1.5. *Peripheral Expression of Kisspeptin Receptor*

Among peripheral regions, expression of Kiss1r has been observed in liver and intestine of rat (Lee et al., 1999); in pituitary of sheep (Smith 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, pancreas and in low levels in the spinal cord, 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 also expressed in peripheral organs (pituitary, heart, kidney, liver, muscle, stomach and gonads) of many non mammalian vertebrates. The other non mammals in which Kiss1r expression has been discovered include frog (pituitary, testis, intestine, liver and heart) and bullfrog (pituitary and testis) (Lee et al., 2009; Moon et al., 2009).

1.6. *Kisspeptin Functions*

Kisspeptin signaling is known to play a role in a number of physiological and patho-physiological functions, including, metastasis, metabolism, puberty onset and reproduction (Kirby et al., 2009; Clarke and Caraty, 2013; Colledge et al., 2013; Wahab et al., 2013). There is a huge quantity of supporting literature now available in a host of species, indicating the functional role of kisspeptin. In this section such role of kisspeptin will be briefly described.

1.6.1. Metastasis

Kisspeptin was initially discovered as metastasis- suppressor gene, described in tumor biology (Lee et al., 1996; Miele et al., 1996; Lee and Welch, 1997a). Later, detection in breast cancer changed the views that this anti-metastatic effect is not limited to melanoma cells only (Lee and Welch., 1997b; Martin and Hall, 2005). Kiss1 gene expression and its anti-metastatic activity was assessed in additional tumor specimens and cell lines, such as pancreatic cancer cells, papillary thyroid carcinoma, melanoma, breast cancer and ovarian carcinoma (Lee and Welch, 1997a; 1997b; Ohtaki et al., 2001; Matsui et al., 2004; Jiang et al., 2005). Failure of Kiss1 gene expression was found to be a probable outcome of metastasis and tumor development in gastric carcinoma, esophageal squamous cell carcinoma and bladder cancer (Sanchez-Carbayo et al., 2003; Ikeguchi et al., 2004).

1.6.2. Pregnancy and Lactation

Kisspeptin system is proposed to play some role in gestation physiology. High expression of kisspeptin has been reported in human placenta (Lee et al., 1996; Muir et al., 2001). Kisspeptin concentration increases 1,000 folds during the first trimester and up to 10,000 folds in the third trimester. The main source of rise in kissppetin during pregnancy is placenta, because kisspeptin and its receptor mRNA are detectable in the syncytiotrophoblasts in the placenta (Horikoshi et al., 2003). 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). This circulating concentration of kisspeptin is hugely higher as compared with those observed in normal male and nonpregnant females (Dhillo et al., 2005). The exact physiological relevance of kisspeptin during human pregnancy is unknown. However, as kisspeptin and its receptor has been reported in human trophoblast (Bilban et al., 2004) with higher expression during the first trimester and than in the third trimester, therefore it is possible that kisspeptin regulates the trophoblast invasiveness, the process important for embryonic development (Bilban et al., 2004).

1.6.3. Reproduction

Kiss1-Kiss1r system is the major regulator of reproduction as has been evidenced by the studies in 2003, when concurrent observations of two independent research groups inferred that the loss of function mutations in KISS1R were the cause of idiopathic hypothalamic hypogonadism (IHH) and delayed pubertal maturations, which they observed in their patients (de Roux et al., 2003; Seminara et al., 2003). A mutation in KISS1R gene was found to be responsible for IHH in a family, but 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 cell, with mutant receptor variants was decreased by 65% (Seminara et al., 2003). Another mutation was reported by Lan Franco and his colleagues (2005), where an insertional mutation took place and an elongated Kiss1 protein with an elongation of 43 aa was obtained. This mutation

resulted in delayed puberty, low testosterone and low response of LH to GnRH. In mice, targeted deletions of Kiss1 produced the same phenotypic abnormality of reproductive malfunctions (Funes et al., 2003; Seminara et al., 2003). On the other hand, a heterozygous mutation (Arg386pro) in KISS1R, in a girl 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 et al., 2007). These findings lead to the idea that Kiss1-Kissr1 signaling is critical for the beginning of gonadotropin secretion and 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 hypothalamic GnRH pulse generator. On the contrary, a gain of function mutation in KISS1R may initiate earlier activation of the GnRH pulse generator.

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) suggested that kisspeptin potently increased LH and FSH release (Gottsch et al., 2004; Navarro et al., 2004; Plant et al., 2006). Intravenous or central administration of kisspeptin-10, results in the pulsatile release of 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). It was observed that expression of hypothalamic Kiss1 mRNA increased at the time of puberty in the male agonadal monkeys as compared to juvenile male monkeys. As compared with the juveniles Kiss1 mRNA increased in mid pubertal ovary intact female monkeys (Shahab et al., 2005). Intracerebroventricular (icv) injections of kisspeptin increased 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; Thomson et al., 2004; Navarro et al., 2005a; 2005b). Foregoing observation demonstrates 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 pubertal development and this

signaling shifts the developmental phase from juvenile to pubertal by increasing GnRH pulse frequency. Kisspeptin stimulated LH secretion in sheep, pig, goat, cow and horse (Abbara et al., 2013).

1.6.3.1. Kisspeptin Signaling Stimulates the GnRH Secretion and Potent Regulator of Reproduction

GnRH neurons are found in close proximity with kisspeptin neurons in sheep, primates and humans (Shahab et al., 2005; Franceschini et al., 2006; Smith et al., 2007; Rometo et al., 2007). Kisspeptin receptor expression has been reported on 50-70% of GnRH neurons (Tena-Sempere, 2010). Treatment **o**f mice MBH explants with kisspeptin stimulates GnRH release (d'Anglemont de Tassigny et al., 2008). In juvenile and adult mice, almost 90% of GnRH neurons express Kiss1r mRNA (Han et al., 2005). However, during transformation from juvenile to adult life Kiss1 mRNA increases. The kisspeptin release increases at the onset of puberty Kiss1r signaling also changes within the GnRH neurons. Some 90% GnRH neurons in adult mice 27% in juvenile and 44% in prepubertal mice are depolarized by kisspeptin administration (Han et al., 2005). GnRH neurons co-express Kiss1r mRNA in rat and Kiss1 induces c-fos expression in more than 85% of GnRH neurons (Irwig et al., 2004). Kisspeptin elicited GnRH secretion by rat hypothalamic explants (Thompson et al., 2004; Castellano et al., 2005). Intra-cerebroventricular and peripheral administration of kisspeptin caused a marked rise in the plasma LH and to the lesser extent FSH in several mammalian species including rats, mice, sheep, monkeys and humans (Gottsch et al., 2004; Thompson et al., 2004; Dhillo et al., 2005, 2007; Messager et al., 2005; Seminara et al., 2006, Caraty et al., 2007). Kisspeptin increased GnRH in cerebro-spinal fluid in the sheep (Messager et al., 2005). GnRH neurons of monkey express Kiss1 mRNA (Shibata et al., 2005). The Kisspeptin cell body number decreases during the infant-juvenile transition in rhesus monkey. Thus a decline in pulsatile GnRH release during infant, juvenile transition is related to a decrease in the number of kisspeptin neurons of the arcuate nucleus during this developmental transition (Ramaswamy et al., 2013). GnRH antagonists block the stimulation of the gonadotropin by kisspeptin, in rat, mouse and monkey (Gottsch et al., 2004; Matsui et al., 2004; Navarro et al., 2004; Navarro et al., 2005a, b; Shahab et al., 2005).

Evidence for this includes the reduction of kisspeptin induced gonadotropin rise following pre treatment with acyline, GnRH antagonist (Plant et al., 2006). These findings are consistent with the phenotype of hypogonadotropic hypogonadism of Kiss1-/- and Kiss1r-/- mice (de Roux et al., 2003; Seminara et al., 2003; d' Anglemont de Tassigny et al., 2007). Kisspeptin administration restored the GnRH release in Kiss1-/- mice, but not in Kiss1r-/- mice (de Roux et al., 2003; Seminara et al., 2003; Messager et al., 2005). Foregoing suggests the pivotal role of the Kiss1-Kiss1r system in the stimulation of GnRH release.

In ME (internal zone and to a lesser extent external zone), kisspeptin and GnRH axons are in close and extensive association (Ramaswamy et al., 2008). Kisspeptin is released into ME of the monkey in a pulsatile fashion which matches exactly to the pulsatile release of GnRH in the same region (Keen et al., 2008). Kiss1-immunoreactive (ir) fibers were found very near to GnRH fibers in the ME, and kisspeptin caused GnRH release from rat ME in vitro (Inoue et al., 2008). Recently, it has been observed that GnRH neurons in the ME express Kiss1r (Inoue et al., 2008). The later finding suggested that kisspeptin may regulate GnRH secretion, non-synaptically at the level of ME. These data convincingly suggest that kisspeptins control the mammalian gonadotrophic axis via hypothalamic GnRH neurons, where kisspeptins activate GnRH secretion, which leads to stimulation of LH and FSH release from the pituitary. To date, it is clear from the literature that kisspeptin and its receptor plays major role in the regulation of the hypothalamic pituitary gonadal axis. A few years back, the discovery of kisspeptin receptor antagonist (P234) had unveiled the role of Kiss1/Kiss1r signaling in various functions (Rosweir et al., 2009). Pre treatment with P234 the firing rate of GnRH neurons was reduced as compared with the alone kisspeptin treatment. Central infusion of P234 inhibited pulsatile GnRH release in the pubertal female monkeys. In adult male rats and mice the kisspeptin induced LH release and post castration rise in LH was inhibited by P234. In sheep, LH pulses were also inhibited by P234 treatment. The central administration of the kisspeptin antagonist blocked GnRH secretion. Peripheral administration in rats of the penitratin mixed kisspeptin antagonist blocked the GnRH and LH release (Pineda et al., 2010).

1.6.3.2. Kisspeptin and Pituitary Gonadotropes

Kisspeptin treatment in pituitary cells and tissue explants, *in vivo,* results in dose dependent release of LH. Based on Kiss1r gene in gonadotropes, the kisspeptin treatment results in the gonadotropin secretion from the pituitary explants (Kotani et al., 2001; Navaro et al., 2005; Gutierrez-Pascual et al., 2007; Richard et al., 2008).

Until now, the role of kisspeptin within the pituitary is unclear. Within the anterior pituitary *in vivo* and *ex vivo* studies suggest a stimulatory role of kisspeptin. Kissppetin signaling within pituitary may have a dogmatic role in gonadotropin function, which is diverse to its better characterized role within the hypothalamus.

1.6.3.3. Role of Kisspeptin in Pubertal Development

Expression of hypothalamic Kiss1 mRNA increased at or 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). Intra cerebroventricular injections of kisspeptin increased LH and FSH levels in the blood in 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, b). Continuous intravenous administration of human metastin in male monkeys increased testosterone levels (Ramaswamy et al., 2007). Foregoing observations demonstrate that there is a developmental increase in expression of hypothalamic Kiss1 and release of kisspeptin in hypothalamus which activate Kiss1r. This means that the stimulation of GnRH neurons by kisspeptin increases with pubertal development and this signaling shifts the developmental phase from juvenile to pubertal by increasing the GnRH pulse frequency.

1.6.4. Metabolism

Kiss-1 encoding peptides kisspeptins colors the canvas of the gonadotropic axis by linking reproduction and energy homeostasis in addition to other metabolic and nutritional signals. Among peripheral tissues, KISS-1 and KISS1R are also

expressed in adipose tissue and pancreas. The level of expression is regulated by sex steroids and food availability in adipose tissues. Moreover, hypothalamic expression of KISS-1 and KISS1R mRNA is regulated by leptin (Smith et al., 2006), energy status (Luque et al., 2007) and IGF-1 (Hiney et al., 2009). KISS-1 acts as a whole body energy sensor which acts to modify fertility and puberty by relaying information on nutritional status to GnRH-gonadotropic axis (Castellano et al., 2005). Streptozotocin treated diabetic rats expressed diminished levels of Kiss1 mRNA while Kiss1r remained unaltered (Castellano et al., 2006). Kisspeptin system shows versatility in its job not only regulating reproduction mediating metabolic status of the body to hypothalamic circuitry, but vice versa, it also keeps the focus about body weight, energy budgeting and glucose maintenance at least in female mice (Tolson et al., 2014). The presence of the peptide and its receptor in islets of Langerhans in mouse and humans predicted its role in controlling metabolism via regulating insulin secretion. Exogenous kisspeptin remained unaffected in elevating basal insulin secretion but is capable to stimulate insulin secretion when increasing concentrations of glucose are introduced (Hauge Evans et al., 2006; Brown et al., 2008) suggesting its role in conservation of energy.

1.6.5. Other Related Functions

Over the years, continuous research in kisspeptinology has un-veiled many of the secrets of kisspeptin actions in various organs in many species. A part from central axis it is now known to play roles in peripheral organs as well. Large numbers of pancreatic islet endocrine cells express 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 drops a hint that there is a local autocrine or paracrine mode of action for pancreatic islet kisspeptin (Hauge-Evans et al., 2006). Kisspeptin mRNA and protein have been observed in the anterior pituitary in species ranging from rats (Richard et al., 2008) to primates (Ramaswamy et al., 2009), so Kiss1r could play a paracrine role in this tissue. 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 the 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 patho-physiology of cardiovascular disease.

1.7. *Regulation*

Kisspeptinergic signaling is a complex process and it plays a major role in the regulation of the reproduction (Messager et al., 2005). Various regulatory networks impinge on the kisspeptin neurons and play a role in its regulation (Roa et al., 2008; Oakley et al., 2009; Simonneaux et al., 2009). Kisspeptin signaling has been regulated by the testosterone and sex steroids (Smith et al., 2005 Kisspeptin signaling has been regulated by the metabolic energy balance as the acute fasting in adult male and female rats decreases hypothalamic Kiss1 mRNA (Castellano et al., 2005). Insulin-like growth factor 1 (IGF-1) has no effects on ARC Kiss1 gene but stimulates the expression of AVPV Kiss1 (Hiney et al., 2009). In female rats, ghrelin increases the expression of ARC and AVPV Kiss1 but its receptor on kisspeptin neuron is not reported (Forbes et al., 2009).Such possible interactions are shown in Figure 1.4.

1.7.1. Sex Steroids

Some factors regulate Kiss1 expression p**o**sitively or negatively and thus modulate HPG axis. Sex steroids positively regulate Kiss1r 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, b; 2006).

Kiss1 neurons present in ARC project to POA where these are targeted by estrogen-positive feedback which causes metastin release into POA in female rats (Kinoshita et al., 2005). On the other side, Kiss1 mRNA is increased in ARC of rats after gonadectomy which is decreased when sex steroid are administered (Navarro et

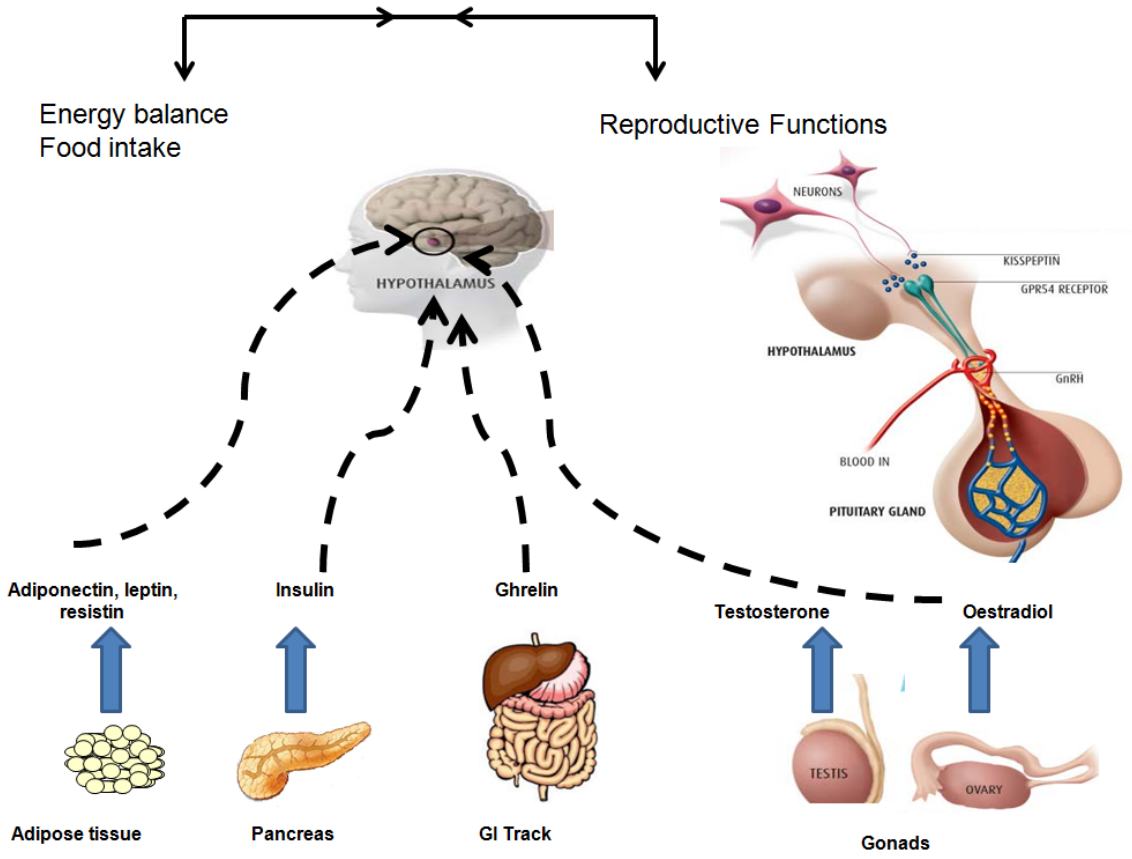

Figure 1.4: **Schematic diagram showing different hormonal signals originating from different peripheral tissues involved in the integral control of food intake, energy balance and reproductive functions.**

al., 2004). Similarly, in sheep ovarian steroid hormone negatively regulate ARC Kiss1 and that ovariectomy increases Kiss1 (Pompolo et al., 2006). In rhesus monkey, surgical isolation of the MBH from rest of the brain does not interfere negative or positive feedback of estrogen (Krey et al., 1975; Hess et al., 1977; Knobil, 1980).While this indicates importance of ARC kisspeptin cells and eradicates a vital role for the POA kisspeptin cells, in sex steroid feedback, yet an active role for POA cannot be ruled out. Positive feedback was compromised in monkeys by bilateral lesions to the POA (Norman et al., 1976). Similar results were also noted after separation of the anterior hypothalamus and MBH (Cogen et al., 1980). In castrated adult male monkeys the testosterone treatment reduces the LH secretion and leads to concurrent reduction in KISS1 mRNA levels in the MBH, but not in POA (Shibata et al., 2007). Thus, in primate, the anterior hypothalamic nuclei (potentially kisspeptin cells in POA) may play a role in mediating some aspects of sex steroid feedback control. Whether there is a sex difference in kisspeptin expression in primates has yet to be determined.

1.7.2. Energy Balance

Hypothalamic expression of KISS1 is reduced by fasting and limited feeding in rodents (Castellano et al., 2005; Luque et al., 2007), sheep (Backholer et al., 2010) and nonhuman primates (Wahab et al., 2011). The negative energy balance associated with lactation also suppresses the kisspeptin expression (Yamada et al., 2007; True et al., 2011). These data support the idea that the kisspeptin neurons in the hypothalamus are an important mediator in interaction of reproduction and the nutritional clues.

1.7.2.1. Leptin

Leptin is a peptide hormone synthesized and secreted by the adipocytes, which conveys information about body energy reserves and nutritional status (Zhang et al., 1994; Pelleymounter et al., 1995). The loss in weight decreases the fat mass and it is directly proportional to the level of circulating leptin (Mafferi et al., 1995). Developed model of hypoleptinaemia is leptin deficient mouse (Zhang et al., 1994). These mice show delayed puberty and are infertile. Interestingly, similar phenotype is also reported in humans with mutations of either leptin or its receptor and in women with

hypoleptinaemia as a consequence of low body weight (Clements et al., 1998; Farooqi et al., 1999; Welt et al., 2004). Hypothalamic GnRH neurons in rat and mice do not express leptin receptor (Ob-Rb) (Quennell et al., 2009). Interestingly, 40% Kiss1 mRNA-expressing cells of the ARC express Ob-Rb mRNA (Smith et al., 2006). Kiss1 expression in the obese (ob/ob) mice is decreased in the ARC which is increased by the leptin treatment (Smith et al., 2006). This indicates the missing link between nutritional clues and reproduction is kisspeptinergic system and leptin positively regulates the kisspeptin induced reproductive activity. Possible impact of leptin on reproductive system has been presented in Figure 1.5.

1.7.2.2. Ghrelin

Ghrelin is a gut derived peptide hormone secreted by endocrine cells of the gastric mucosa in human (Date et al., 2000; Solcia et al., 2000; Rindi et al., 2002), functioning as signal for energy insufficiency (Tena-Sempere et al., 2008). Unlike leptin, ghrelin has been reported as a negative regulator of puberty and GnRH secretion in a variety of species, including human, monkeys, sheep and rodents, acting at the central level (Fernandez-Fernandez et al., 2005;Tena-Sempere et al., 2008). Recently, in female rats ghrelins has been reported to inhibit hypothalamic Kiss1 mRNA expression (Forbes et al., 2009). In mice, Kiss1 neurons in ARC and POA are co-express the ghrelin receptor (GHSR) (Frazao et al., 2014). This phenomenon still needs to be confirmed. However, it may contribute to the suppression of HPG-axis. Ghrelin action on the kisspeptinergic system is modulated by estrogen as it has been reported the high levels of E2 in ARC increase GHSR mRNA expression, modifying the co-localization rate with ERα and Kiss1 and the proportion of Kiss1 neurons acutely responding to ghrelin (Frazao et al., 2014).

1.7.2.3. Neuropeptide Y, Proopio-Melanocortin and Insulin

In addition to leptin and ghrelin there are some other metabolic regulators acting afferent to kisspeptin neurons. ARC nucleus contains discrete sub-populations which express the orexigenic peptide neuropeptide Y (NPY) (Ichimaru et al., 2001) and

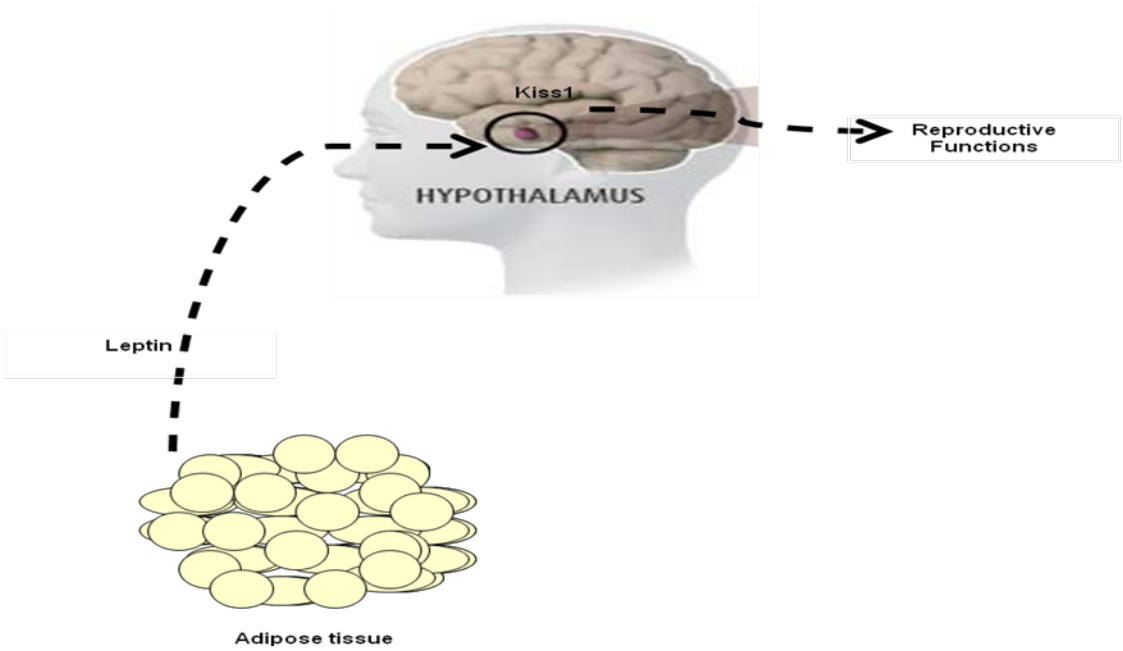

Figure 1.5: Schematic presentation of possible leptin action

anorectic peptide α-melanocyte-stimulating hormone (α-MSH), derived from proopio-melanocortin (POMC: Fu and Van den Pol, 2010; Backholer et al., 2009). NPY acts as a stimulatory factor for Kiss1 expression. The Kiss1 expression was decreased in the NPY knockout (KO) mice while NPY increased the Kiss1 expression in the hypothalamic cell line, N6 (Luque et al., 2007). Such stimulatory effects are counterintuitive. As, NPY expression in response to negative energy balance is increased, while the kisspsptin and GnRH axis are suppressed, which indicates the negative effect of NPY on the reproductive axis (Xu et al., 2009). At certain experimental and physiological conditions, NPY may drive a stimulatory indication to the GnRH system (Kalra and Kalra, 2004); circumstances where Kiss1 pathways might function as putative effector.

In any event, since leptin has been shown to reduce the expression of NPY at precise neuronal populations in ARC (Schwartz et al., 1996), NPY acts as independent modulator instead of driving the stimulatory effects of leptin on Kiss1 neurons. In addition, melanocortins, which are anorectic neuropeptide products of proopio-melanocortin (POMC) neurons in the ARC, have been suggested to stimulate hypothalamic Kiss1 gene expression at the POA in the sheep (Backholer et al., 2009). In contrast, the orexigenic neuropeptide, melanin-concentrating hormone (MCH), which is prominently expressed in the lateral hypothalamus, suppressed kisspeptin-induced stimulation of GnRH neurons (Wu et al., 2009).To date the effects of MCH on Kiss1 expression have not been reported. Finally, insulin does not appear to significantly contribute to the regulation of Kiss1 expression, as suggested by studies in models of diabetic male rats centrally infused with insulin, which displayed persistently reduced Kiss1 mRNA levels despite insulin administration. In addition, insulin failed to stimulate Kiss1 expression in the hypothalamic cell line, N6, *in vitro* (Castellano et al., 2006; Luque et al., 2007).

1.7.3. Photoperiod

Day length (photoperiod) is an important factor which modulates Kiss1 signaling to control reproduction in seasonal breeders. In Syrian hamsters, the expression of Kiss1 is increased in ARC nucleus during long days (LD) and reduced

in the short days (SD). The chronic administration of kisspeptin restores the testicular activity of SD hamsters (Revel et al., 2006a). The Kisspeptin peptide expression is higher in an ARC of SD as compared with the LD Siberian hamsters. Acute injection of kisspeptin in the Siberian hamster stimulates LH release in both LD and SD conditions in male (Grieves et al., 2007) but only SD conditions in female (Mason et al., 2007). Kiss1 mRNA expression is increased during breeding seasons as compared with non breeding seasons in the ovine ARC nucleus. In ovarectomized sheep and ovarectomized sheep with estrogen replacement Kiss1 is increased during the breeding seasons (Pompolo et al., 2006; Smith et al., 2007; 2008). Interestingly, LH/GnRH response to kisspeptin is increased during non breeding seasons (Smith et al., 2009; Li et al., 2011). Expression of Kiss1r on the GnRH neurons is increased during non breeding seasons (Li et al., 2011)**.**

1.8. Objectives of the Study

The first study of this thesis was designed to investigate the role of the peripheral administration of kisspeptin antagonist in the nonhuman primate models. In the second study, we determined whether season regulates the endogenous kisspeptin tone (release of kisspeptin from with in the body). Thirdly, we determined the effect of kisspeptin in the differentiation and motility of rhesus monkey derived GFP-Lyon ES cells

1.8.1. Study of the Role of Endogenous Kisspeptin Signaling in Regulating HPG Axis in Adult Male Rhesus Monkeys

1a) To determine the effect of peripheral administration of P234 on plasma testosterone levels.

1b) To study the effect of peripheral administration of P234 on kisspeptin-10 induced testosterone release.

1c) To study the effect of peripheral administration of peptide 234 on plasma adiponectin level. Previously it was observed that kisspeptin positively regulate the adiponectin release (Wahab et al., 2010). There must be some link between

kisspeptinergic signaling and adiponectin release therefore we determined the effect of Kiss1r blockage on adiponectin release.

1.8.2. Study of the Role of Season in the Regulation of Kisspeptin Signaling in the Adult Male Rhesus Monkeys

In wild and domestic non human mammal's season plays major role in the regulation and alteration of reproductive activity (Robinson, 1959). Alteration in the day length is a key environmental factor; the animals are either long day breeders or short day breeders, such that the reproductive activity is / activated by increasing day length or decreasing day length respectively (Kennaway, 2005; Boden and Kennaway, 2006). Many studies carried out in hamster and sheep where the effect of the season has been studied (Revel et al., 2006a,b; Smith et al., 2007; 2008). Kisspeptin signaling is also involved in regulating the reproductive cycle of species that show seasonal breeding. In hamsters (Greives et al., 2007; Revel et al., 2006a) and sheep (Smith et al., 2007) kisspeptin expression has been found to be decreased in the nonbreeding seasons. The role of kisspeptin in the seasonal breeding was confirmed as the kisspeptin administration during the nonbreeding season stimulated the reproductive axis and induce testicular growth in hamsters (Revel et al., 2006a) and ovulation in ewes (Caraty et al., 2007). The inhibitory effects of estrogen on the kisspeptin expression and Kiss1 mRNA in ARC are greater during the non breeding season (Smith et al., 2008). These studies report a remarkable change in the factors involved in the breeding of the animals specially linked with the reproductive activity to date, no specific study has been designed or performed to determinethe effect of the season's in higher nonhuman primates. We designed this study to determine the effect of the season in the regulation of the hypothalamic pituitary axis in the male rhesus monkey. The following were the main objectives of the study:

2a. To determine the alteration in central and peripheral kisspeptin release in breeding and non breeding season.

2b. To study the expression analysis of kisspeptin its receptor and GnRH mRNA in the MBH by reverse transcriptase PCR, and quantitative RT-PCR.

2c. To study the change in the peripheral testosterone release during breeding and non breeding season.

2d. To determine the seasonal change in immunohistochemical expression of and interaction of GnRH, Kisspeptin receptor in the ARC and MBH of adult male rhesus monkeys.

1.8.3. Study of the Role of the Kisspeptin in Increasing GnRH Expression in Rhesus Monkey Derived RNCs

Kiss1/Kiss1r signaling also appears to be involved in cell growth and differentiation. For example, in R366.4 cell line kisspeptin-10 treatment has been reported to play anti-proliferative role and leads to differentiation of the cells to more specific cell types consistent with the morphological changes (Huma et al., 2013). Kisspeptin-10 treatment has also been reported to play a role in the GnRH neutrite growth *in vitro* (Fiorino and Jasoni, 2010). However, the activation of Kiss1r by kisspeptin-10 has been shown to inhibit cell motility, proliferation, invasion, chemotaxis and metastasis (Kotani et al., 2001; Ohtaki et al., 2001). Kisspeptin-10 treatment has been observed to increase the GnRH mRNA level in GnRH secreting cell line (Novaira et al., 2009).

To gain a clearer understanding of how kisspeptin signaling is related to stem cell growth and GnRH induction in embryonic stem cells (ESC) may provide some novel insights. ESCs are unique in that they are capable of indefinite self renewal and multi-lineage multifunctional segregation. (Evan et al., 1981, Martin et al., 1981, Svendsen et al., 1999; Li et al 2005, Kuai et al., 2009). ESCs can differentiate into numerous cell types, *e.g.* heametopoetic, epithelial, neuronal, vascular, cardiac muscles, smooth muscles, condrogenic and adiponergic lineages (Czyz et al., 2001). Accordingly, ESCs has increasingly become a target research area as well as a valuable molecular biology research tool allowing greater insights into many fields, including development toxicology, and pharmacology (Byrne et al., 2006; Wobus et al., 2011; de Peppo et al., 2012). To date, ESCs have been isolated or derived from mouse embryos (Evan et al., 1981; Martin et al., 1981; Li et al 2005), non-human primates (Thomson et al., 1995) and humans (Thomson et al., 1998).

This study was designed to study the effect of kisspeptin on the monkey derived R366.4 and Lyon ES cells. In respect to neuronal phenotype and GnRH expression development.

3a) To determine the effect of kisspeptin-10 on the proliferation and differentiation of Lyon ES cells.

3b) To determine the expression of GFAP, nestin, β tubulin III and GnRH by using ICC in the Lyon ES cells.

Chapter 2: Role of Endogenous Kisspeptin Signaling in the Regulation of HPG-axis

2.1. Abstract

Kisspeptin signaling is a potent regulator of the reproductive axis and an elicitor of GnRH secretion. The present study assessed the effect of the peripheral administration of a kisspeptin antagonist (P234) on testosterone secretion in the adult male rhesus monkey. Five adult male rhesus monkeys were administered with either P234 (38.8 µg / kg body weight) or vehicle (0.9% saline). Plasma testosterone and adiponectin levels, as a positive control, were measured using specific ELISA in blood samples collected sequentially at 15 min intervals during a 60 min pre to until 360 min post P234 / vehicle injection period. In three monkeys, the experiment was repeated where the animals received a kisspeptin-10 (50 µg) challenge 30 min after the P234 or vehicle treatment. No effect on basal testosterone levels was found after the systemic administration of P234. Plasma adiponectin levels were found to be briefly decreased by P234. P234 administration was found to suppress kisspeptin dependent testosterone and adiponectin release. The present results suggest that the systemic administration of P234 was effective peripherally. However, the minimal peripheral effects on testosterone secretion in the male macaque suggested that P234 did not cross the blood brain barrier and blocked the peripheral kisspeptin receptors.

2.2. Introduction

Kisspeptin neurons have been identified as the key neuroendocrine system in the regulation of the hypothalamic–pituitary-gonadal (HPG) axis, that act one step upstream of gonadotropin releasing hormone (GnRH) neurons (Smith et al., 2005; Oakley et al., 2009). GnRH neurons express GPR54, a G-protein coupled receptor targeted by kisspeptin (Lee et al., 1999; Muir et al., 2001; Brailoiu et al., 2005). A sufficient kisspeptinergic drive to GnRH neurons has been found to be necessary to maintain fertility in human males (Dhillo et al., 2005) and a disruption of this system has been associated with a wide variety of reproductive development problems. For example, inactivating mutations in GPR54 lead to a normosmic form of hypogonadotropic hypogonadism (HH) in humans (de Roux et al, 2003; Seminara et al., 2003) and an activating mutation of GPR54 was found to cause precocious puberty (Teles et al., 2008). In addition, GPR54 knock-out mice fail to make a pulsatile leutinizing hormone (LH) and follicle stimulating hormone (FSH) secretions and do not undergo puberty (Funes et al., 2003).

Kiss1 (encoding kisspeptins) and Kiss1r gene expression has been noted in the hypothalamus of non-primates (Smith et al., 2005), non-human primates (Shahab et al., 2005) and humans (Muir et al., 2001) and has been found to be regulated by a number of mechanisms. For example, season and metabolic status have been shown to regulate the expression of Kiss1 (Simonneaux et al., 2009; Wahab et al., 2011).Sex hormones have also been found to regulate the expression of Kiss1 mRNA in the PeN and AVPV of both male and female mice (Smith et al., 2005). Interestingly, gonadectomy was found to increase Kiss1 mRNA levels in the ARC of rats, and that this increased transcription was lowered with sex steroid replacement (Navarro et al., 2004). This foregoing evidence suggested the existence of a feedback mechanism involved in the regulation of kisspeptin transcription, expression and secretion, which might ultimately regulate reproductive function.

The pivotal role of Kiss1-Kiss1r signaling in reproductive function has been demonstrated in a number of pharmacological (Shahab et al., 2005; Wahab et al., 2010) and gene expression studies (Brown et al., 2008). However, the physiological

significance of this signaling has not been well characterized. This may be due to the lack of physiological evidence, which had been difficult to ascertain because of the absence of a potent kisspeptin antagonist. In recent years, the discovery of a kisspeptin antagonist, peptide 234 (P234), and its use in animal investigations has shed light on the role of endogenous kisspeptin signaling in regulating the reproductive axis. These studies provided detailed evidence for a physiological role of kisspeptin signaling in regulating GnRH secretion in mammals (Shahab et al., 2005; Brown et al., 2008; Roseweir et al., 2009; Wahab et al., 2010; Pineda et al., 2010; Guerriero et al., 2012). However, few studies have been directed at examining testosterone release in primates in response to peripheral kisspeptin. A few studies that have used P234 have predominantly administered the antagonist centrally within the nervous system. Although these studies have providedinformation into the role of kisspeptin signaling, yet, these have limited limited focus on kisspeptin's peripheral effect within the body. Roseweir et al. (2009) found that the central administration of the kisspeptin antagonist inhibited the firing rate of GnRH neurons in the mouse brain slices, reduced pulsatile GnRH secretion in female pubertal monkeys, inhibited kisspeptin induced release of LH in rats and mice and that P234 administration blocked castration-induced rises of LH levels in sheep, rats and mice. Furthermore, the central infusion of P234 inhibited the onset of puberty and blocked the pre-ovulatory surge of LH in female rats (Pineda et al., 2010).

Only a few studies have used the peripheral administration of kisspeptin antagonists (P234, P271). These include the peripheral administration (intra peritoneal) of P234 and P271 (P234 conjugated to penitratin to enhance transport across the blood brain barrier) in rats, which showed that P271 blocked kisspeptin dependent LH release (Pineda et al., 2010). In another study P271 blunted gonadotropin response to kisspeptin-10 in rats (Pineda et al., 2010) and suppressed GnRH release in pre-pubertal female monkeys (Guerriero et al., 2012). None of these studies examined testosterone release in response to the peripheral action of kisspeptin.

The present study was specifically designed to investigate the physiological role of endogenous kisspeptin in the regulation of testosterone secretion in the adult

male rhesus monkey, using the intravenous injection of P234. The effect of the Kiss1r blockade on the plasma adiponectin levels was used as a positive control for the peripheral effectiveness of P234 as kisspeptin had been found reported to act peripherally to increase adiponectin secretion (Wahab et al., 2010).

2.3. Materials and Methods

2.3.1. Animals

Five intact adult male rhesus monkeys (*Macaca mulatta*), 6-8 years old, weighing 8.5 -15.5 kg were used for the current study. These animals were housed in individual cages, under standard colony conditions in the primate facility of the Department of Animal Sciences, Quaid-i-Azam University. The animals were fed daily with fresh fruits (09:00-09:30 h), hard boiled eggs at 11:00 h and bread at 1300-1330 h. Water was available *ad libitum*. Appetite was monitored for a month prior to the beginning of the experiments. In order to reduce the effects of stress on the blood sampling, the animals were habituated to the chair restraint two months prior to commencing the experiments. The duration of restraint was gradually increased until a daily period of 3 h was attained. The animals were sedated with ketamine hydrochloride (Ketler, Astarapin, Germany; 5 mg/kg BW, i.m) for placement in and removal from the restraining chair. All experimental protocols of the present study were approved by the Department of Animal Science Committee for Care and Use of Laboratory Animals, Quiad-i-Azam University.

2.3.2. Venous Catheterization

To permit sequential withdrawal of blood samples and i.v. administration of kisspeptin-10 and the kisspeptin antagonist, the animals were anesthetized with ketamine (10 mg/kg BW, i.m) and a Teflon cannula (Vasocan Branule, 0.8 mm/22 G O.D, B. Braun, Melsungen AG, Belgium) was inserted in the saphenous vein. The distal end of the cannula was attached to a syringe via a butterfly tube (Length 300 mm, volume 0.29 ml, 20 GX3/4", JMS, Singapore). The experiments were not initiated until the animals had fully recovered from sedation.

2.3.3. Pharmacological Agents

Ketamine and heparin (Rotex Medica, Trittau, Germany) were purchased from local distributors. Human kisspeptin-10 (112-121) was purchased from Calbiochem (La Jolla, CA, USA). The kisspetin antagonist - P234- was purchased from Sigma-

Aldrich (St. Louis, MO, USA). Working solutions of kisspeptin-10 and P234 were made in normal saline (0.9% NaCl).

2.3.4. Experimental Design

The first experiment was designed to measure the effect of a kisspeptin receptor blockade on basal plasma testosterone levels in adult male rhesus monkeys. The animals (n=5) were blood-sampled at 15-min intervals for a total period of 420 min. The animals were injected with saline (0.9 % NaCl) as a vehicle after getting 60 minutes sample. One week later, the same animals were injected with P234 (using a similar protocol and were sampled as described above) as a single bolus i.v. injection (38.8µg/kg body weight) after 60 min. The dose of P234 was calculated based on a previously administered dose of P234 applied centrally in the female monkey (Roseweir et al., 2009). The peripheral dose used here was ten-fold higher than the previous dose administered centrally.

In a second experiment, the effect of the P234 on testosterone response to kisspeptin-10 was evaluated in adult male rhesus monkeys (n=3) by systemically delivering the P234 prior to kisspeptin-10 stimulation. Each animal was given a bolus i.v. injection of P234 (38.8 µg/kg BW) followed by a kisspeptin-10 injection (50 µg; iv) 30 min later. For control sampling, one week earlier than the treatment, the same animals received vehicle followed by a kisspeptin-10 injection. Blood samples were collected at 15-min intervals for 60 min before P234/vehicle administration and until 210 min after the kisspeptin-10 administration. As mentioned above, the doses of P234 and kisspeptin-10 were calculated based on previous work in adult female (Rosweir et al., 2009) and adult male rhesus monkeys (Wahab et al., 2010), respectively. Figure 2.1 shows the detailed experimental design.

2.3.5. Blood Sampling

Sequential blood samples (2 ml) were obtained in heparinized syringes. Following withdrawal of each sample an equal amount of heparinized (5 IU/ml) normal saline was administered to avoid blood loss and to keep the cannula patent. Blood sampling was conducted between 10:00-18:00 hrs. Blood sampling for the first

experiment was done in Oct 19th, 2010 and Oct 27th, 2010, while for the second experiment it was done on Nov 3rd, 2010 and Nov 10th, 2010. Blood samples were centrifuged at 3,000 rpm for 15 min at 4 ºC, and plasma was separated and stored at -20 °C until hormonal analysis.

2.3.6. Hormone Assays

Plasma testosterone levels were measured using a commercial testosterone ELISA kit (BioCheck, Inc, Foster City, CA, USA). The sensitivity of the assay was 0.025 ng/ml. Intra and inter assay co-efficient of variation for the testosterone assay were 4.4% and 10%, respectively. Plasma adiponectin levels were measured using the AssayMax human adiponectin ELISA kit (Assaypro, St. Charles, MO, USA). The sensitivity of the assay was 0.5 ng/ ml. Intra and inter assay co-efficient of variation of the adiponectin assay were 4.1% and 7.2%, respectively. The assays were performed following the kit manufacturer's instructions.

2.3.7. Statistical Analyses

Statistical comparisons for the mean plasma levels of testosterone and adiponectin during pre and post P234 periods were made by paired student's t-tests. Changes in testosterone concentrations observed after administering P234 were assessed by a repeated measures ANOVA followed by Dunnett's multiple comparison test. All data are presented as mean (± SEM). Differences were considered statistically significant at $p<0.05$.

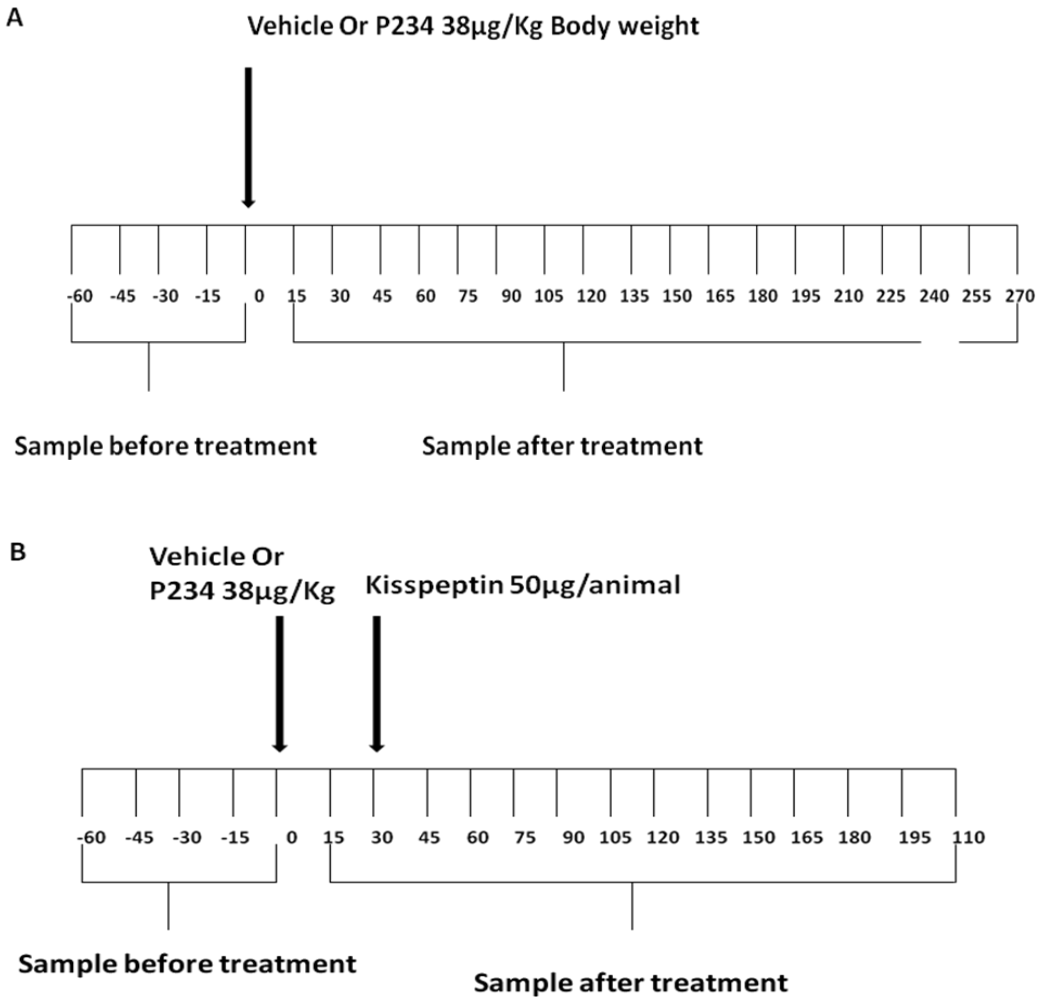

Figure 2.1: Experimental design of experiment 1 (A), Experimental design of experiment 2 (B)

2.4. Results

2.4.1. Effect of Peripheral Administration of P234 on Plasma Testosterone Levels

Changes in plasma testosterone levels in pre and post P234 injection periods are shown in Figure 2.2a. A repeated measure ANOVA showed no effect of vehicle or P234 on mean testosterone levels. Mean plasma testosterone levels observed before and after i.v. injection of P234 were comparable as there was no significant difference (Figure 2.2b).

2.4.2. Effect of Peripheral Administration of P234 on Kisspeptin-10 Induced Testosterone Release

Temporal changes in plasma testosterone concentrations after the kisspeptin-10 administration in P234 or vehicle pre-treated monkeys are shown in Figure 2.3. Mean plasma testosterone levels were significantly increased ($p<0.001$) in the vehicle treated animals at 105, 120, 135, 150,180, 195 and 210 min following kisspeptin-10 administration, whereas, the plasma testosterone levels were found to be significantly increased ($p<0.001$) in the P234 treated group at 90 and 105 min only. Comparison of mean pre and post kisspeptin-10 testosterone levels by t-test revealed that the kisspeptin-10 injection significantly ($p<0.05$) increased the mean plasma testosterone levels in the vehicle treated animals, but that no significant ($p>0.05$) changes were found in the mean plasma testosterone levels after a kisspeptin-10 injection in P234 treated animals (Figure 2.4).

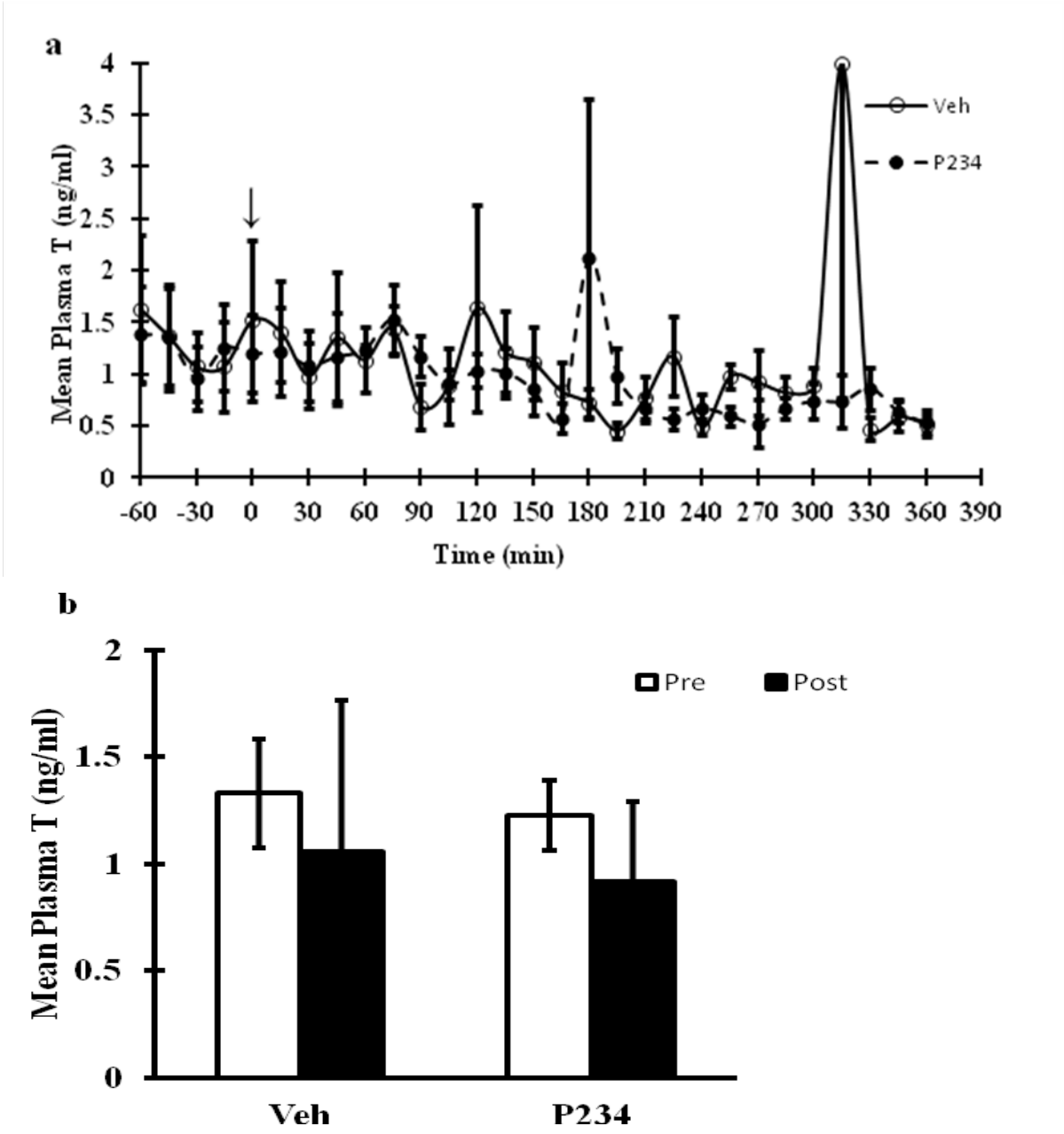

Figure 2.2: Time course of plasma testosterone (T) levels (mean ± SEM) before and after vehicle or P234 i.v. bolus injection (38.8µg/kg BW; arrow) in adult male rhesus monkeys (n=5). A repeated measure ANOVA showed no effect of the vehicle or the antagonist on the mean(a) Comparison of mean ± SEM testosterone levels prior to (-60-0 min) and post (15-360 min) injection of P234 or vehicle (b).

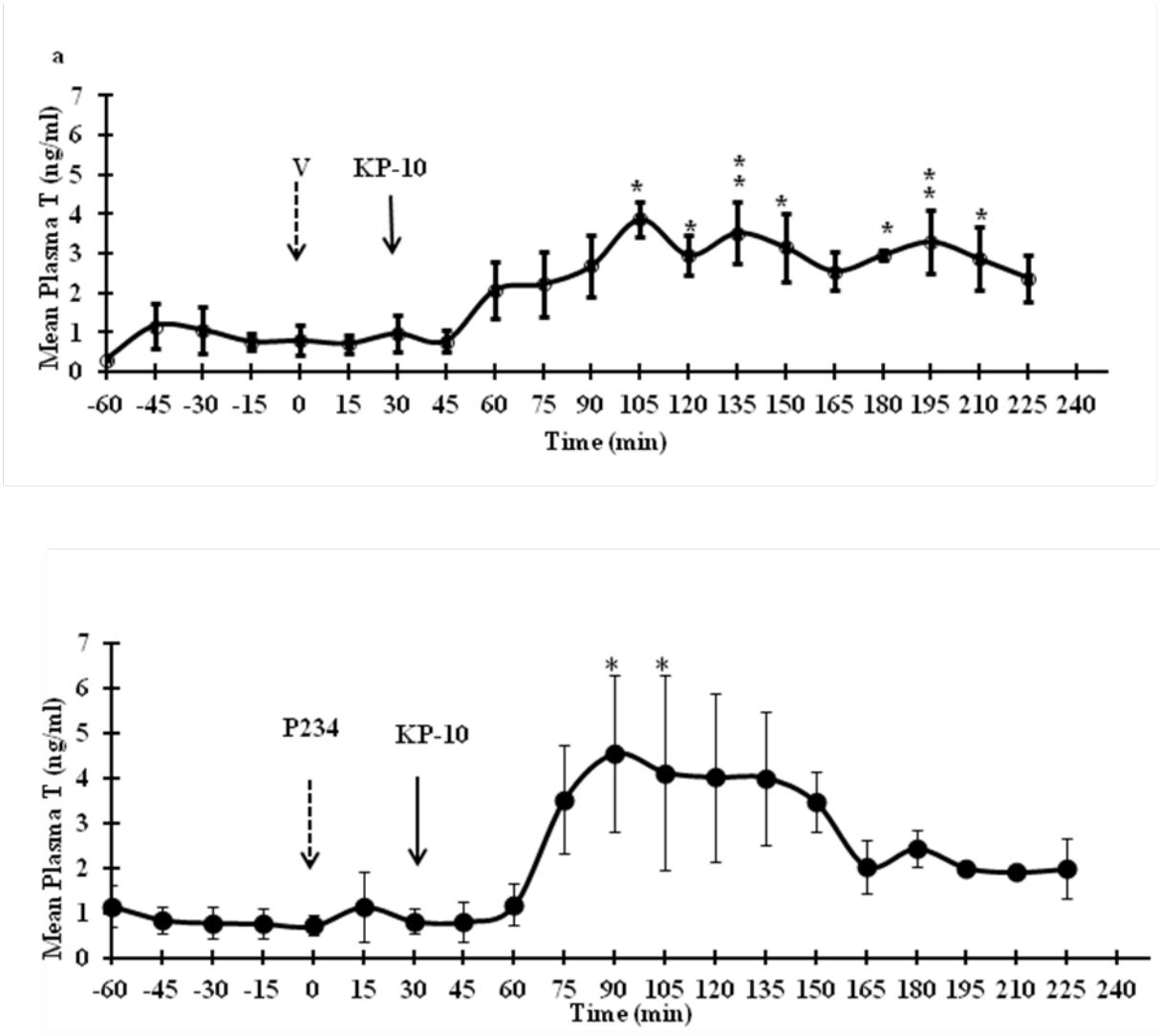

Figure 2.3: A kisspeptin-10 (50 µg) i.v. bolus injection significantly increased the basal levels of plasma testosterone (mean ± SEM) in the vehicle treated adult male rhesus monkeys (a, n=3). Conversely, only a slight and short, albeit significant, elevation change was observed in the plasma testosterone levels in P234 (38.8 µg/kg BW) treated animals b, (n=3), (*$p<0.01$, ** $p<0.001$ vs 30 min).

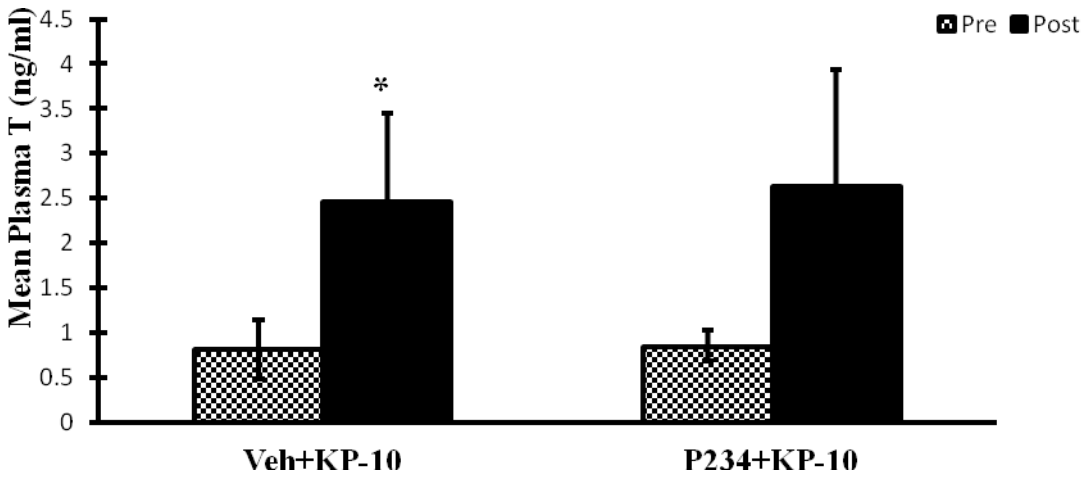

Figure 2.4: Comparison of mean ± SEM plasma testosterone observed before and after kisspeptin-10 (50 µg iv) in vehicle or P234 treated adult monkeys (n=3) revealed that the stimulatory effect of kisspeptin-10 on plasma testosterone was no longer significant following P234 pre-treatment. (* $p<0.05$).

2.4.3. Effect of Peripheral Administration of Peptide 234 on Plasma Adiponectin Level

Mean basal plasma adiponectin levels obtained during -30 to 150 minutes were found to be significantly changed in the P234 treated animals, while no significant decrease in plasma adiponectin levels was observed in the vehicle treated animals (Figure 2.5a). Circulating adiponectin concentrations in blood found after kisspeptin stimulation were significantly reduced following P234 antagonist treatment in comparison to vehicle pre-treatment ($p<0.01$) (Figure 2.5b).

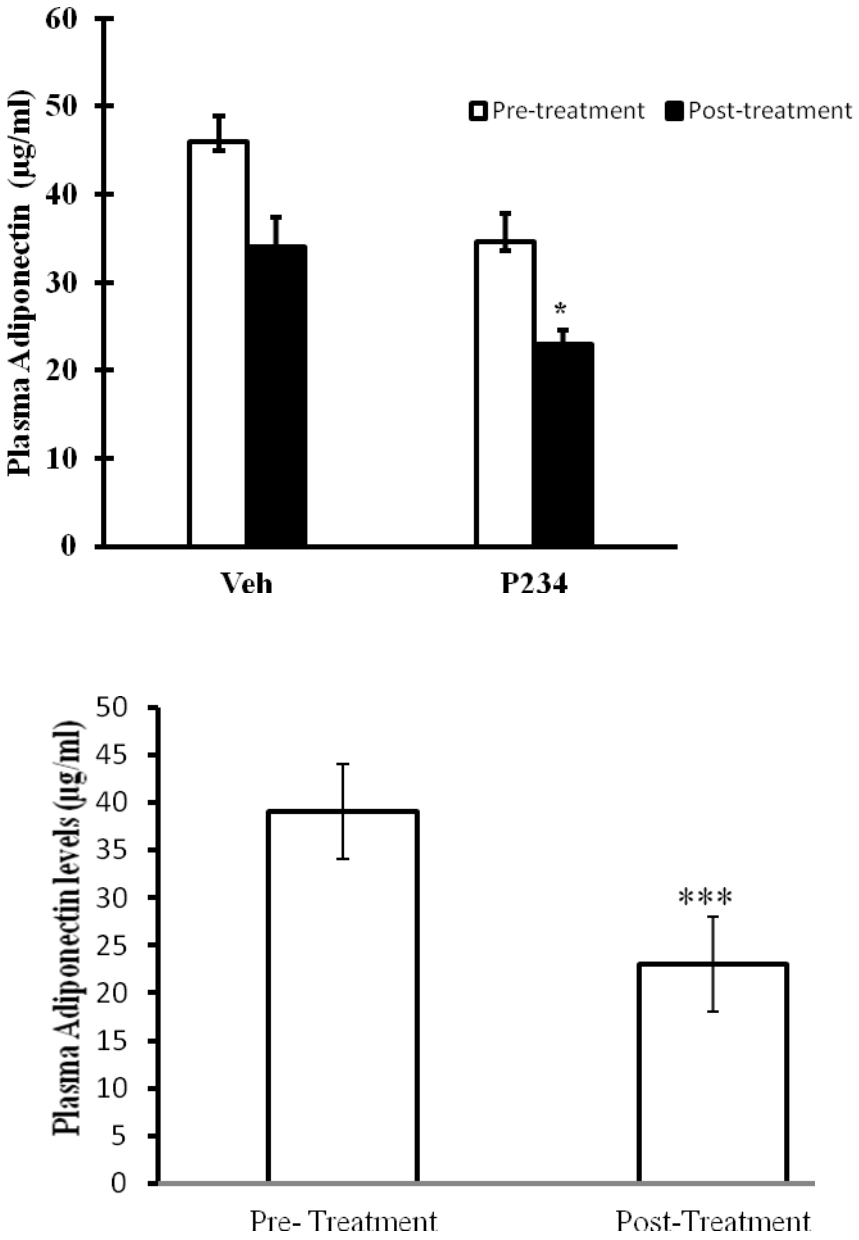

Figure 2.5: Mean ± SEM plasma adiponectin concentration observed in the male rhesus monkeys before (white bars) and after (black bars) vehicle or p234 (38.8 µg/kg BW) i.v. administration (n=3). A significant reduction (*p<0.01) of plasma adiponectin level was evident after P234 injection (a). Mean ± SEM plasma adiponectin concentration observed in the male rhesus monkeys treated with vehicle / P234 followed by kisspeptin-10 stimulation (n=3) (***p<0.0001), (b).

2.5. Discussion

This study assessed, for the first time, the effect of the peripheral administration of P234, a Kiss1r antagonist, in adult male rhesus monkeys on plasma levels of testosterone, the terminal endocrine signal of the HPG-axis. The experimental results demonstrated that the bolus peripheral administration of P234 did not affect the basal testosterone release in the monkeys. These results are in line with previous data demonstrating that basal LH levels in adult male rats and that basal GnRH level in adult female rhesus monkey were not affected by the central administration of the P234 (Roseweir et al., 2009).

The results presented here also indicated that the systemic delivery of P234 ablated the testosterone response to kisspeptin; although P234 did not fully abolish kisspeptin dependent testosterone release. Forgoing demonstrates that P234 was indeed antagonizing the Kiss1r in this study an acute central bolus dose of P234 has been shown to similarly inhibit the kisspeptin dependent LH release in intact male rats and mice (Roseweir et al., 2009). LH is subsequently responsible for the testosterone release from the Leyding cells of the testis (Saez, 1994). In castrated male rats and mice, P234 was found to inhibit the post-castration LH increase, which showed that P234 blocked Kiss1r receptors centrally. Additionally, penitratin mixed P234 (P271) administered peripherally abolished the kisspeptin dependent LH release in adult male rats (Pineda et al., 2010), which suggested that peripheral administration of P234 could cross the blood brain barrier (BBB) in the presence of penitratin, an agent which helps transport macromolecules across the BBB. In this study, kisspeptin induced testosterone secretion was also found to be considerably reduced by the peripheral administration of P234 without penitratin. However, this reduction was not a complete inhibition of testosterone release, which suggested that the P234 had very minimal effect. Although, there have been preliminary reports of the presence of Kiss1r in the monkey testis (Irfan et al., 2008; Tariq et al., 2010), initial findings from our laboratory have indicated that kissppetin does not stimulate testosterone release from monkey testicular tissue fragments *in vitro* (Tariq and Shahab, unpublished data). This indicated that kisspeptin may not directly interact with testicular tissue and

that the regulation of testosterone release was independent of any peripheral action by kisspeptin.

Therefore, the moderate inhibitory effect of P234 on kisspeptin dependent testosterone release observed in the present study was likely due to a central perturbation; despite it not being certain to what extent the P234 peptide crossed the BBB. Kisspeptin receptors have been shown to be located on the GnRH fibers in the ME region outside the BBB (Inoue et al., 2008). Similarly, GnRH fibers originating in the monkey hypothalamus that project outside the BBB to the ME have been shown to be contacted by kisspeptin fibers in the ME (Ramaswamy et al., 2008). Therefore, it is possible that the P234 could access kisspeptin receptors in the ME without passing the BBB in this study. The finding that a partial inhibition of testosterone release was found in P234 treated monkeys suggested that some of the P234 acted on the median eminence and blocked Kiss1r stimulation, which was potentially responsible for the impaired testosterone release. It is likely that the peripherally administered P234 might not have crossed the BBB and could not block kiss1r at sites afferent to GnRH neurons, hence, P234 having no effect on basal testosterone secretion in the present study and / or on basal LH secretion in other studies (Roseweir et al., 2009). On the other hand, the peripherally administered P234 may have blocked kiss1r on GnRH fibers in ME, which may have provided enough of an effect to significantly reduce kisspeptin-induced testosterone release, but not strong enough to completely abolish the stimulated testosterone release.

Our previous study indicated that the kisspeptin significantly increased plasma adiponectin levels in the adult male rhesus monkey (Wahab et al., 2010). This was a novel finding and suggested that kisspeptin acted peripherally on adipose tissues and stimulated adiponectin release. In this study, consistent without earlier finding, it was observed that basal and kisspeptin stimulated adiponectin levels were decreased following a peripheral administration of P234. These findings suggested that peripheral P234 could access adipocyte Kiss1r and was able to reduce adiponectin release accordingly. The potential for the P234 bolus injection to cause a reduction in plasma adiponectin levels and to impair the kisspeptin dependent adiponectin release

in the current study is indicative that P234 did not need to cross the BBB in order to produce antagonistic effects on the kisspeptin-adiponectin system.

Overall, this study described the effect of peripheral administration of P234 on plasma testosterone and adiponectin levels in adult male rhesus monkeys. The results indicated that P234 moderately reduced the testosterone response to kisspeptin but did not affect basal testosterone levels. These findings suggested that P234 administration modulated median eminence kisspeptin-kiss1r signaling involved in kisspeptin stimulated testosterone release and may have not crossed the BBB. In addition, peripheral kisspeptin signaling could be modulated by P234 and was found important for adiponectin secretory function in adult male monkeys as the P234 administration was also found to decrease adiponectin levels and kisspeptin-dependent adiponectin response. In summary, current results suggest that kisspeptin signalling provides regulation of testosterone dependent reproductive function in male primates in a manner that is regulated predominately through the central neuroendocrine system. It is also suggested that peripheral kisspeptin signaling plays an important role in the regulation of adipokine secretion in higher primates.

Chapter 3: Evidence for Increased Hypothalamic Kisspeptin Signaling During Breeding Season in the Free Ranging Adult Male Rhesus Monkeys

3.1. Abstract

Reproductive activity in the free ranging rhesus monkeys is seasonal (November-February). Kisspeptin is known as one of the fundamental regulators of the neuroendocrine reproductive axis. We hypothesized that the expression of hypothalamic Kiss1, Kiss1r, GnRH, pDYN, KOR, NKB and NK3R underlies the seasonal changes of reproduction in this higher primate. To prove our hypothesis, we examined the expression of Kiss1, Kiss1r, GnRH, pDYN, KOR, NKB and NK3R (mRNA levels using RT-qPCR) and Kiss1, Kiss1r and GnRH, protein expression by IHC/ICC techniques in the medio-basal hypothalamus (MBH) of adult male rhesus monkeys during breeding season (BS; January; n=3) and non-breeding season (NBS; July; n=3). The animals were maintained under free ranging conditions in the primate breeding colonies of Kunming Institute of Zoology (102.71º longitude, 25.03º latitude). Additionally, we measured CSF (four samples collected at 30 min interval/animal from the lumbar vertebrae; n=3) kisspeptin and peripheral testosterone levels (1000-1600) in BS (November) and NBS (August) (n=4) by using specific RIA's.

The relative mRNA expression showed significant increase in the breeding season for kisspeptin ($p<0.01$), Kiss1r ($p<0.0005$) and GnRH ($p<0.0001$) while no observable change was seen in the expression of pDYN, KOR, NKB, NK3R in the MBH. The number of kisspeptin cell bodies significantly increased in the arcuate nucleus (ARC) during the BS ($p<0.0001$). The number of GnRH, Kiss1r positive and Kiss1r positive GnRH cell bodies was significantly increased ($p<0.01$; $p<0.01$ and $p<0.001$, respectively) in the BS. The number of contacts between the GnRH and kisspeptin cell bodies was also enhanced in the BS ($p<0.01$). The CSF kisspeptin levels were significantly increased during the breeding season ($p<0.0001$). Peripheral testosterone concentrations ($p<0.0001$) and paired testis weight ($p<0.0001$) were also increased in the BS monkeys.

The present data suggest that in higher primates, season related reproductive activity is associated with increased hypothalamic kisspeptin signaling impinging on GnRH neurons.

3.2. Introduction

There are daily and annual changes in light, temperature, and humidity, which in turn cause cyclic changes in food availability. Organisms therefore must anticipate these predictable changes and adapt their biological functions to ensure their survival. Specifically, most mammals restrict their fertility to a specific time of the year to guarantee that the birth and weaning of the progeny occur during the most favorable season (Lincoln and Short, 1980; Karsch et al., 1984). Although a variety of environmental factors are vital to consider, a majority of mammalian species uses the highly predictable annual variations in the light interval (or photoperiod) to ascertain the time of year and acclimatize their reproductive activity accordingly. In response to seasonal changes supra-chiasmatic nucleus (SCN), adjusts these signals through the retinohypothalamic track (RHT) (Reppert and Weaver, 2002). Lesions in the SCN abolish the circadian rhythms in rhesus and squirrel monkeys (Reppert et al., 1981). Melatonin is secreted in response to photoperiodic signals by the pineal gland in both long day and short day animals (Challet, 2007). Many animals use variations in the melatonin secretion as seasonal clock (Lincoln et al., 2003). Melatonin acts at the pre mammilary area of the posterior hypothalamus and the pars tuberalis of the pituitary plays role as mediator of seasonal changes (Malpaux et al., 2001). The alteration in the period of secretion of melatonin, thus serves as a biological indicator for the organization of day-length-dependent cyclic functions such as reproduction, actions, coat growth and camouflage coloring in seasonal animals (Arendt et al., 2005). In mammals, pineal gland acts as a transducer of photoperiodic signals into hormonal signal melatonin, which controls hypothalamic pituitary gonadal (HPG) axis by acting on hypothalamus (Malpaux et al., 1998).

Rhesus macaques are seasonal breeders, with the majority of mating happening in October till December and births coinciding with the end of the rainy season, or during the time of maximum food availability (March-July) (Lindburg, 1971; Qu et al., 1993). Circanual variations have been reported in various species of monkeys with regards to testicular endocrine and exocrine functions that indicate divergent seasonality in testicular volume size, sperm production, semen quality, sexual behavior and hormonal milieu (FSH, LH and testosterone) having highest

responses during the breeding seasons (Lodge et al., 1970; Plant et al., 1974; Michael et al., 1977; Wickings et al., 1980).

Research during the last 40 years has firmly established that GnRH plays major and important role in reproduction (Schally et al., 1971). Peptidergic signaling mechanism modulates the GnRH pulse generator activity (Lehman et al., 2010) and therefore is considered as an important regulatory component of the HPG axis. Later, the discovery that kisspeptin and its receptor are important for normal reproduction (de Raux et al., 2003; Seminara et al., 2003), the kisspeptin pathways gained importance in regulating GnRH secretion. In 2009, the NKB was described as a critical player in gonadotropin release (Topaloglu et al., 2009). NKB is a member of the substance P-related tachykinin family (Krajewski et al., 2005). Tachykinin neurokinin 3 receptor (NK3R) is the receptor for NKB, and is expressed on GnRH neurons (Todman et al. 2005). Another study reported that the patients with inactivating mutations of NKB or NK3R display hypogonadotropic hypogonadism, which closely resembles the phenotype of patients with loss of function mutations in KISS1 or KISS1R genes (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2009; Fukami et al., 2010; Gianetti et al., 2010; Young et al., 2010; Francou et al., 2011; Topaloglu et al., 2012). The KNDy neurons with cell bodies found in the ARC and co-synthesize kisspeptin, NKB and dynorphin (Goodman et al., 2007). These KNDy neurons have been reported for their role in negative feedback control of the reproductive activity (Smith et al., 2005; Goodman et al., 2007; Smith et al., 2007) and also serve as pacemakers for the GnRH pulse generator (Navarro et al., 2009; Wakabayashi et al., 2010; Navarro et al., 2011; Gottsch et al., 2011). Particularly, kisspeptin (de Raux et al., 2003; Seminara et al., 2003) and NKB (Topaloglu et al., 2009) have revealed to play key roles in the physiological development and reproductive functions. Kisspeptin signaling is also involved in regulating the reproductive cycle of species that show seasonal breeding. The role of kisspeptin in the seasonal breeding was confirmed as the kisspeptin administration during the non breeding season stimulated the reproductive axis and induced testicular growth in hamsters (Revel et al., 2006a) and ovulation in ewes (Caraty et al., 2007). In hamsters (Revel et al., 2006b; Greives et al., 2007) and sheep (Smith et al., 2007) kisspeptin expression has been found to be decreased in the non breeding season. The inhibitory

effects of estrogen on the kisspeptin expression and kiss1 mRNA in ARC are greater during the non breeding season (Smith et al., 2008). All the previous studies done in sheep and rodents indicated that kisspeptin plays important role in regulating the seasonal reproduction in these species. The increase in the negative feedback of the estrogen in the non breeding seasons is specially the main key point. Signals controlling these seasonal alterations in kisspeptin expression may possibly include day length acting by means of melatonin (Revel et al., 2006; Chalivoix et al., 2009) and food limitation (Paul et al., 2009).

In primates, it is well recognized that seasonal reproduction is the norm rather than the exception (Lancaster and Lee, 1965; Lindburg, 1987). Of all the non-human primates, none has been as extensively studied as the rhesus macaque. It has been described as the species most importantly featured in a debate on seasonality in primates: however, while much is known about rhesus behavior, reproductive biology, and endocrinology, seasonality in rhesus reproduction is poorly understood (Lindburg, 1987).

Many studies have been conducted on seasonality in monkeys and various parameters have been studied. The role of kisspeptinergic system has also been very well know in reproduction (Seminara et al., 2003; Rosweir et al., 2009) and seasonal reproduction in hamsters (Revel et al., 2006a), sheep (Smith et al., 2008) and ewe (Caraty et al., 2007). There is a gap as in non human primates neuroendocrine control of seasonality less has been studied. Until now there has been no study which examines the role of the kisspeptinergic system in seasonal breeding of nonhuman primates. The present study was designed to test the hypothesis that kisspeptin plays a key role in maintaining and activating reproduction in response to changes in photoperiod in a seasonally breeding higher primate. We choose rhesus monkeys (*Macaca mulatta*) because the role of kisspeptinergic signaling in seasonal breeding of rhesus monkeys has not been studied before.

3.3. Materials and Methods

3.3.1. Animals

Ten adult intact male rhesus monkeys (*Macaca mulatta*), age 7-9 years, weighing 6.5-10 kgs were used for this study. The animals were maintained under free ranging environment in the primate breeding colonies of Kunming Institute of Zoology, Kunming, China (102.71º longitude, 25.03º latitude). The breeding season of the rhesus monkeys of Kunming, China lasts from September to February and non breeding season from March to August (Zheng et al., 2001). The animals were fed daily with fresh fruits and monkey chow. Water was available *ad libitum*. Four animals were habituated to chair restraint, several weeks prior to commencing of the experiments in order to reduce the effects of stress on blood sampling. The time of restraint was gradually increased until a daily period of 3-6 h was attained. The animals were again released to the free ranging environment and were brought back one day before the sampling was conducted. The animals were sedated with ketamine hydrochloride (Rotex media, Trittau, Germany; 5 mg/kg BW, im) for placement in and removal from the restraining chair. Other six animals which were used for CSF and tissue collection were not chair restrained. The sampling was done for the breeding season in November-January and for a non breeding season in July-August. All the experiments were approved by the Departmental Committee for Care and Use of Animals.

3.3.2. Venous Catheterization

To allow sequential withdrawal of blood samples the animals were sedated with ketamine (10 mg/kg BW, im) and fixed to a retrained chair. A Teflon cannula (BD Vialon, 22 G *1. 16” 1.1mm * 30mm; Becton Dickinson, Medical Devices Co., Ltd. Suzhou, P. R. China) was inserted in the saphenous vein. The distal end of the cannula was attached to a butterfly tube (Length 300 mm, volume 0.29 ml, 20 GX3/4”, JMS, Singapore) and blood was drawn to haparinized vacutainer tubes (Sanli, Liuyang, Hunan, China) Experiments were not initiated until the animals had fully recovered from sedation.

3.3.3. Pharmacological Agents and Antibodies

Ketamine and pentabarbitol (Rotex media, Trittau, Germany) were purchased locally. The primary antibodies rabbit anti-metastin (1:500, Phoenix Pharmaceutical Inc, Washington, AZ, USA); mouse anti GnRH (1:500, Millipore, Billerica, Massachusetts, USA) and rabbit anti- KISS1R (1:100; Santa Cruz, Heidelberg, Germany) were used for immunocytochemistry. Goat anti rabbit IgG-TR (Santa Cruz, Dallas, Texas, USA) and goat anti mice IgG FITC (Santa Cruz, Dallas, Texas, USA) were used as secondary antibodies.

3.3.4. Blood Sampling

Sequential blood samples (2 ml) were obtained in heparin coated vacutainer tubes (Sanli). Following withdrawal of each sample an equal amount of heparinized (5 IU/ml) normal saline was administered to avoid blood loss and to keep the cannula patent. Blood sampling was conducted between 1000-1600hrs for 6 hrs duration. Blood sampling for the non-breeding season was done on 8th and 9th August, 2011 while blood sampling for the breeding season was done on 15th and 16th November, 2011. Blood samples were centrifuged at 3000 rpm for 15 min, and plasma was separated and stored at -80°C until hormonal analysis.

3.3.5. CSF Collection

CSF was collected from the caudal vertebrae of the rhesus monkey (n=3). For CSF collection, animals were anesthetized using ketamine HCl (intramuscular: 10 mg/kg). One ml of CSF was obtained from the lumbar vertebrae using a 22-gauge needle and 5-cc syringe within 15 minutes of anesthesia. Four samples were collected with the interval of 20 minutes starting from 9 am. The CSF was collected on 9th January 2012 for breeding season and on 9th July 2012 for non breeding season. CSF samples were immediately placed in liquid nitrogen and later transferred to a –70° freezer.

3.3.6. Hormonal Analysis

Plasma testosterone levels were measured using a commercial testosterone RIA kit (Immunotech, Marseille Cedex 9, France). Sensitivity of the kit was 0.025 ng/ml. Inter and intra assay coefficients of variation were 8.6% and 11.9%, respectively. Metastin levels were measured using the KiSS-1 (112-121) -NH2/Kisspeptin-10/Metastin (45-54) -NH2 RIA kit (Phoenix Pharmaceutical, Inc., AZ, USA). Sensitivity of the kit was 10 pg/ml. Inter and intra assay coefficients of variation were 5-7% and 12-15%, respectively. Assays were done following the instructions of the kit manufacturers.

3.3.7. Hypothalamus Location and Tissue Collection

The animals were anesthetized using ketamine HCl (im, 10 mg/kg BW). The heart was exposed by making anterior in situ exposure. The right ventricle was punctured to drain the blood at the same time pericardial infusion of normal saline was instituted to wash out of blood. After that the animals were decapitated and the brain was removed, divided into two hemispheres. The MBH was located as the area caudal to the optic chiasm, rostral to the mammillary body, medial to the hypothalamic sulcus, and the ventral half of the hypothalamus and dissected out. Figure 3.1 shows the diagrammatic presentation of the MBH location.

One MBH hemi area was snap-frozen on dry ice in RNAlater (Ambion, Austin, TX, USA), and stored in liquid nitrogen until processed for RNA extraction (up to 2-3 months). The time between the decapitations of animals to the tissue dissection was less than 1 h.

3.3.8. Testicular Tissue Collection and Paired Testes Weight

Paired testes were collected immediately after decapitation and were weighed using manual weighing machine.

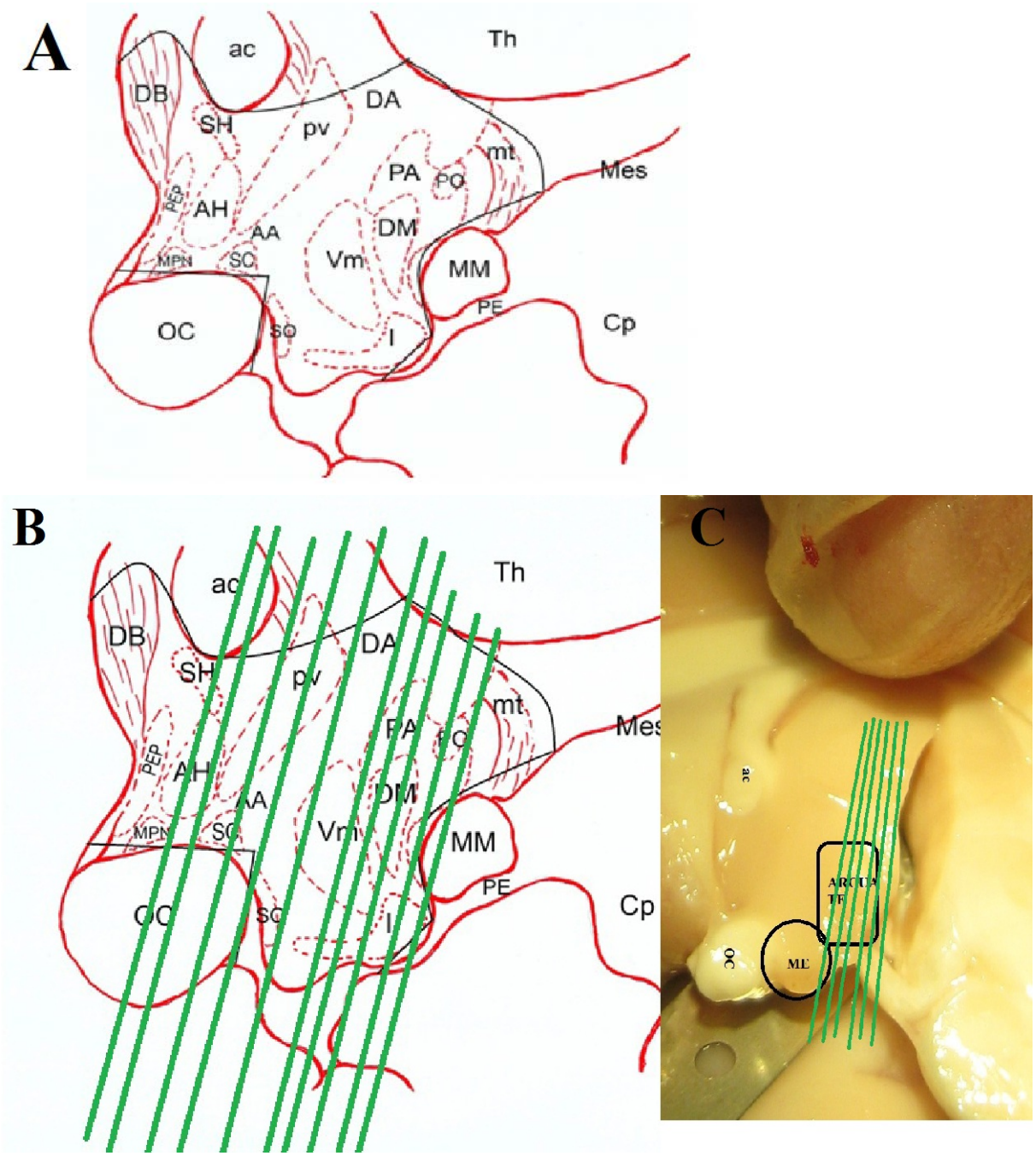

Figure 3.1: Diagrammatic presentation of the hypothalamic location (A). Orientation of sectioning (B). Original picture of the tissue block with plane of sectioning (C).

3.3.9. MBH Tissue Fixation Procedure

One hemi MBH area was immediately placed in 4% para-formaldehyde following MBH dissection and kept for 4-6 hrs. Then the tissues were transferred to the 20% sucrose until they sank down to the bottom. The tissues were washed with the PBS three times and transferred to 30% sucrose and waited till these sank down to the bottom. After that the OCT (opti-mum cutting temperature compound) was used to cover the tissue. The tissues were kept at -20ºC until the sections were made Figure 3.1.

Later on 20 µm thick serial section was made using Leica CM 1850-Cryostat (Leica Microsystems Nussloch GmbH, Nussloch, Germany) keeping the temperature at -28ºC. The sections were fixed on the poly-lysine labeled slides.

3.3.10. Total RNA Extraction and Reverse Transcription

Total RNA from the tissue MBH was extracted using the tRNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Prior to conducting quantitative real-time reverse transcriptase-PCR (RT-PCR), the total RNA was digested with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) to remove possible contamination of genomic DNA. Final RNA concentration was assessed with a Beckman DU 650 (Fullerton, CA, USA) spectrophotometer. The GeneAmp RNA PCR Core kit (Applied Biosystems, Branchburg, NJ, USA) was used for reverse transcription with 2 µg of total RNA and random hexamer primers according to the manufacturer's specifications.

3.3.11. Primers for Real Time qPCR

The Kiss1, Kiss1r and GnRH1 mRNA expression in hypothalamus tissues of malerhesus monkeys were quantified by real-time PCR. GAPDH was used as internal control. Primers used for real time qPCR for the above genes are described in Table 3.1. The real time qPCR was performed in a total volume of 20 µl. Each reaction contained 1 µl of reverse transcribed sample (1, 0.5, 0.25 and 0 dilutions of one of the hypothalamic sample) 10 µl of BioRad PCR master mix, 0.3 µl of each primer and 8.4

µl of DEPC water. The initial step was done at 95ºC for 5min, followed by 40 cycles of a 95 ºC denaturing step for 30 Sec, annealing was done at 55 ºC. For kisspeptin the annealing temperature was 65 ºC for 30 Sec, and an elongation step at 72 ºC for 30 Sec. After the amplification dissociation curve analysis was performed to insure the purity of PCR product. GAPDH was used as a reference gene. Relative mRNA expression was calculated by using ΔΔCT method (Lapatto et al., 2007). The ΔCT for each sample was determined by obtaining the difference between the average CT of the reference gene (GAPDH) and the average CT of the gene of interest (Kiss1, Kiss1r, GnRH1, pDYN, KOR, NKB and NK3R). The ΔCT of the calibrator (the sample with the lowest count) was subtracted from the ΔCT of the each sample to determine the ΔΔCT. This number was then used to determine the amount of mRNA relative to the calibrator and normalized by the GAPDH, or the n-folds difference. The n-fold difference was calculated by the equation 2-ΔΔCT.

3.3.12. Fluorescence Immunocytochemistry

Fluorescence immunocytochemistry for kisspeptin, GnRH and kiss1r was performed on frozen sections. Dual labeling was done for kisspeptin/GnRH and kiss1r/GnRH. Sections were rinsed at room temperature in 50 mM PBS (PH 7.3, 4×10 min), incubated for 30 minutes with 3% hydrogen peroxidase, and rinsed again with PBS (3×5 min). Sections were then incubated overnight at 4ºC in PBS buffer containing 5% normal goat serum (Sigma Chemical Co., St. Louis, MO, USA), 0.05% Triton X-100 and 0.1% BSA (Sigma Chemical Co.) to block non specific binding. For dual label immunoflouresence staining, the sections were incubated for 48 hrs at 4ºC with cocktail of primary antibodies prepared in PBS goat serum buffer at a dilution of 1:200- 1:500. After that sections were brought to room temperature and washed with PBS (3×5 min). Then sections were incubated with cocktail of respective secondary antibodies for 1.5 h at room temperature. Again (3×5 min) washes with PBS were done. The slides were incubated with the DAPI for 15 minutes. Again (3×5 min) washes were done with PBS, and then the slides were cover slipped using GEL/MOUNT aqueous mounting media (Sigma-Aldrich). They were stored at 4ºC until the microscopy and analyses were performed.

For single labeling same procedure was repeated but only one primary antibody was used and then corresponding secondary antibody was used. Single labeling was done for kisspeptin, GnRH and Kiss1r.

3.3.13. Confocal Microscopy

Imaging of fluorescence labeling for kisspeptin, kiss1r and GnRH was performed using an Olympus FV1000 confocal microscope dual laser (Olympus America Inc., Melville, NY, USA). Optical sections along the z-axis were collected at. 0.5-1-µm intervals. Composite digital images were then converted to JPG format, imported into Adobe Photoshop (Adobe Photoshop CS2, version 9.0; Adobe Systems Inc., San Jose, CA, USA), and color balance were adjusted for presentation.

3.3.14. Statistical Analyses

Statistical comparisons for the mean plasma levels of testosterone, CSF kisspeptin and kisspeptin perikarya, GnRH, Kiss1r cell body count, and number of cell to cell contacts during breeding and non breeding season were made by unpaired student's t-tests. Two way ANOVA was also used for metastin like ir and testosterone, time and season were factors. Differences were considered statistically significant at $p < 0.05$.

Table 3.1: Primers used for the RT-qPCR

S. No	Gene	Accession No.	Forward Primer 5'-3'	Reverse Primer 5'-3'	Prod. Size (bp)
1	Kiss1	XM_001098284.2	ACCGAGAGGAAGCCGTCTGCTA	AGTTGTAGTTCGGCAGGTCCTTCT	181
2	Kiss1r	XM_001117198.1	CTCGCTGGTCATCTACGTCA	CGAACTTGCACATGAAATCG	173
3	GnRH	NM_001195436.1	CGGCTGGAGGAAAGAGAGATGCCG	GCCAGTTGACCAACCTCTTTGA	76
4	NKB	XM_001115535.1	GAGCCACAGGAGGAGATGGTT	TCCAGAGATGAGTGGCTTTTGA	101
5	NK3R	NM_001037862.1	TCT GGT CCC TGG CGT ATG	AAG TTC TGG AAG CGG CAG TA	229
6	pDYN	NM_001257884.1	TCC TGA ACTCCC AGT CAT GTT GGT	AAA TTG GTTTGC CTT GCT CCC TCC	122
7	KOR	NM_001032824.1	GCTGGACCCCCATTCACATA	GCCTAAGGCGATGCAGAAGT	101
8	GAPDH	NM_001195426.1	CTC CTG TTC GAG AGT CAG CC	GCC CAA TAC GAC CAA ATC CG	105

For single labeling same procedure was repeated but only one primary antibody was used and then corresponding secondary antibody was used. Single labeling was done for kisspeptin, GnRH and Kiss1r.

3.3.13. Confocal Microscopy

Imaging of fluorescence labeling for kisspeptin, kiss1r and GnRH was performed using an Olympus FV1000 confocal microscope dual laser (Olympus America Inc., Melville, NY, USA). Optical sections along the z-axis were collected at. 0.5-1-µm intervals. Composite digital images were then converted to JPG format, imported into Adobe Photoshop (Adobe Photoshop CS2, version 9.0; Adobe Systems Inc., San Jose, CA, USA), and color balance were adjusted for presentation.

3.3.14. Statistical Analyses

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3.4. Results

3.4.1. Season Affected the Kisspeptin and Testosterone Release, and Altered the Paired Testes Weight

A clear difference was observed in the kisspeptin release in adult monkeys during the breeding season where it was increased significantly ($p<0.0001$) as compared with the non breeding season (Figure 3.2).

The peripheral plasma testosterone levels showed a significant increase in the breeding season and a decrease in the non breeding season (Figure 3.3). There was significant time related changes in the secretion of testosterone with respect to time and season effect. Overall, mean testosterone values were significantly increased during the breeding season. Paired testicular weight was also increased during the breeding seasons ($p<0.0001$).

3.4.2. Season Modulated Gene Expression

Measurable seasonal changes were observed in the MBH mRNA expression of kisspeptin, Kiss1r and GnRH in adult male monkeys (Figure 3.4). Overall, there was a significant increase in the mean relative mRNA expression of kisspeptin, kiss1r and GnRH in the breeding season. The NKB, pDYN expression remained unchanged in both season. Also, no significant increase was observed in receptor NK3R and receptor KOR expression (Figure 3.5).

3.4.3. Season Influenced Immunocytochemical Expression

The immunocytochemistry analysis showed an increase in the number of kisspeptin cell bodies in the ARC region during the breeding season as compared with the non breeding season (Figure 3.6, Figure 3.7). Similarly the kisspeptin-ir fibers were also increased during breeding season (Figure 3.8).

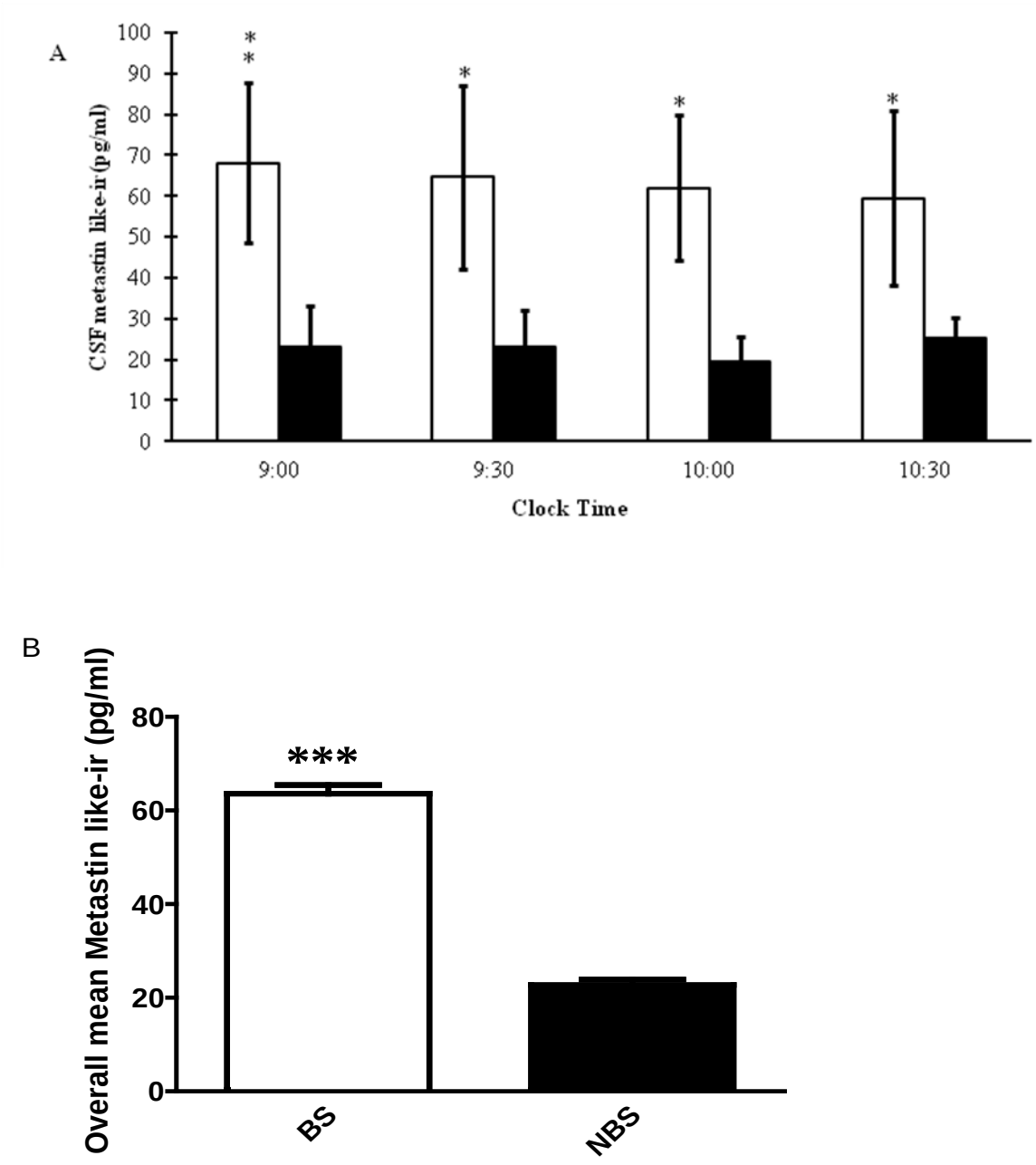


Figure 3.2: The data presented here represent mean ±SEM CSF metastin like-ir value of three male rhesus monkeys at different times during the breeding (BS) and non breeding season (NBS). Two way ANOVA indicated significant effect of ($p<0.0001$) and time ($p<0.0001$). At all given points CSF metastin was significantly elevated in BS (*$p<0.01$,**$p<0.005$) (A). The data presented here are overall mean ± SEM CSF metatstin like-ir value of three adult male rhesus monkeys, the unpaired t-test indicated a highly significant high CSF metastin like ir in BS (*** $p < 0.0001$), (B).

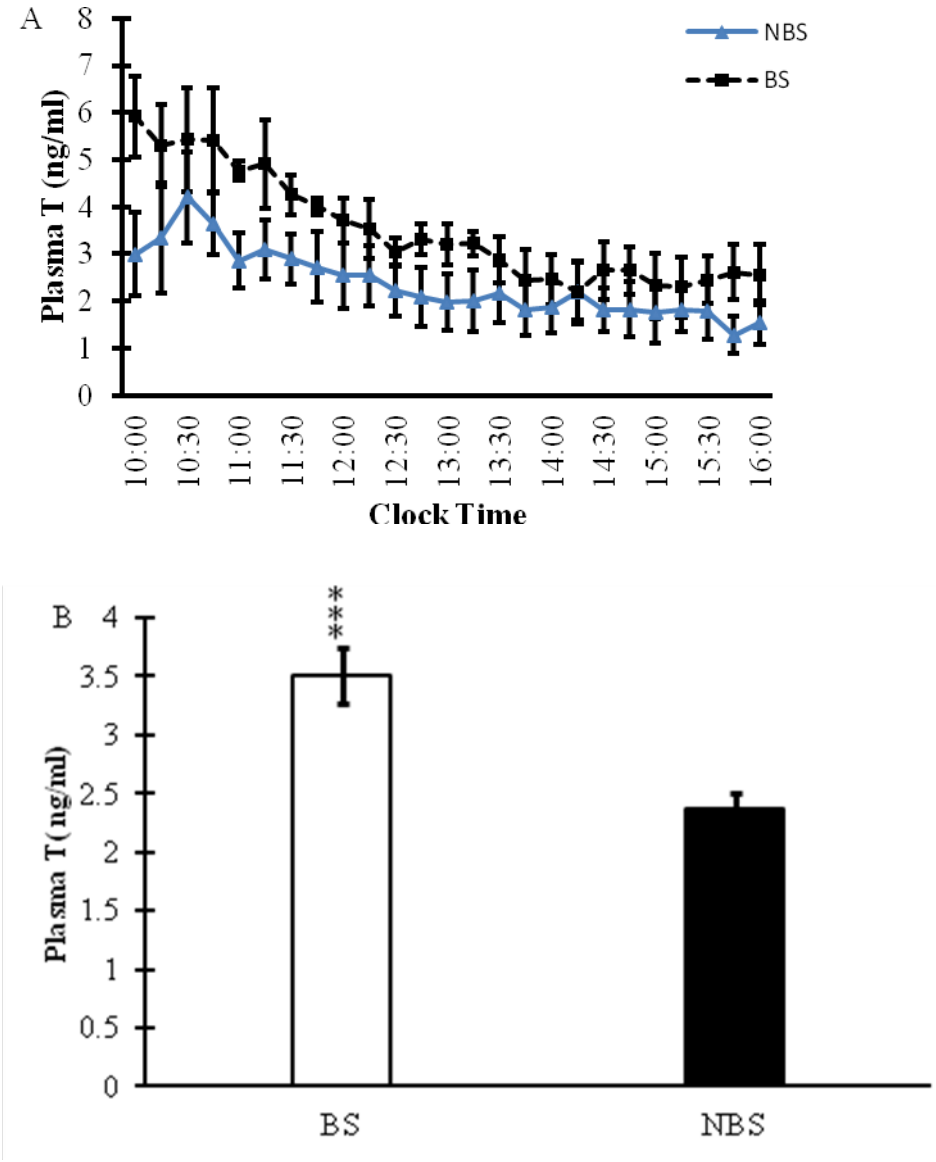


Figure 3.3: Time related change in mean ± SEM plasma testosterone levels of four adult male rhesus monkeys. Two way ANOVA followed by F-crit as a post test indicated highly significant effect with respect to time ($p<0.0001$) and season ($p<0.0001$), (A). Overall mean ± SEM plasma testosterone levels were increased (***$p<0.0001$) during the breeding season (B).

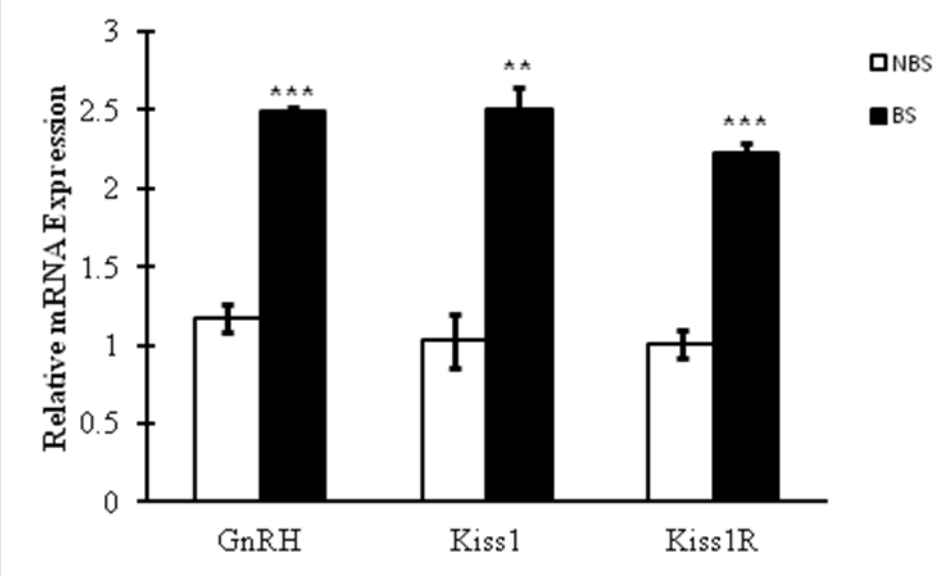


Figure 3.4: The relative mRNA expression of GnRH, Kiss1 and Kiss1r in the adult male rhesus monkey MBH (n=3) quantified using RT-qPCR. GnRH (***$p<0.0001$), kisspeptin (**$p<0.01$) and Kiss1r (***$p<0.0005$) expression was increased during the breeding season.

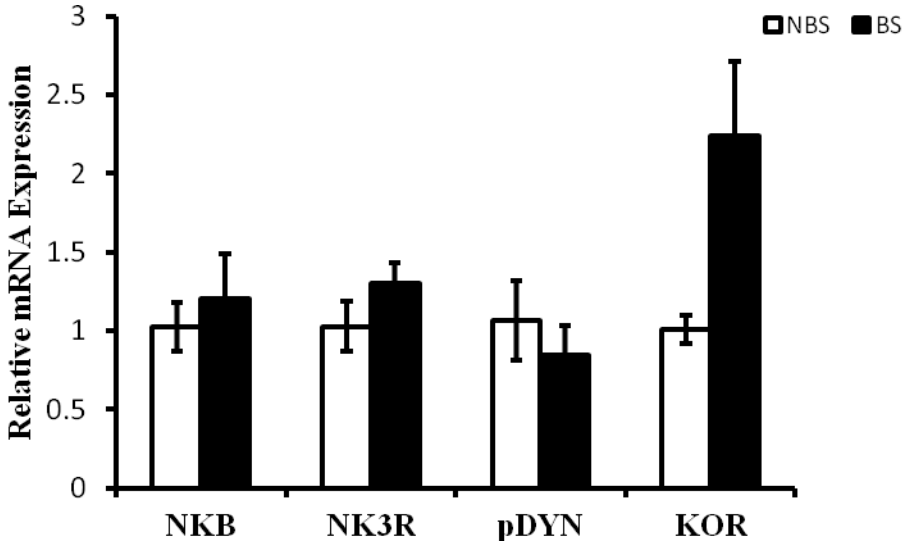
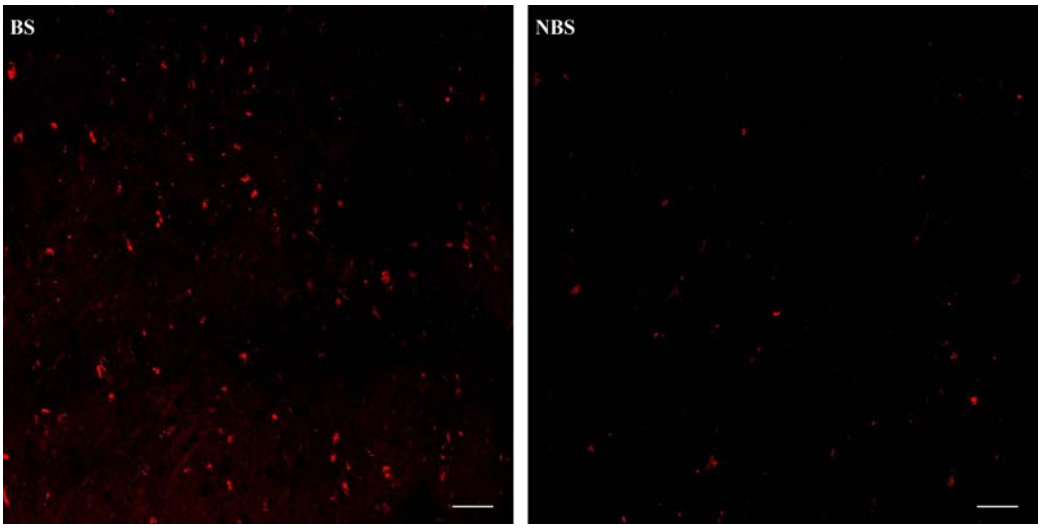


Figure 3.5: The relative mRNA expression of NKB, NK3R, pDyn and KOR in the adult male rhesus monkey MBH (n=3) quantified using RT-qPCR. No observable difference in NKB, pDYN, NK3R and KOR mRNA expression occurs during both seasons.

A



B

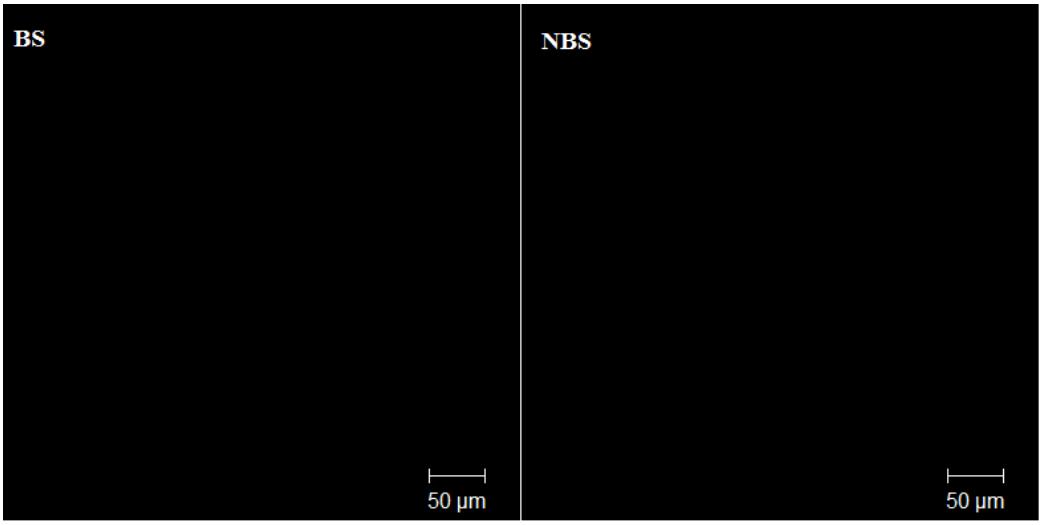


Figure 3.6: Photomicrograph shows kisspeptin ir perikrya in the breeding and non breeding season in the ARC of the adult male rhesus monkeys (A). Antibody omitted control for the breeding and non breeding season (B). The scale bar is 50 µm.

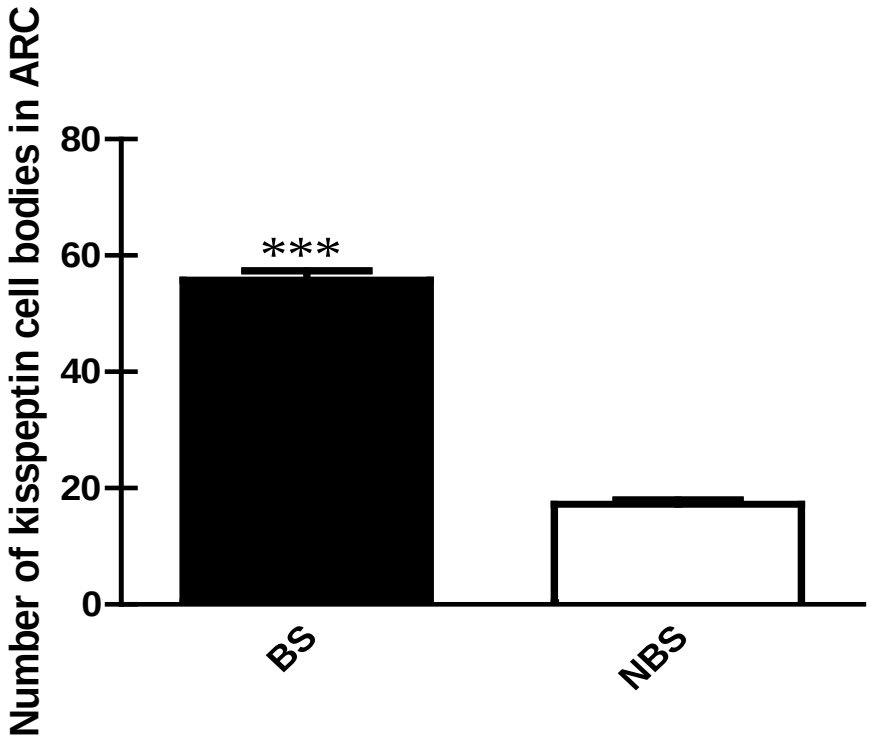


Figure 3.7: Semi quantitative data of kisspeptin cell bodies count in the ARC. The unpaired t - test was applied to compare the mean ± SEM of number of kispeptin cell bodies in an ARC (***p<0.0001).

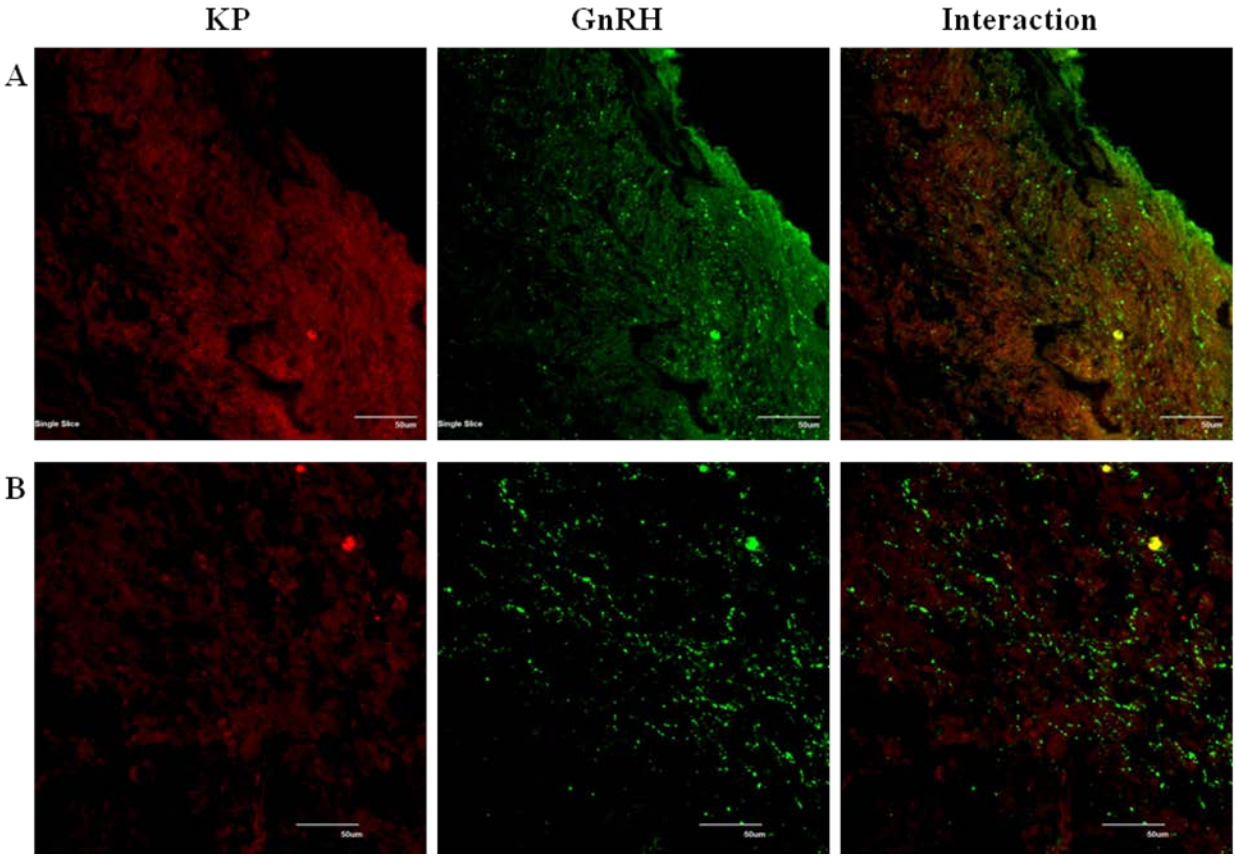


Figure 3.8: Kisspeptin-ir fiber and GnRH ir fibers expression and there colocalization as observed in the ME of adult male rhesus monkeys. Photomicrograph at 40x magnification is shown here. The arrows indicate the area which is magnified. Kisspeptin ir-fibers and the interaction with the GnRH ir-fibers was observably increased in in the ME during the breeding season (A) , while no observable difference was observed in the non breeding season (B).

No change in the number of GnRH cell bodies was observed during both breeding and non breeding season (Figure 3.9). The kisspeptin and GnRH cell to cell interactions were increased in the breeding season as compared with the non breeding season (Figure 3.10, 3.11).

The kisspeptin receptor expression was also increased in the breeding season. Further Kiss1r expression specifically on the GnRH neuronal cell bodies was also increased during the breeding season (Figure 3.12). Semi quantitative data on the number of Kiss1r, GnRH, Kiss1r positive GnRH cell bodies and contacts between kisspeptin and GnRH cell bodies are shown in Figure 3.13.

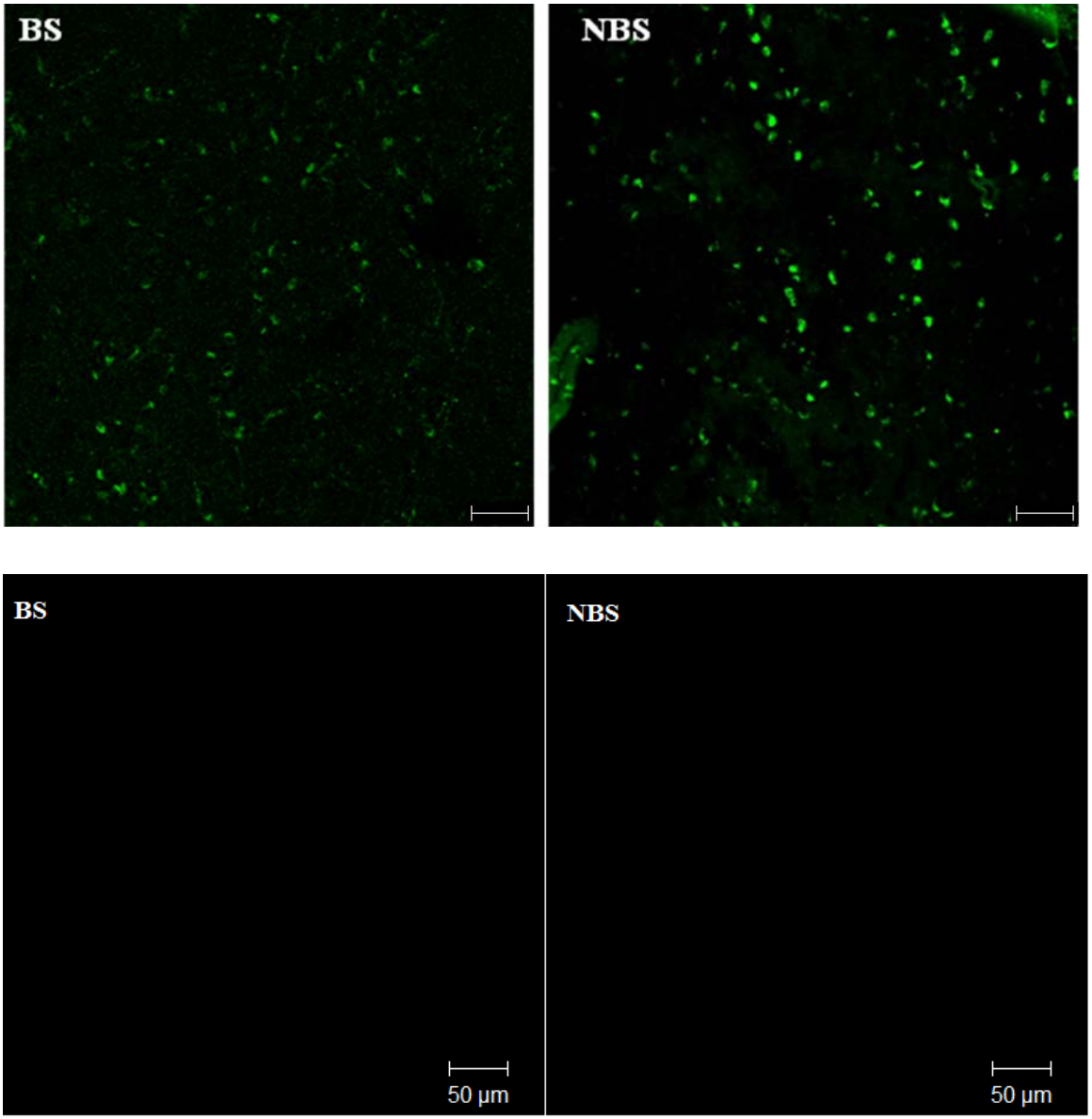


Figure 3.9: GnRH-ir cell bodies as observed in the MBH of adult male rhesus monkeys during breeding season and non breeding season. Controls for GnRH immunoreactivity are also shown for both seasons. No observable change was noticed in the GnRH ir cell bodies in the breeding and non breeding season.

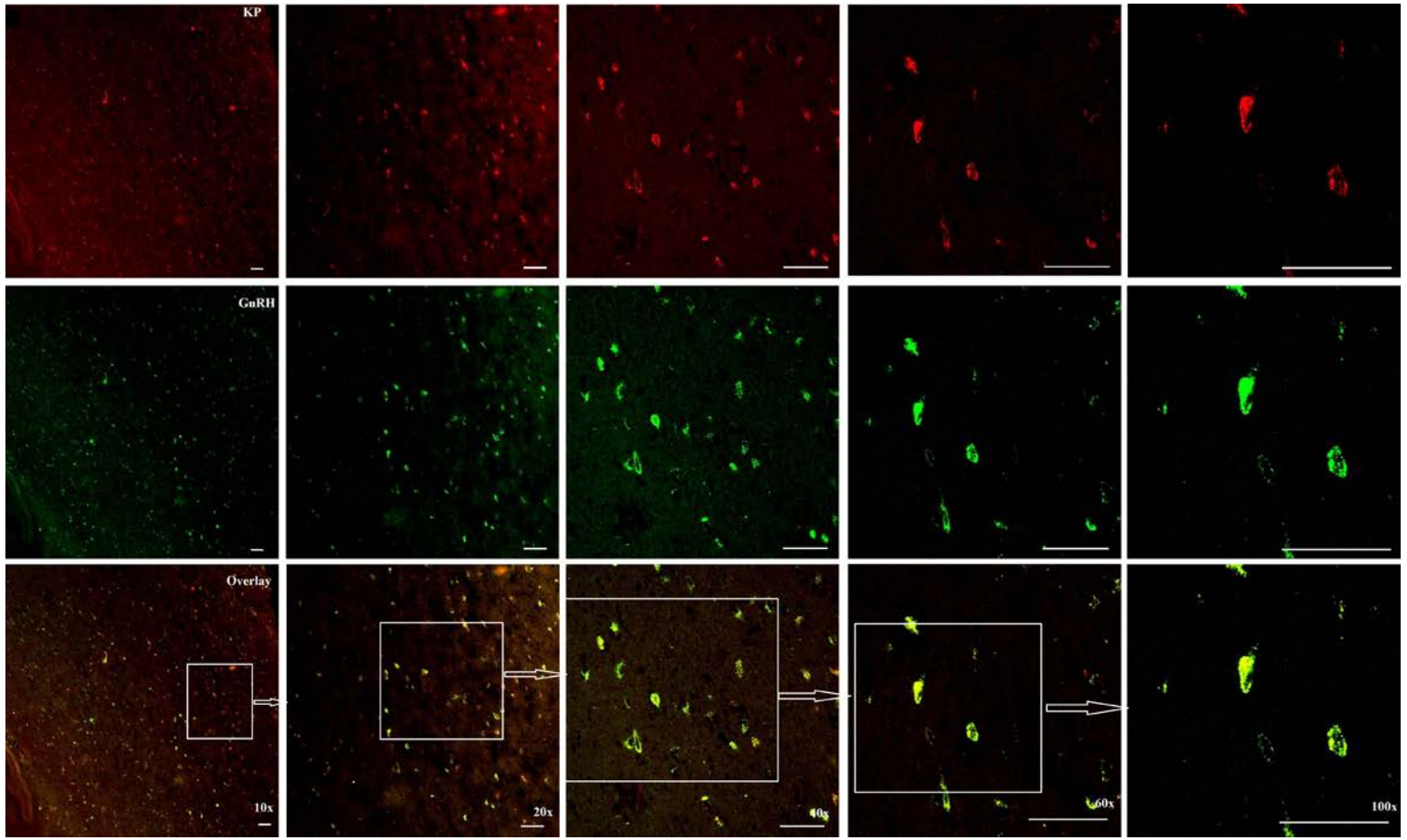


Figure 3.10: Photomicrograph at 10x, 20x, 40x, 60x and 100x (left to right) showing the observed kisspeptin ir cell bodies, GnRH ir cell bodies and their interaction in the MBH of the adult male rhesus monkeys. The kisspeptin ir cell bodies and also the interaction of kisspeptin and GnRH ir cell bodies were increased during the breeding season.

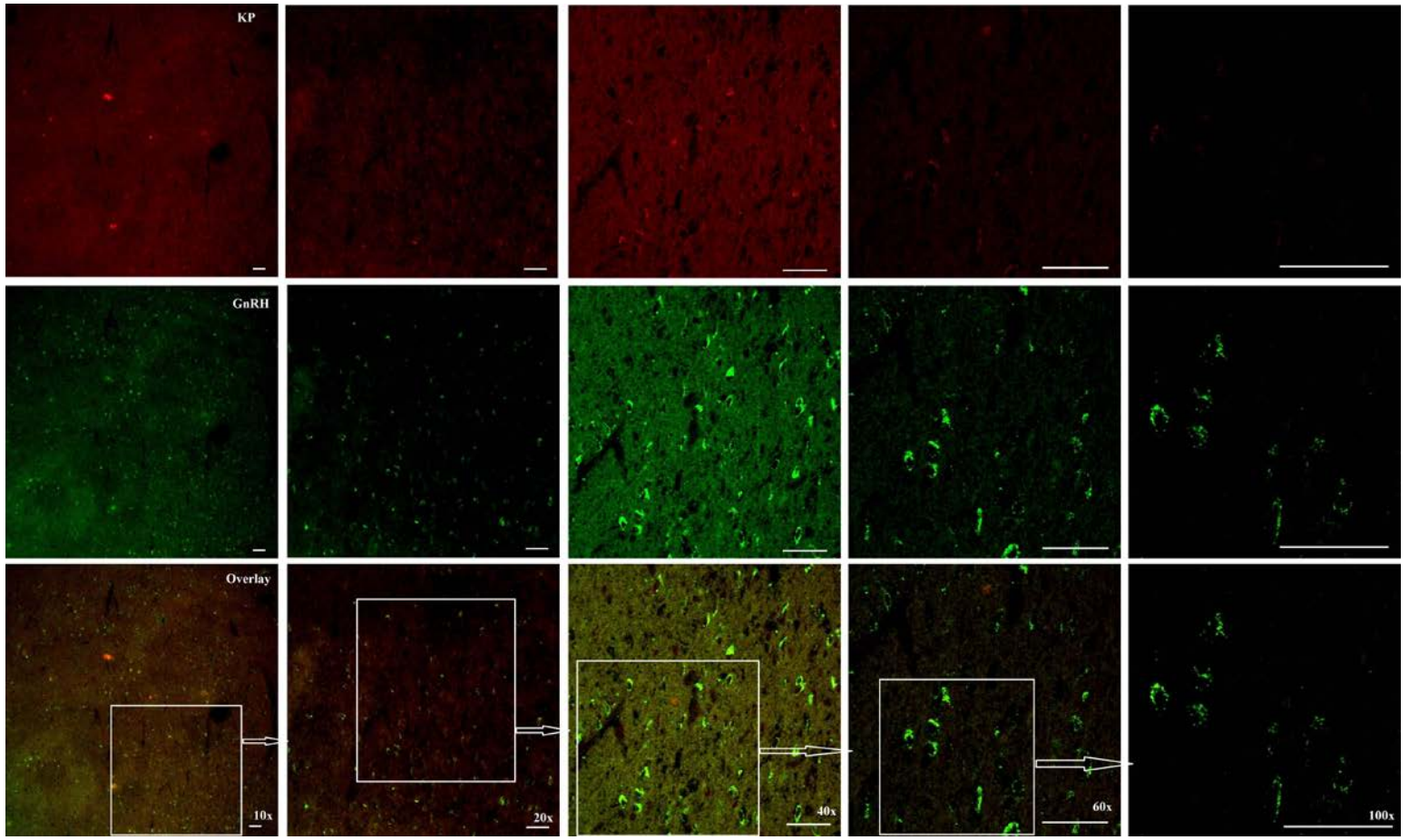


Figure 3.11: Observations as described in Figure 3.10. The kisspeptin ir cell bodies and their interaction with the GnRH ir cell bodies was decreased during the non breeding season.

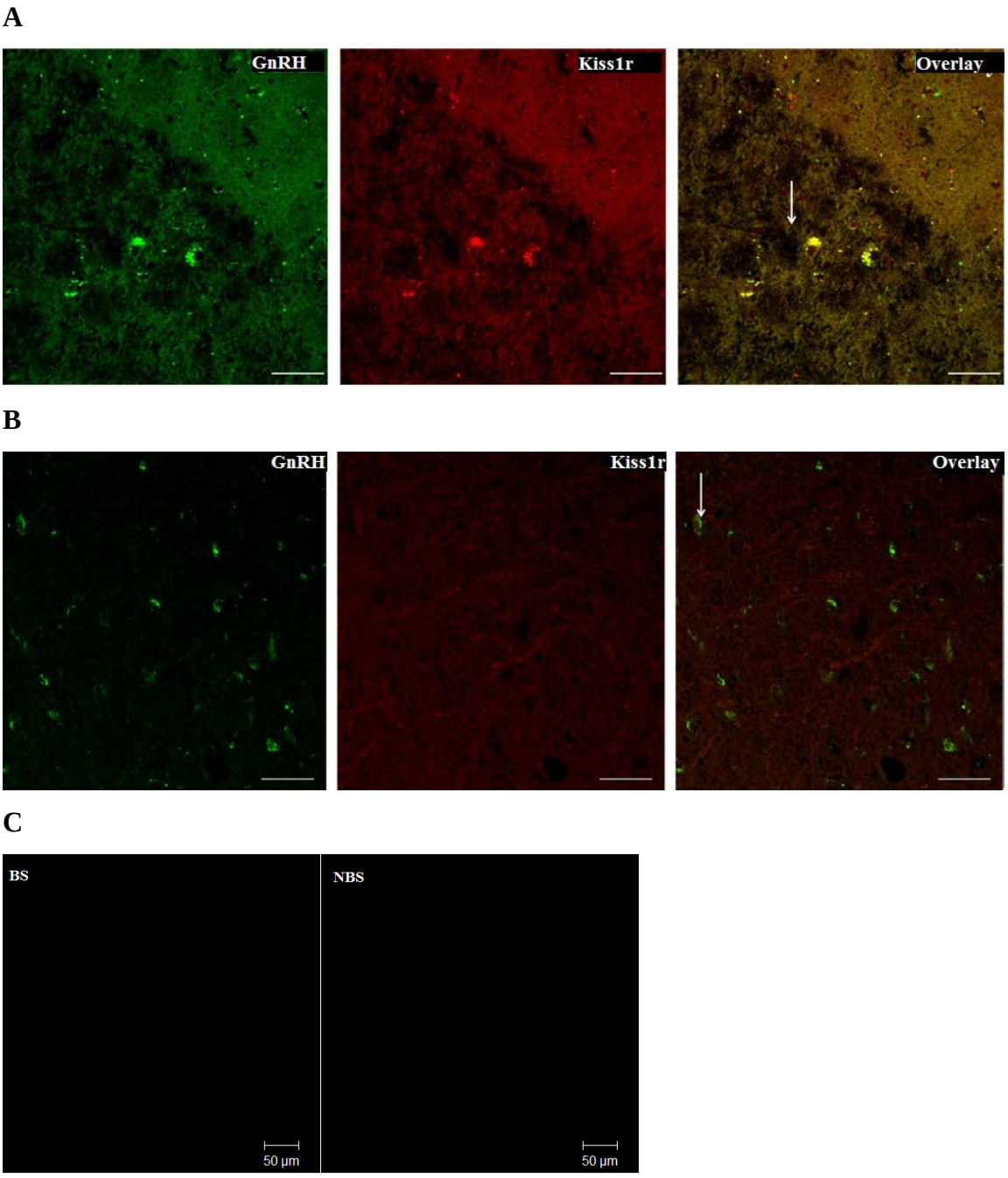


Figure 3.12: Photomicrograph showing the GnRH, Kiss1r immunoreactivity and their interaction in the MBH of the adult male rhesus monkeys during breeding season (A) and non breeding season (B). The Kiss1 receptor expression and its interaction with GnRH neuronal cell bodies was observed to be increased in the breeding season while no observable expression was observed during non breeding season. Antibody omitted controls are also shown for kiss1r. Controls for both breeding and non breeding seasons (C).

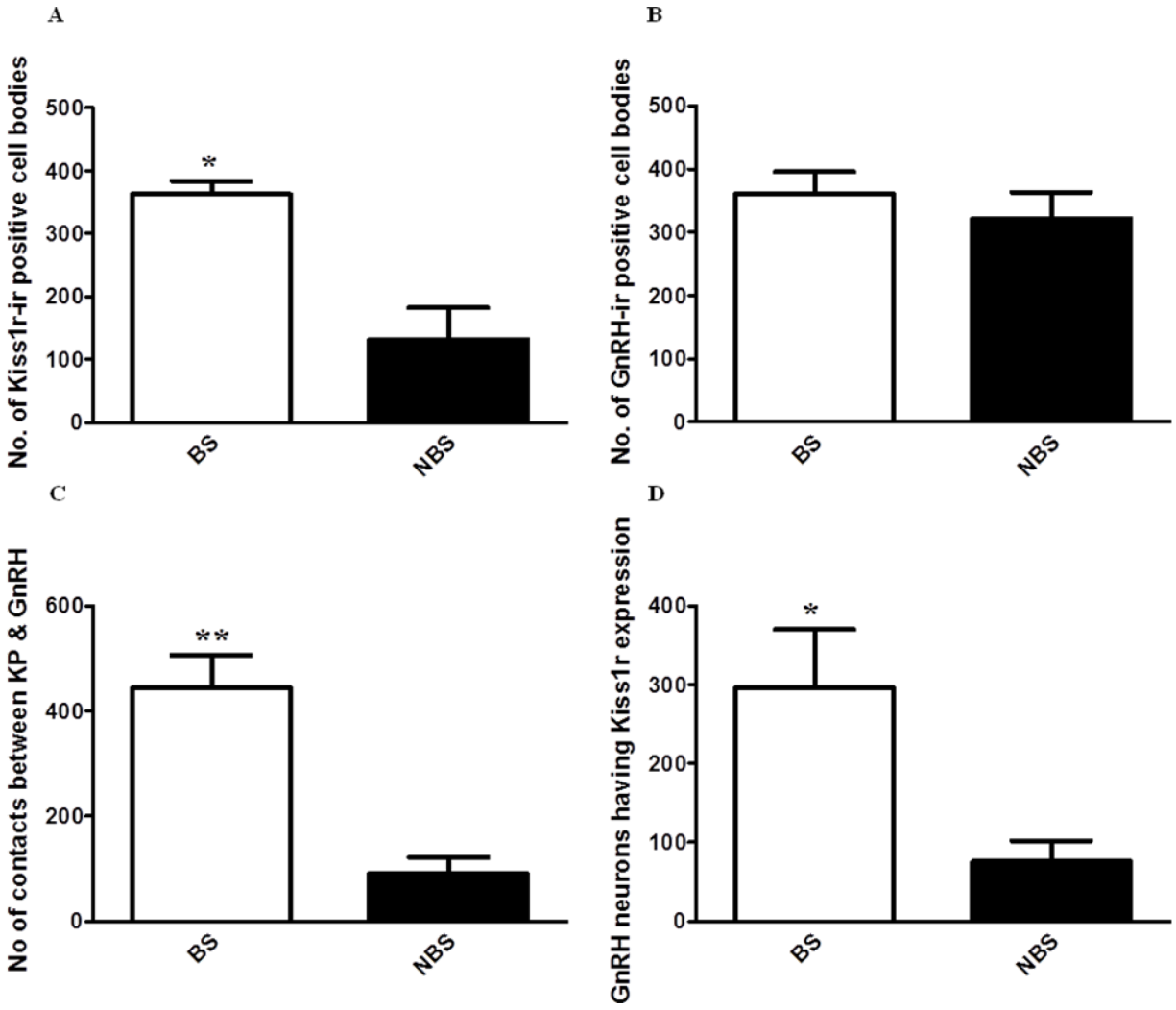


Figure 3.13: Data presented is mean ±SEM values of A) No of kiss1r-ir cell bodies, B) No of GnRH positive cell bodies. C) No of contacts between kisspeptin and GnRH D) GnRH neuronal cell bodies expressing kiss1r.

3.5. Discussion

Seasonal reproduction involves complicated neuroendocrine processes of the hypothalamo-pituitary-gonadal axis. It is dominantly regulated by photoperiod, a crucial environmental cue. GnRH, kisspeptin and Kiss1r are well known to play a role in regulating the reproductive activity in various mammals and non mammals (Rosweir et al., 2009). Since the discovery of reproductive roles of kisspeptin in 2003 (Seminara et al., 2003), substantial amounts of efforts has been put in to discover its role in the seasonality of reproduction. In recent years, it has been found that Kiss1/Kiss1r system, which influences GnRH secretion avidly, is regulated by both melatonin and the feedback action of gonadal steroid hormones (Revel et al., 2006b). Consequently, Kiss1/Kiss1r system may play a key role in seasonal reproduction (Huang et al., 2011). The kisspeptin signaling is reported to play a role in passing photoperiodic and environmental signals to increase the reproductive activity during the breeding seasons in hamster and sheep (Ansel et al., 2010; Revel et al., 2006a; reviewed by Clarke et al., 2013). The present study describes the qualitative and quantitative expression of the kisspeptin and Kiss1r changes during the breeding and non breeding season in adult male rhesus monkeys.

We observed the increase in the peripheral release of testosterone during the breeding season compared to the non breeding season along with an increase in the paired testis weight of the rhesus monkeys. Our observations are consistent with the previous data in several seasonal breeders (Pelletier et al., 1982; Revel et al., 2006b; Chiver et al., 2014). In this study, we for the first time report an increase in the central release of the metastin like-or during the breeding season as compared to the non breeding season. Kisspeptin signaling plays key role in regulating the reproductive activity. These findings indicate seasonal transition of the monkeys from the non breeding to the breeding season, leading to an increase in the reproductive activity.

An important finding of this study was the observation of the season related changes in the expression of kisspeptin and its receptor in the MBH of the adult male rhesus monkeys. A significant increase was noted in the mRNA expression of the Kiss1 and Kiss1r during the breeding season as compared to the non breeding season. This observation about the increased Kiss1 mRNA seems to be in line with the previous

observations in other seasonal breeders like Syrian hamsters, sheep soya ewes (Revel et al., 2006a; Smith et al., 2007; Wagner et al., 2008) but is in contradiction with the observation in the Siberian hamsters (Greives et al., 2007; Mason et al., 2007). We observed an increase in the Kiss1r mRNA expression during the breeding season in the rhesus monkeys these results are contradictory with the data observed in sheep (Smith et al., 2007; Li et al 2012). This change in the observation can be due the species differences observed. The GnRH mRNA expression was also increased in the breeding season as compared with the non breeding seasons.

A significant increase in kisspeptin perikarya was also noted in the ARC of the adult male rhesus monkey during the breeding season compared to the non breeding season. It is interesting to note that increased MBH Kiss1 mRNA and number of ARC kisspeptin cell bodies, was also associated with the elevated CSF metastin like-ir levels during the breeding season. Together, these reflect increased kisspeptinergic activity in the hypothalamus of breeding season monkeys. This finding is supported by the previous study done in sheep which described that the Kiss1 mRNA expression is regulated by seasons (Smith et al., 2007). Another important finding of this study was the observation of an increase in the number of close contacts between kisspeptin and GnRH cell bodies during the breeding season as compared to the non breeding season. This observation is in agreement with previous observation in rodents (Clarkson and Herbison, 2006) and sheep (Clarkson et al., 2009).

In the current study, we also quantified the expression of Kiss1r on the GnRH neurons for the first time during different seasons in rhesus monkeys. There was a marked elevation of the expression of Kiss1r on the GnRH neurons during the breeding season compared to the non breeding season leading to activation of the GnRH neuronal system and an increase in the reproductive activity. This result is, however, contrary to what was observed in ewe, the Kiss1r expression on the GnRH neurons was decreased during the breeding seasons (Li et al., 2012). This difference can be due to the different technical approaches or the species variation that in the nonhuman primates the Kiss1r expression is differently expressed than in the ewe.

The kisspeptin perikarya contacts with the GnRH neurons were also increased during the breeding season, this observation is consistent with the previous observation

in sheep (Smith et al., 2008; Smith, 2012). Overall, the above mentioned observations indicate that the connectivity of the Kiss1 and Kiss1r receptor was increased during the breeding season. This is very strong and convincing evidence that kisspeptinergic signaling plays a very important role in the seasonal variation in the rhesus monkeys.

Recently, NKB, pDyn and kisspeptin were found to be localized as sub population of neurons in the human ARC (Rance , 2009). It has also been demonstrated in other species like sheep (Foradori et al., 2006) and ewe (Goodman et al., 2007). These three neuropeptides are secreted by this subpopulation of neurons. In this study, we are reporting for the first time the effect of seasons on the NKB, pDYN and their NK3R and KOR respectively. We semi quantified the mRNA expression of NKB, pDYN, NK3R and KOR. We did not observe any significant change in the relative mRNA expression during both seasons. Our data about the pDYN is contrasting with previously studied in ram it was reported that pDYN was decreased in the breeding season (Scott et al., 2008). This difference in result could be due to species differences. Previously the regulation of these peptides by the seasonal changes has not been studied much. In Syrian hamsters, NKB DYN along with Kiss1 increased during the short day period in the ARC but decreased in the dorsomedial hypothalamus during the short day period (Bartzen-Sprauer et al., 2014). This indicates that NKB and DYN expression is photoperiod dependent in seasonal rodents but not in rhesus monkeys. The NKB/DYN/kisspeptin neurons express the NK3R and KOR the high affinity receptors for NKB and pDYN (Krajewski et al., 2005; Navarro et al., 2009). Recently, it has been documented that Kiss1 neurons in the ARC of male mice are nearly devoid of KOR (less than 6%; Navarro et al., 2011), while females displayed a slightly greater percentage of colocalization (~20%; Navarro et al., 2009), demonstrating the possible presence of a minor subset of Kiss1 neurons in the ARC expressing KOR There is possibility that NKB and pDYN are playing auto-regulatory role to control their own secretion. NKB overcomes the inhibitory effect of the pDYN and positively regulate the GnRH secretion along with kisspeptin (Figure 3.14). The GnRH neurons express both kiss1r and NK3R (Irwig et al., 2004; Krajewski et al., 2005) but do not express KOR (Mitchell et al., 1997; Sannella and Petersen, 1997). So, it is possible NKB might limit GnRH release while acting on GnRH fiber.

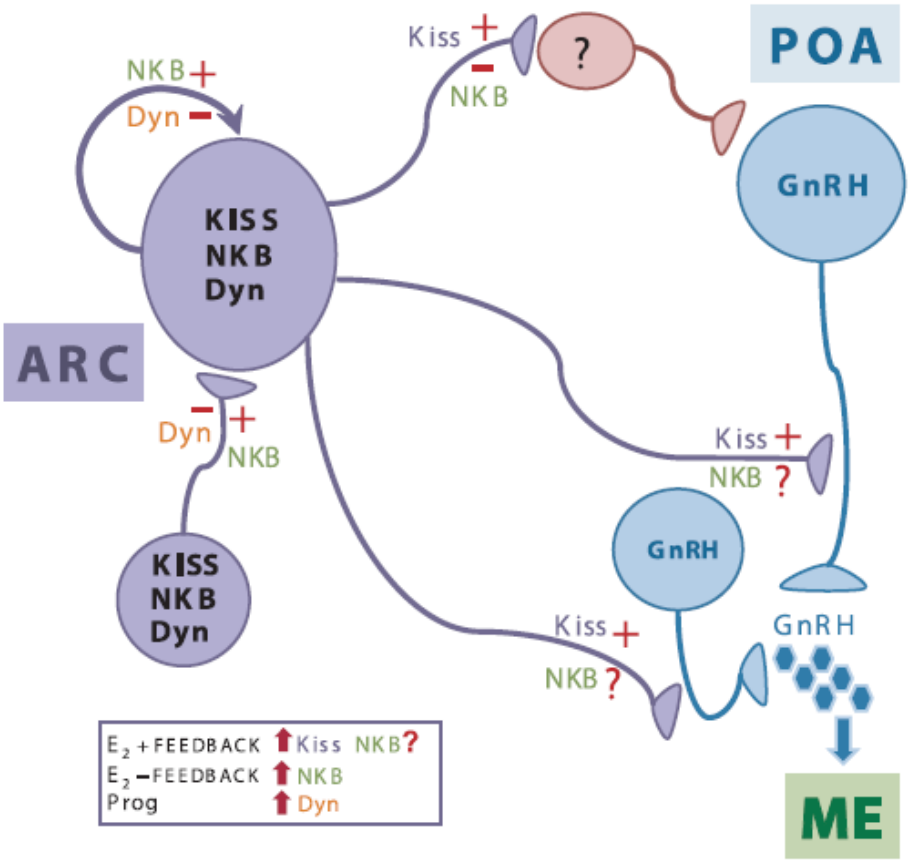


Figure 3.14: Schematic presentation of the NKB neurons that co express kisspeptin and Dyn in the ARC and their hypothetical role in GnRH secretion in the ME (Lasaga and Debeljuk et al., 2011).

In addition to activating kisspeptin neurons, NKB also acts on the GnRH nerve terminals in the median eminence. While the kisspeptin activates the GnRH terminals. Navarro et al. (2009) also presented the same idea. Over all, our data suggest that the NKB, pDYN and their receptor NK3R and KOR do not change under the effect of seasons in nonhuman primates.

All the foregoing findings of this study provide an important clue towards the seasonal switch of the HPG-axis in adult male rhesus monkeys. A possible mechanism for the transition between breeding and non breeding seasons can be inferred from these observations. Onset of breeding season in the adult male rhesus monkeys leads to an increase in Kiss1 and Kiss1r mRNA expression in the ARC, causing an increase in the central kisspeptin signaling. Among the seasonal species the kisspeptin expression appears different because of the kisspeptin synthesis is regulated by other seasonally regulated factors like leptin and sex steroids (Smith et al., 2008; Wagner et al., 2008; Quennell et al., 2009).These seasonal changes might be due to the increased negative feedback effect as the inhibitory effect of long term estrogen treatment on kiss1 mRNA and kisspeptin expression on ARC are greater during the non breeding season (Smith et al., 2008). However, what environmental/ neural/ neuroendocrine signals stimulate a seasonal increase in kisspeptinergic signaling remains to be elucidated. Kisspeptin cells in ovine do not express melatonin receptors (Li et al., 2011). There must be some interneurones or other factors which convey the photoperiod clues to the kisspeptin neurons and regulates their expression (Figure 3.15).

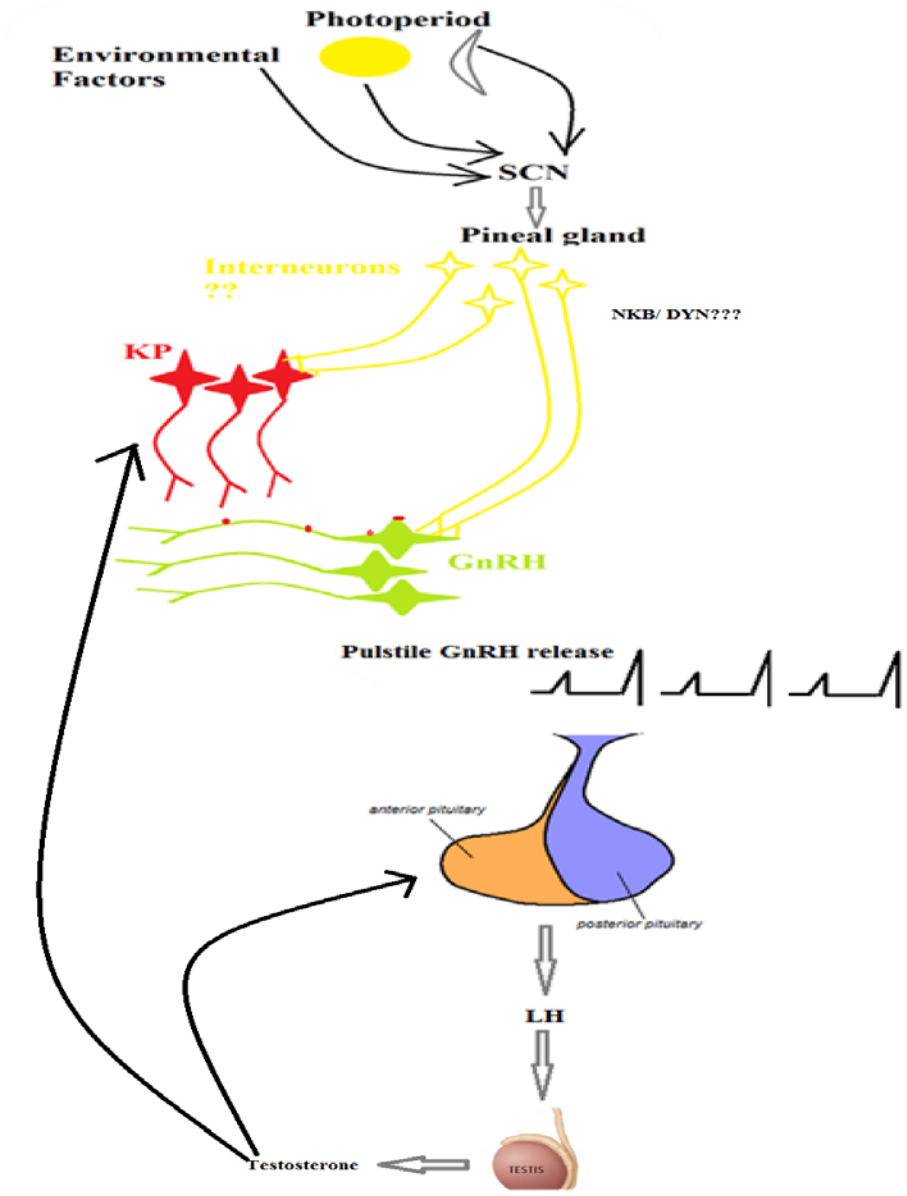


Figure 3.15: Schematic presentation of the photoperiodic regulation of kisspeptinergic signaling to regulate the hypothalamic pituitary gonadal axis.

There are various neurons, which act upstream to kisspeptin neurons. Kispspetin cells in the ewe do not express melatonin receptors (Li et al., 2011). So there must be some international network, which conveys these photopheriodic signals to kisspeptinergic system. The first possibility is that, RFRP neurons work upstream with kisspeptinergic system and its infusion, increase the kisspeptin expression in the ARC. So, possibly these may be the neurons, which convey the photoperiodic signals to kisspeptinergic systems (Simonneaux et al., 2013). The second possibility is that melatonin is directly acting on the pituitary as the pituitary cells do express the melatonin receptor (Hazlerigg, 2001). Melatonin most probably might be playing a role by regulating the negative feedback mechanism conveying signals back to hypothalamus and controlling kisspeptin expression and release (Figure 3.16). So from all this information we can say that Signals which control these seasonal changes in kisspeptin expression may include photoperiod acting via melatonin (Revel et al., 2006b; Chan et al., 2009) and food restriction (Paul et al., 2009). Expression of Kiss1r on GnRH neurons also increases during the breeding season leading to the augmented binding of the kisspeptin to its receptor. This ultimately results in activation of the GnRH neurons and the HPG-axis, which enhances the reproductive activity and leads to an increase in the testicular weight and plasma testosterone levels seen in breeding season.

To assure the validity of out immunocytochemical findings, several steps were taken during this study. Primary antibody omitted control sections were used to assess the nonspecific Kiss1, Kiss1r and GnRH like-ir. Absence of the discrete immunoreactivity showed a lack of nonspecific binding of the fluorescent labeled secondary antibodies.

In conclusion, the present study demonstrates the semi quantitative differences in the MBH expression of the kisspeptin mRNA and protein during the breeding and the non breeding seasons in adult male rhesus monkeys. The expression of hypothalamic Kiss1, Kiss1r and their close contacts with GnRH fibers were found to be higher during the breeding season.

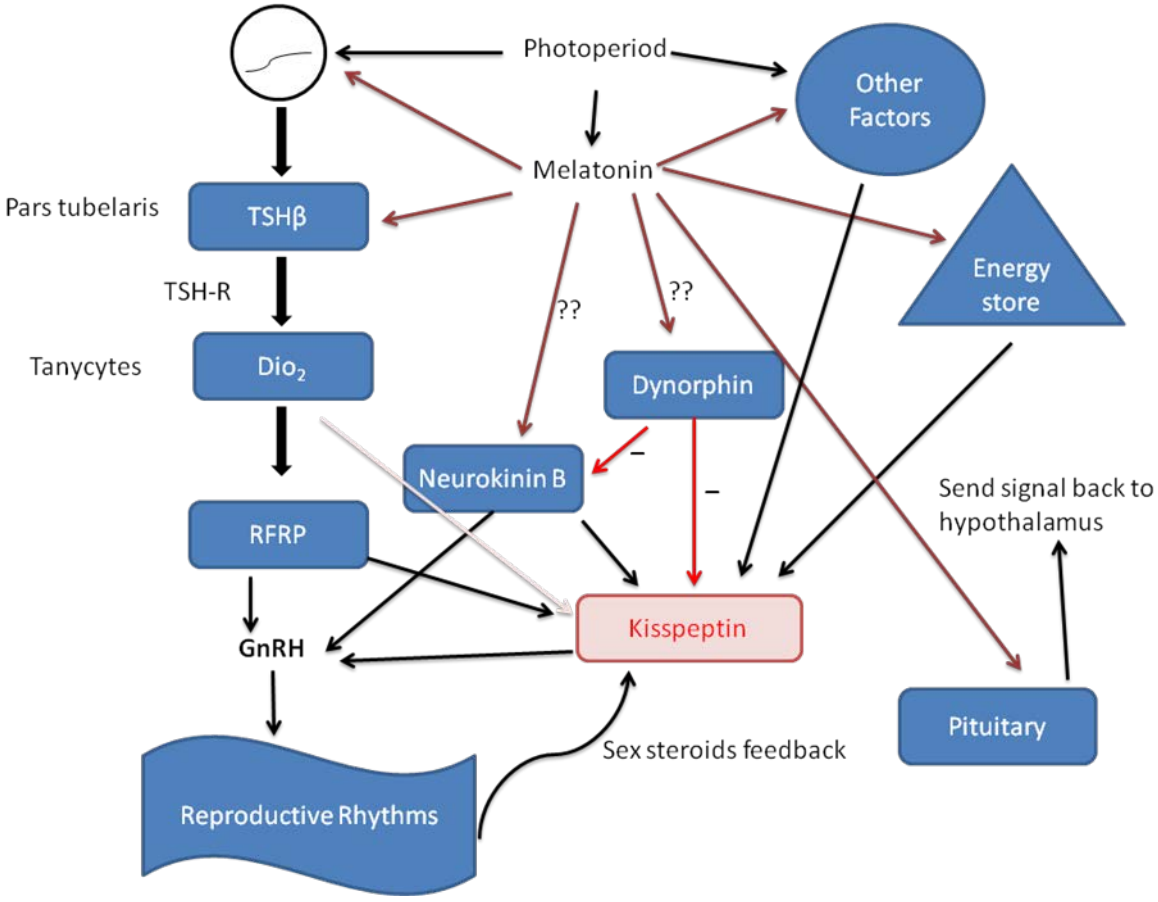


Figure 3.16: Schematic diagram presenting the possible photoperiodic pathways which may regulate the seasonal reproduction in the rhesus monkeys.

Further, the present study establishes the season related changes in the expression of kiss1r on GnRH neurons for the first time. Based upon these findings, we postulate that kisspeptin acts as an important arbitrator of the various environmental cues on the reproductive axis. It acts as an excitatory factor to enhance the GnRH pulse generating activity during breeding season and a decrease in kisspeptin signaling leads to commencement of the non breeding season in higher primates.

Chapter 4:Kisspeptin-10 Treatment Induces GnRH Expression in the Rhesus Monkey Derived Lyon RNCs

4.1. Abstract

Embryonic stem cells carry enormous potential in cell based therapies due to their unique ability of indefinite self-renewal and multi-lineage differentiation. Unfortunately, stem cell differentiation depends on specific cell signaling, and a lack of precise information limits the ability for researchers to move forward with developing embryonic stem cell models. For example, recently kisspeptin treatment was shown to cause dendritic extension / neurite growth of GnRH neurons in brain slices *in vitro,* while Kiss1-Kiss1r signaling has been shown to act as a potent regulator of the reproductive axis, as well as an elicitor of GnRH and gonadotropin secretion. Kisspeptins may then play major roles in reproductive axis and working upstream to GnRH. In the present study, we tested how different doses of kisspeptin-10 treatment led to specific neuronal differentiation among. At the early rosette stage, we administered 0.1, 1, 10, 100 nM of kisspeptin-10 and ddH_2O as a control to rhesus macaque derived tau GFP-Lyon ES cells. The results of our analyses showed that kisspeptin-10 had an anti-proliferative effect on the Lyon ES cell line leading to a differentiation state and morphological changes consistent with neuronal stem cell development. Moreover, the Lyon-ES stem cell line differentiates into GnRH-neuronal types based on kisspeptin signaling, a novel finding consistent with recent connections observed between kisspeptin signaling and GnRH neurons observed in some congenital disorders, such as idiopathic hypogonadotropic hypogonadism. Future transplantation studies among rhesus macaques may offer *in vitro* verification on the effectiveness of these cells grown *in vivo* with kisspeptin-10 administration.

4.2. Introduction

Embryonic stem cells (ESCs) are unique in that they are capable of indefinite self renewal and multi-lineage multifunctional segregation. (Evan and Kaufman, 1981, Martin et al., 1981, Svendsen et al., 1999; Li et al 2005, Kuai et al., 2009), allowing them to differentiate into numerous cell types, *e.g.* hematopoietic, epithelial, neuronal, vascular, cardiac muscles, smooth muscles, condrogenic and adiponergic lineages (Czyz and Wobus, 2001). Accordingly, ESCs have become a target research as well as a valuable molecular biology research tool allowing greater insights into many fields, including developmental toxicology, and pharmacology (Wobus et al., 2011, de Peppo et al., 2012, Byrne et al., 2006). One particular area in which ESCs may soon play a critical role is development of novel cell-based therapies which may be able to treat a variety of previously untreatable diseases and disorders. While such therapeutic are theoretically possible, to date, there are no approved treatments employing ESCs, both as a result of ethical concerns as well as inherent difficulty attached in working with cells. Being pluripotent, ESCs requires specific signals to control cell differentiation. If these signals are not precisely controlled, ESCs can continue to uncontrollably differentiate into many different types of cells and in medical treatments, cause correspondingly severe complications. The real challenge of working with ESCs then becomes learning how to control cell differentiation.

In exploring the relationship between signaling and ESCs differentiation, there are some neurological disorders which may serve as excellent starting points to explore the basic possibilities of an ESCs-based treatments as well as the current difficulties faced in using ESCs. For example, hypogonadism occurs when either male testes or the female ovaries produce little or no sex hormones (Yialamas et al., 2003), resulting in a variety of complications, including delayed or no onset of puberty, early onset of menopause, low-bone density, sexual dysfunction, or even infertility. While some forms of this disorder are treatable notably those where physical problem lies in the gonads other forms are less likely or even impossible to be treated (Kumar et al., 2010). A particularly difficult variant of hypogonadism, which has few treatment options is hypogonadotropic hypogonadism (HH; also known as secondary hypogonadism), which occurs when the underlying cause of hypogonadism originates

in the brain, at hypothalamus or pituitary level (Styne et al., 2011). In hypothalamus, gonadotropin-releasing hormone (GnRH) is synthesized and then released by GnRH neurons (Krsmanovic et al., 1993). GnRH plays major role in reproduction and sexual function (Mead et al., 2007). During childhood, GnRH level is low, which is associated with low reproductive function (Ritzen, 2003). Deficiencies in GnRH can then accordingly lead to a type of hypogonadotropic hypogonadism known as idiopathic hypogonadotropic hypogonadism (IHH), which is accompanied by a spectrum of olfactory sensing phenotypes ranging from complete anosmia (i.e., Kallmann Syndrome) to normosmia where the sense of smell remains intact.

Different studies have proposed several underlying mechanisms involved in IHH and its characteristic olfactory phenotypes, *e.g.*, aging (Araujo et al., 2004; Wang et al., 2009; Bhasin et al., 2010), neurodegenration (Margolin et al., 2003), as well as improper / no migration of GnRH neurons during embryonic stages (Gibson et al., 1994). However, there is no agreement on a single cause. Earlier studies found that some forms of IHH result from dysfunction of GnRH neurons that have developed and migrated properly (reviewed by MacColl et al., 2002). Likewise, a genetic investigation into olfactory spectrum that accompanies IHH noted that rather unique patho-physiological association between IHH and impaired olfaction resulted from a defect in olfactory and GnRH neurons, which originate from an undefined stem cell population within the embryonic olfactory placode (Lewkowitz-Shpuntoff et al., 2012). Lewkowitz-Shpuntoff et al. (2012) reported association between several gene sequences and different phenotypes of IHH including *KAL1* (anosmin-1, MIM: 308700), *GNRH1* (GnRH 1, MIM: 152760), *GNRHR* (GnRH receptor, MIM: 138850), and most interestingly, *KISS1 (*KISS-1 metastin, MIM: 603286) and *KISS1R* (KISS1 receptor, MIM: 604161).

The proteins controlled by Kiss1 gene, kisspeptins (Lee et al., 1996; Ratnasabpathay et al., 2013) are endogenous ligands for the G-protein coupled receptor GPR54, also known as hOT7T175 or AXOR12 (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). The C-Z terminal end of kisspeptin peptides binds and activates Kiss1r signaling (Kotani et al., 2001), which has a number of downstream effects. Roseweir et al. (2009) discovered a potent antagonist of kisspeptin receptor,

revealing that kisspeptin play a crucial role in reproduction an association similar to those of GnRH noted in numerous studies on IHH. Further studies on monkeys, rats and mice were done to check the role of central administration of P234 and it was found to reduce pulsatile GnRH secretion in female pubertal monkeys and it also inhibited the kisspeptin-induced release of luteinizing hormone (LH) in rats and mice and blocked the post-castration rise in LH in sheep, rats, and mice (Roseweir et al., 2009). The peripheral administration of P234 also inhibited kisspeptin dependent adiponectin release (Huma et al., 2014).

The role of Kiss1 /Kiss1r signaling is well known with regards to the functional analysis, qualitative and semiqualitative studies in different animal species. In recent few years scientists are focusing to know the role of this signaling on different stem cell lines. For example, Kiss1/ Kiss1r signaling appears to beinvolve in cell growth and differentiation. In R366.4 cell line kisspeptin treatment has been reported to play anti-proliferative role and leads to differentiation of cells to more specific cell types, consistent with the morphological changes (Huma et al., 2013). In some studies kisspeptin treatments have been reported to play a role in the GnRH nitrite growth *in vitro* (Fiorino and Jasoni, 2010). However, the activation of Kiss1r by kisspeptin has been shown to inhibit cell motility, proliferation, invasion, chemotaxis and metastasis (Ohtaki et al., 2001; Kotani et al., 2001).

While the anecdotal overview of IHH is tangential, the aims of the present study, it does highlight the type of complex relationships at work between cell signaling and cell differentiation a key problem in developing novel ESC-based cell therapies. In the present study, we used kisspeptin drug treatment on a particular ESC line (Lyon ES cell line) to examine the effect of kisspeptins on stem cell growth and differentiation, as well as some of the mechanisms at play in neuronal cell fate. Though a variety of intrinsic and extrinsic clues have been implicated as playing potentially important roles in regulating neuronal stem cells (McKay, 1997; Okano et al., 2002), till now the intrinsic factors, such as, proneural neurogenins (Sommer et al., 1996; Sun et al., 2001) and b-HLH transcription factors, have been more clearly established as playing a role in acquisition of a neuronal fate (Nieto et al., 2001). Likewise, transcription factor Lmx1 is believed to be sufficient and is required for

formation of dopaminergic neurons (Andersson et al., 2006). A number of transcription factors that efficiently induce astrocytic and oligodendrocytic fate are also known, which include Olig1 and 2, Hes,15, and Ngn3 (Kageyama et al., 2005; Rowitch et al., 2010). However, no extrinsic factor has been yet described. Previously, kisspeptin was shown to increase the GnRH mRNA and protein expression in GT1-7 cell line in a concentration dependent manner (Terasaka et al., 2013). Kisspeptin also increased GnRH expression in GT1-7 cell lines (Sukhbaatar et al., 2013). It then stands to reason that there might be a possibility that kisspeptins play role in inducing GnRH expression in pluripotent cells. In present study we evaluate the affect of different exposure times and doses of kisspeptin-10 on Lyon ES stem cell differentiation by treating GFP labeled Lyon ES cells with kisspeptin-10 to measure the proliferation, differentiation and morphological changes to the cells.

4.3. Materials and Methods

4.3.1. Lyon ES Cells

To date, ESCs have been derived or isolated from embryo of mouse (Evan and Kaufman, 1981; Martin et al., 1981; Li et al 2005), humans (Thomson et al., 1998), and non-human primates (Thomson et al., 1995). As a potential precursor to future primate-model studies, we used monkey derived tau GFP-Lyon ES cells (gifted by the Lyon Stem Cell Research Institute). Lyon ESCs were cultured in Knockout-DMEM (Gibco, Invitrogen China Limited, Beijing, China) containing 20% knockout serum replacer (KO-SR:Invitrogen China Limited, Beijing, China) supplemented with 1% nonessential amino acids (NAA) (Invitrogen China Limited), 0.1 mM β-mercaptoethanol (β-ME) and 5 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen China Limited). Mechanical passaging of undifferentiated colonies was performed manually every 5-7 days by cutting the colonies into large clumps using a flamed-pulled Pasteur pipette and then plating them onto a feeder layer of mitomycin-C-treated mouse embryonic fibroblasts (MEF, CF-1, ATCC). Mouse embryonic fibroblast cells (CF-1) were grown in DMEM with 4 mM L-glutamine supplemented with 15% fetal bovine serum (FBS) (Invitrogen China Limited).

4.3.2. Formation of Embryoid Bodies (EBs)

We used adherent monolayer protocol, established by Ying et al. (2003), which was further modified by Conti et al. (2005). In brief, embryonic stem cell (ESC) colonies were digested with dispase (Invitrogen China Limited, Beijing, China; 1 mg/ml) at 37 °C for 5-8 minutes and washed with phosphate buffer saline (PBS: Invitrogen China Limited) to remove dispase, then suspended in the N/M medium (1 : 1 DMEM/F12; Gibco, Invitrogen China wqLimited, Beijing, China): Neural Basal Medium (Gibco, Invitrogen China Limited, Beijing, China) containing 1xN2 supplement, 1xB27 (Gibco, Invitrogen China Limited, Beijing, China) and 2 mM glutamine (Sigma-Aldrich China Inc. Shanghai, China). Cells were plated on 15 × 30 mm wells coated with agar (Sigma-Aldrich China Inc. Shanghai, China) and allowed

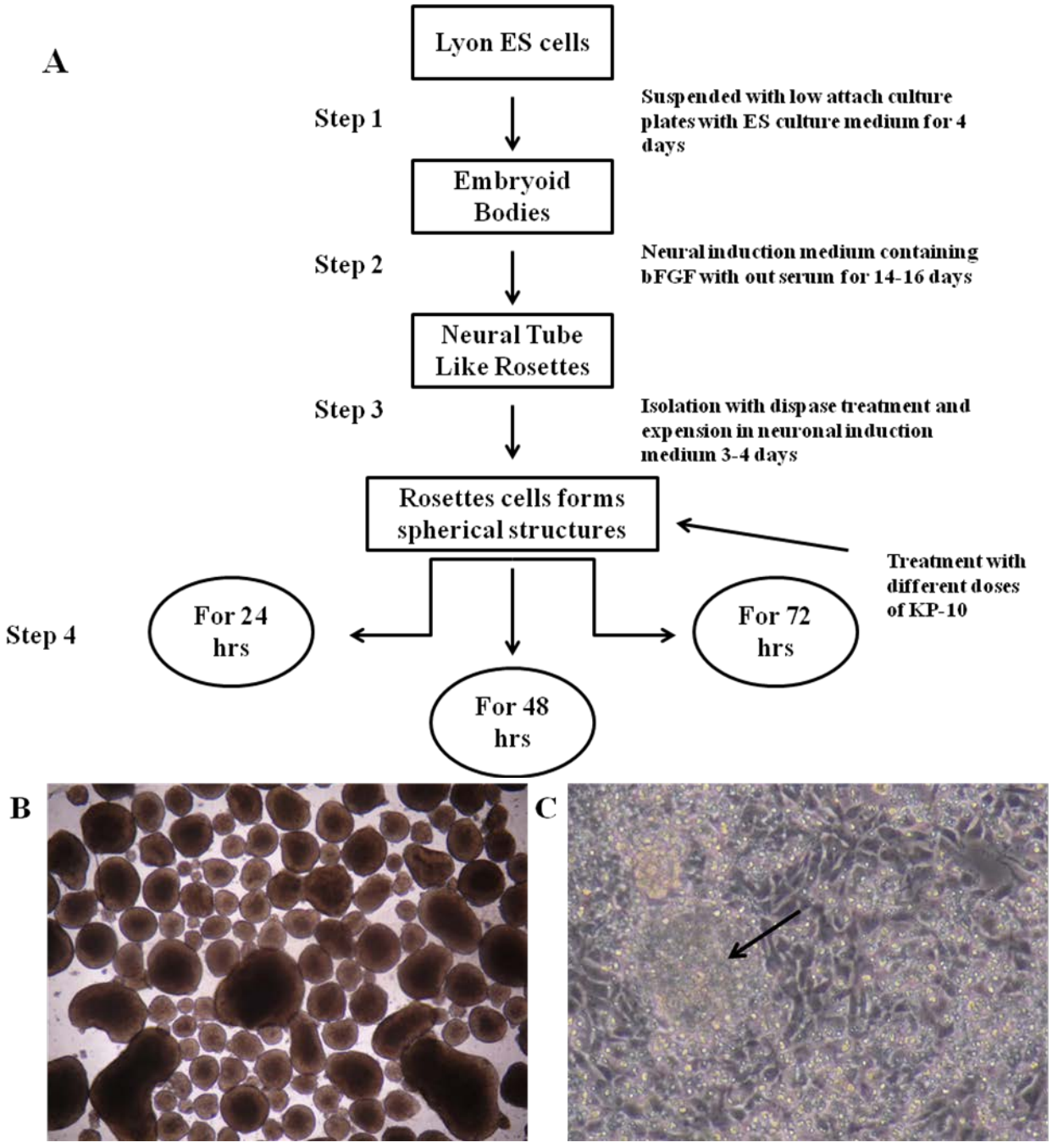


Figure 4.1: Diagrammatic presentation of experimental procedure (A). Photoplate of embryoid bodies (EBs), (B). Photoplate of early rosette stage (arrow) when kisspeptin-10 was added (C).

to aggregate for 4 days to form Embroid Bodies (EBs) (Figure 4.1B).

4.3.3. Neural Progenitor Cell Induction

After aggregation, EBs were selected and cultured in neuronal progenitor media (1:1 DMEM/F12: 1X ITS-x; Gibco), containing 2 ug/ml heparin and 2 mM glutamine in 4 well plates coated with extracellular matrix media (ECM; Sigma-Aldrich China Inc., Shanghai, China) for 3-4 days till rosettes appeared (Figure 4.1C). Rosette-neuronal stem cells (R-NSC) represent the first characterized neuronal stem cell stage at which cells are capable of responding to patterning cues that direct differentiation of different specific regions (Darmon et al., 1981). The detailed experimental design is presented in Figure 4.1A.

4.3.4. Kisspeptin-10 treatment

On day 12 (early rosettes stage - Figure 4.1B), cells were treated with vehicle (ddH_20), 0.1 nM, 1 nM, 10 nM and 100 nM kisspeptin-10 (Phoenix Biotech Co., Ltd., Beijing, China). The doses were decided based on findings of previous studies (Cho et al., 2009; Novaira et al., 2009; Fiorini and Jasoni, 2010; Milton et al., 2012; Huma et al., 2013; Terasaka et al., 2013). Media was changed daily and fresh drug was added immediately after changing the media. The treatment was done at intervals of 24 hrs, 48 hrs and 72 hrs.

4.3.5. Antibodies

Rabbit anti-Nestin (Millipore, Billerica, MA, USA; 1:200)), mouse anti-GFAP (Convance Inc., Beijing, China: 1 : 1000), mouse anti β-tublin-III (Millipore: 1:200) and mouse anti-GnRH1 (Sigma-Aldrich China Inc. Shanghai, China: 1:150)) were used as primary antibodies. Secondary antibodies used included, IgG-Texas Red (Santa Cruz Biotechnology Inc., Shanghai, China) for Nestin, and IgG1-Texas Red (Santa Cruz Biotechnology Inc.) for β-tublin-III, GFAP and GnRH. All experiments were repeated 3-4 times .

4.3.6. Cell and Rosette Count

Rosettes were counted manually under a light microscope (10X; CKX41; Olympus, Tokyo, Japan) every 24 hrs, up to 72 hrs after kisspeptin-10 treatment. Cells were counted at the same time points using the haemocytometric method at 20 X.

4.3.7. Flow Cytometry

Flow cytometry-side population assay was performed for GFP marked rhesus monkey neural progenitor cells were trypsinized and suspended in 1.9×10^6 cells/ ml in NPM (50% F12; Gibco), 50% neural basal medium (Gibco), 1X ITS-x (Gibco), 2 mM Glutamine (Gibco), 2 µg/ ml heparin (Sigma) and 50 units/ ml penicillin and streptomycin (Sigma). For cell proliferation and apoptosis (or cell death) assays, cells were treated either alone with 1 mg/ml propidium iodide (Sigma) for 10 minutes at 37 ºC. The cell suspensions were then analyzed with a flow cytometer (BD influx TM cell sorter, CA, USA).

4.3.8. Tunnel Assay

Cells were fixed in 24 well plates with 4% para-formaldehyde and apoptosis were measured with the In Situ Cell Death Detection Kit, POD (supplemented with 0.1 % sodium citrate), according to the manufacturer's instructions (Roche, Shanghai, China). Cells were then exposed to DNase I 10 min prior to the assay (BioLabs, Inc, Ipswich, Massachusetts, USA) to serve as a positive control. Terminal transfers were omitted to provide a negative control. Tunnel positive and the total number of cells were counted at four randomly selected locations in each plate well. The percentage of Tunnel positive to total cell count was calculated by using formula (tunnel positive cells/ total number of cells*100).

4.3.9. MTT Assay

MTT (3-[4,5-dimethylthiazol-2-yl] -2,5 diphenyl tetrazolium bromide) (Amresco, Solon, OH, USA) was used to test the viability of the Lyon ES cells (Sobottka et al., 1992). LYON-ES cells cultured in 96-well plates were incubated with

10 µl MTT (5 mg/ml) in a CO_2 incubator for 4 hrs. After that the medium was discarded using a suction pump and 100 µl dimethylsulfoxide (DMSO) was added to each well to dissolve the MTT Formosan crystals. The optical density at 570nm was then measured by the plate reader (BIO-RAD, Shanghai, China).

4.3.10. Morphological Changes

Immunocytochemical study was performed for four parameters. GFAP: Astroglia, the star shaped glial cells in the brain and spinal cord, many of which express the intermediate filament glial fibrillary acidic protein (GFAP) a marker for the astroglia (Baba et al., 1997; Venkatesh et al., 2013)**,** Nestin (a neural progenitor marker) (Tonchev et al., 2003)**.,** β-tublin-III**:** The neuronal marker (Ketsetos et al., 2003;Dehmelt and Halpain, 2005) and GnRH**:** kisspeptin has been reported to play major roles in reproduction and it is working upstream to the GnRH neurons (Mead et al., 2007).

Cells were fixed in 4% para-formaldehyde for 20 minutes and then washed with (0.01M) PBS 3-5 times for 5 minutes each. Cells were then treated with 0.4% triton X-100 for 15 minutes, followed by 3-5 times PBS washes, blocked with 5% BSA for 40 minutes, and then washed with PBS as before. Following this process, cells were treated with the primary antibody (mouse anti GFAP: Convance Inc, Beijing, China; 1 : 1000)), rabbit anti nestin (Millipore, Billerica, MA, USA; 1:200), mouse anti β-tublin-III (Millipore; (1:200)) and mouse anti GnRH1 (Sigma, 1:150)) then kept at 4°C overnight (12 hrs) in a sealed humidified black chamber. The cells were washed with PBS three times and incubated at room temperature on a shaker for 2 hours with the respective secondary antibodies, followed again by 2-3 times PBS washes in dark room. The cells were incubated with DAPI (4', 6-diamidino-2-phenylindole; used as counter stain for nucleus) for ten minutes(Bexell et al., 2009). Cells were observed at 488 nm Alexa flour for eGFP and 533 nm Texas Red for Nestin, GnRH, Tubulin and astrolgial marker glial fibrillary acidic protein (GFAP). Four random positions were selected for GFAP, nestin, β-tublin-III and GnRH labeled cell and total numbers of the cells bodies were counted manually under an Olympus FV1000 fluorescent microscope (Olympus America Inc., Melville, NY, USA). The

percentage of the respective positive cells were counted using the formula (positive cells/Total number of cells*100).

4.3.12. Statistical Analyses

Unless otherwise noted, repeated measured two-way ANOVA's followed by Bonferroni post hoc tests was performed for all data using Graphpad 5 (GraphPad Software, Inc, La Jolla, USA). Significance was set at $p<0.05$. In case of tunnel assay, the data were analyzed using one way-ANOVA and students t-test.

4.4. Results

4.4.1. Proliferation Rate, Number of Rosettes, Viability and Apoptosis rate of Lyon Neuronal Progenitor Cells

Administration of kisspeptin affected proliferation rate of the cells in a dose and time dependent manner. Flow cytometry data indicated a well marked increase in proliferation rate of the cells under the effect of kisspeptin doses ($p<0.003$), time ($p<0.0001$) and the interaction between both kisspeptin doses and time was also significant ($p<0.04$) shown in Figure 4.2, 4.3. Higher doses of kisspeptin (100 nM) significantly decreased the number of the cells. As indicated in the Figure 4.4, the cell number was observed to be similar to the control in response to 0.1 nM kisspeptin treatment. Conversely, as expected increases in either dose concentration of kisspeptin or exposure time resulted in a significant ($p<0.0001$) decrease in the number of cells.

Treatment with kisspeptin affected formation of rosettes in a significant dose dependent manner, i.e., at 100 nM dose the number of rosettes was significantly decreased as compared with the control (Figure 4.5). All other doses of kisspeptin also decreased the number of rosettes. MTT was used to check the viability of the cells (Figure 4.6), and results of the tunnel assay showed no significant effect of either dose or time (Figure 4.7).

4.4.2. Morphological Changes

All four kisspeptin-10 doses affected the morphology of the growing neuronal progenitor cells (Figure 4.8). Generally, cell body shape changed to an elongated form like a pendulum. Likewise, cell-cell interactions were also increased. The dendritic extensions or/and stress fibers were longer and larger and had an increased number of cell to cell interactions after kisspeptin-10 treatments (Figure 4.8). These changes were more pronounced with increase in kisspeptin-10 dose, indicating that kisspeptin-10 leads to differentiation of neuronal stem cells into a specific neuronal cell form.

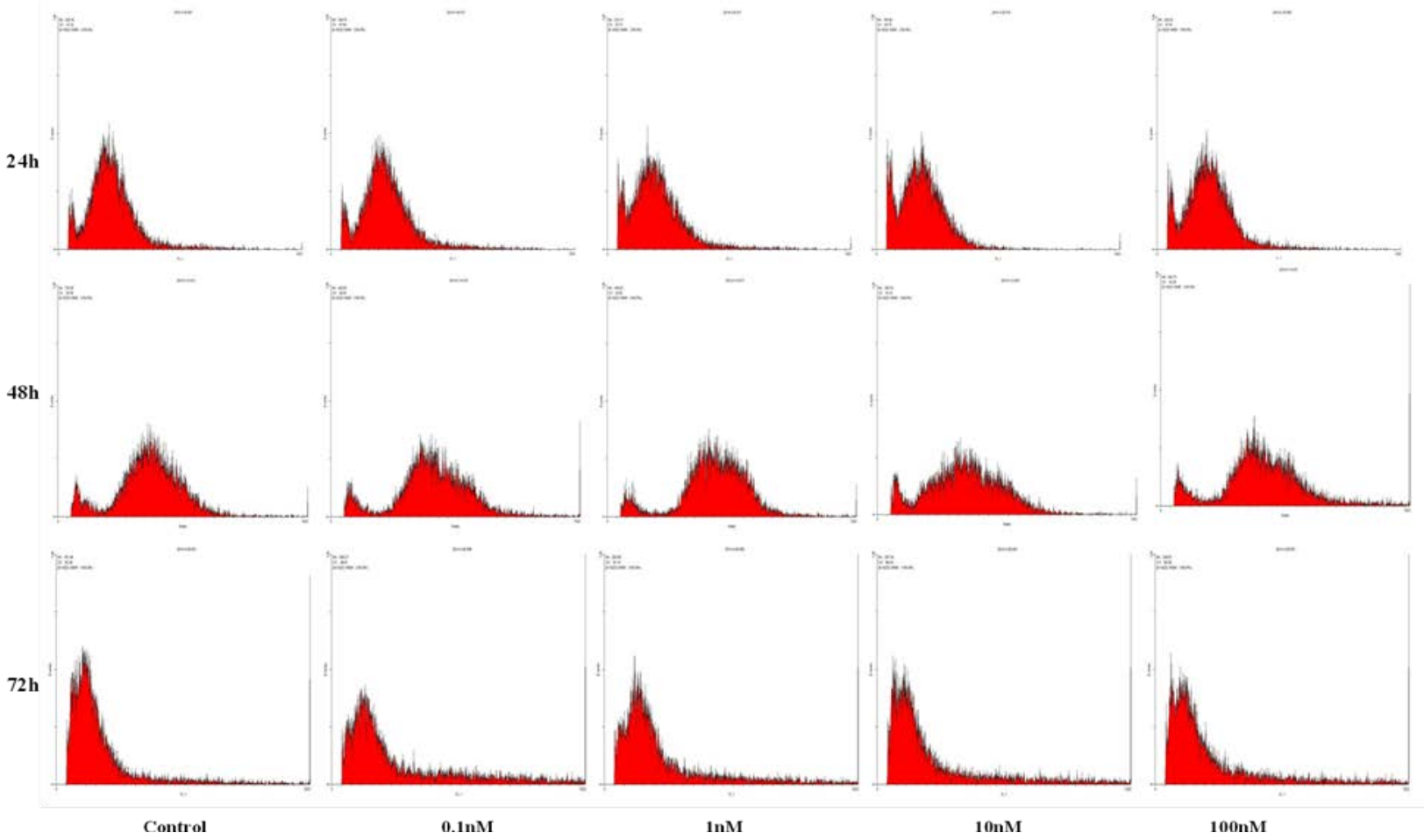


Figure 4.2: Flow cytometry charts for different doses of kisspeptin-10 and incubation time,indicating the proliferation rate of Lyon RNSCs ES cells.

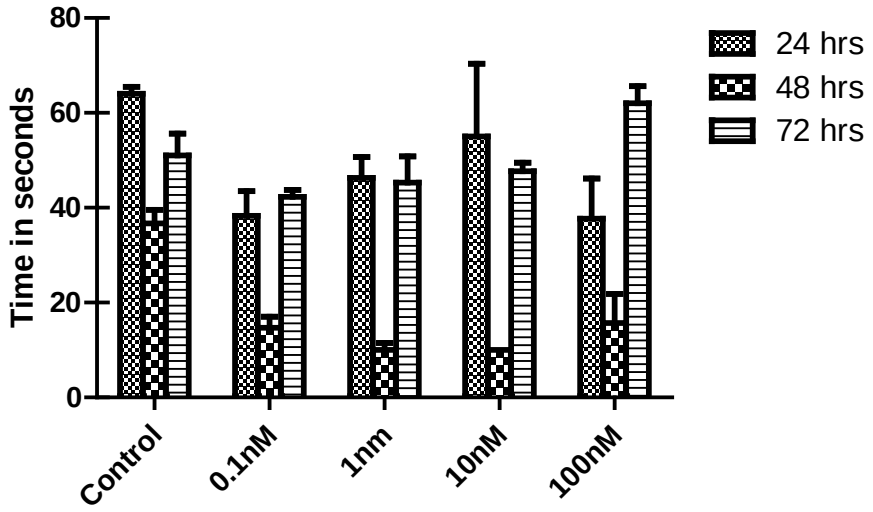


Figure 4.3: The effect of kisspeptin-10 treatment on the proliferation rate of Lyon RNSCs. Time mentioned here indicates the time utilized for counting 10.000 cells. Mean time ± SEM to count 10,000 cells.

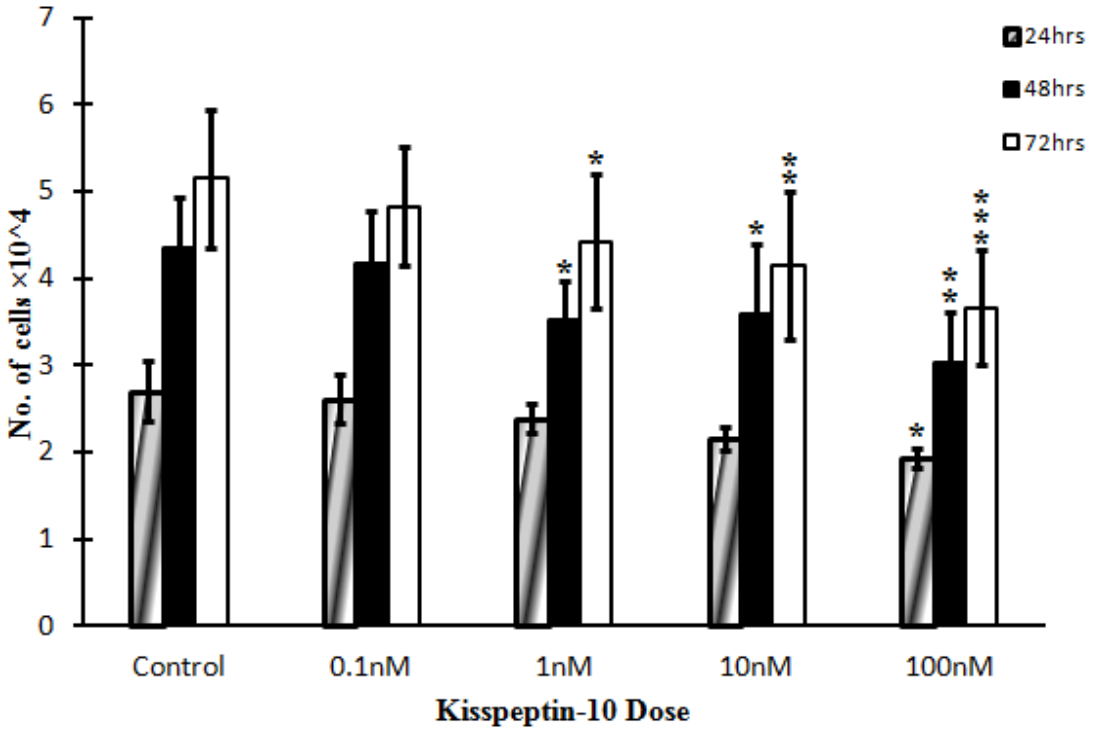


Figure 4.4: Effects of kisspeptin doses and time on the cell proliferation rate. Bars indicate the mean ± SEM number of cells (*p<0.05, **p<0.01, ***<0.001) as compared with control. Significant difference for Dose p<0.02, Time p<0.01 (Two-way ANOVA followed by Bonferroni as a post hoc test).

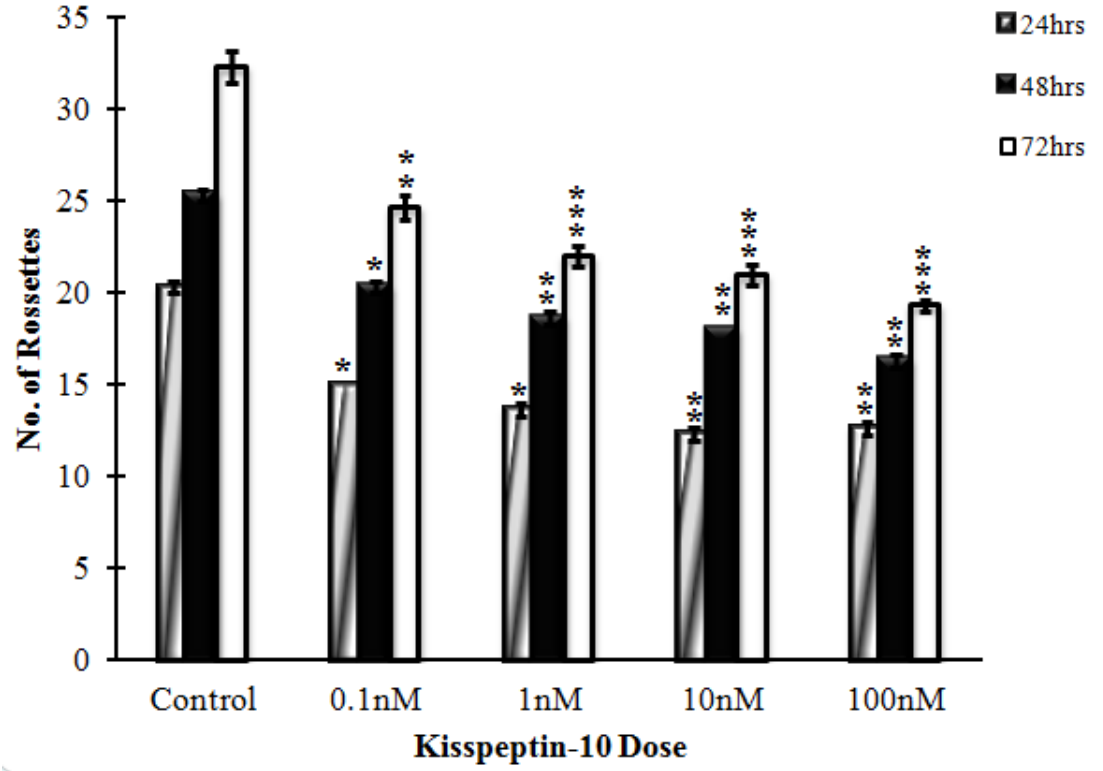


Figure 4.5: Effect of kisspeptin dose and time on the number of rosettes of Lyon ES cells; Bars indicate the mean number of rosettes ± SEM (*p<0.05, **p<0.01, ***p<0.001) compared with control. Significant difference for Dose $p < 0.0001$, Time $p < 0.0001$. (Two way ANOVA followed by Bonferroni correction).

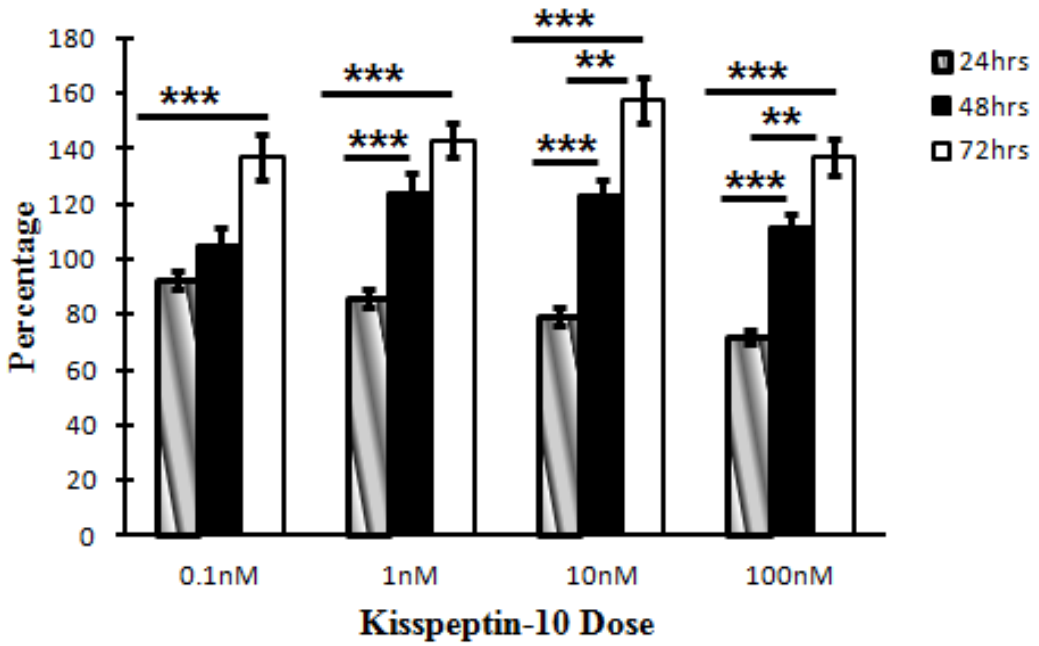


Figure 4.6: Effect of kisspeptin-10 dose and time on the proliferation rate of Lyon RNSCs. MTT data in percentage. All 4 doses showed significance differences ($p < 0.0001$), (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

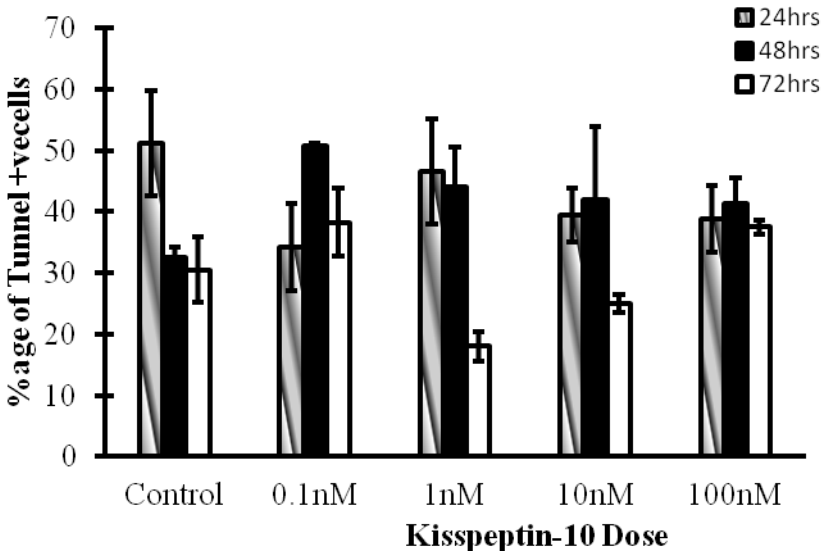


Figure 4.7: Effect of kisspeptin-10 dose and time on the apoptosis. The Figure here represents the tunnel data, which was non-significant for both effects of dose or time.

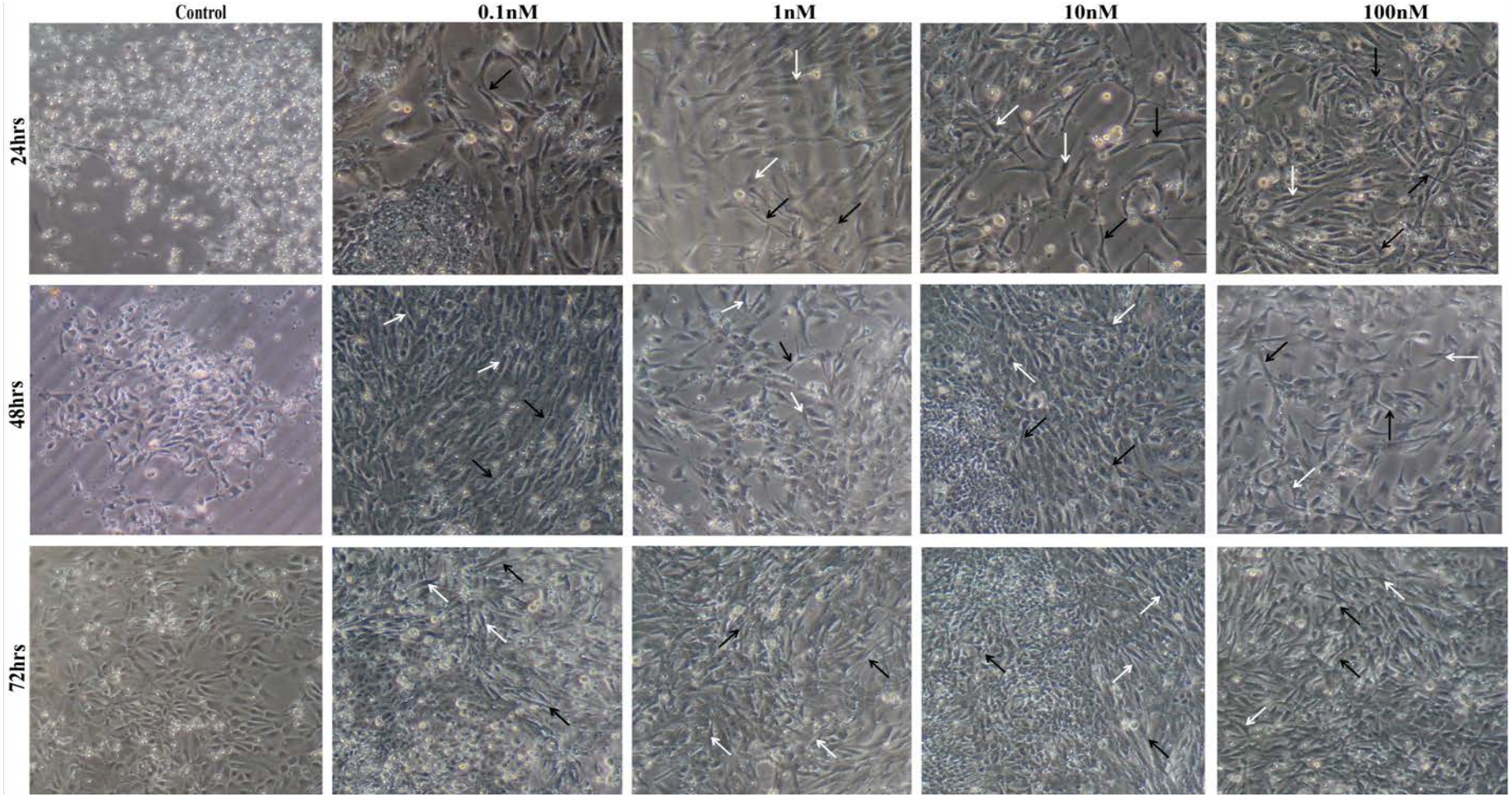


Figure 4.8: Photomicrograph of morphological changes caused by kisspeptin-10 treatment. Kisspeptin-10 treatment caused the change in the cell body shape (white arrows) and cell-to-cell cell interaction was also increased (black arrows).

4.4.4. Kisspeptin-10 Treatment Induced GnRH Neuronal Expression In Vitro in Lyon RNSCs

After exposure to the chosen kisspeptin-10 doses a significant decrease in the astroglia was observed as compared with the controls, and the effect was both in terms of dose and time was noted. Higher doses and longer treatment resulted in the decreased astroglia formation (Figure 4.9, 4.10, 4.11). The percentage of the glia positive cells were determined using the formula (glia positive cells/DAPI positive *100), (see Figure 4.12).

Likewise, for nestin expression, after 72 hrs treatment with 0.1nM of kisspeptin, there was a significant decrease, while 10nM concentration of kisspeptin decreased the nestin expression after 48 hrs and 72 hrs (Figure 4.13, 4.14, 4.15). The percentage of nestin positive cells was calculated as mentioned above for the GFAP (Figure 4.16).

To determine the potential of these cells to differentiate following treatment with different doses of kisspeptin-10, we used immunofluorescence analysis, and found that the cells differentiated into mature neuronal cells (Figure 4.17, 4.18, 4.19). This shows that the differentiation of the Lyon RNSCs into mature neurons occurs in both a dose and time dependent manner, as the differential β-tubulin III expression was observed. It is clearly illustrates that administration of higher doses of kisspeptin-10 increases the expression of β-tubulin III. Additionally, with time, the dendritic extension of these mature neurons increased and the cell to cell interaction was also increased. In brief, the neuronal dendritic extension was increased with time with the highest expression was observed with 100 nM treatment for 24 hrs (93.61 ± 3.9 of DAPI) (Figure 4.20).

Given the observed differentiation of the cells into mature neuronal cells, we tried to determine which types of specific mature neurons were formed. kisspeptin has

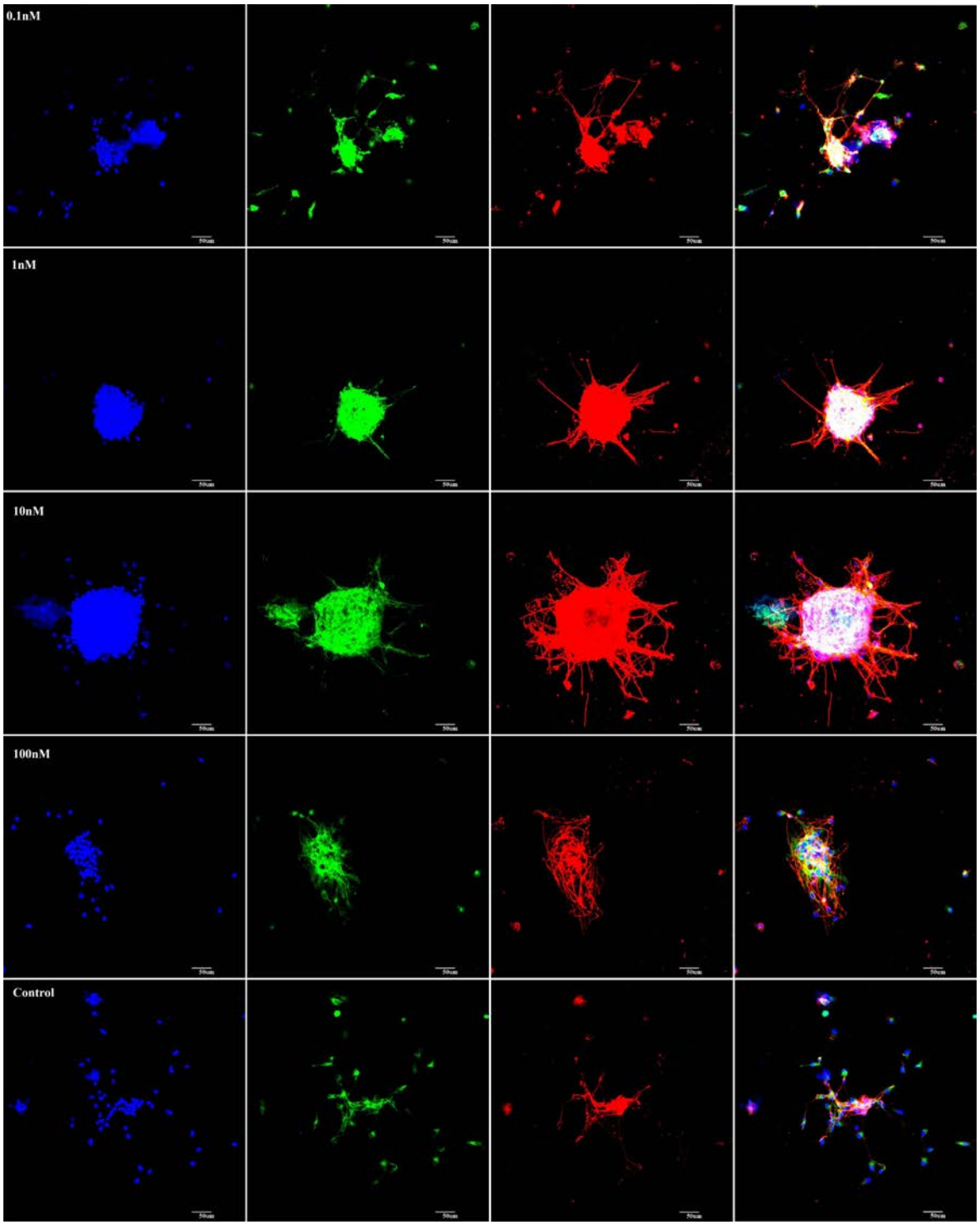


Figure 4.9: Effect of kisspeptin-10 treatment on RNSCs for 24 hrs. Column from left to right (DAPI, eGFP, GFAP and Overlap). The kisspeptin treatment was effective to decrease the astroglia number at lower and higher doses i.e 0.1 nM and 100 nM while the astroglia expression showed a marked increase at 1 and 10 nM.

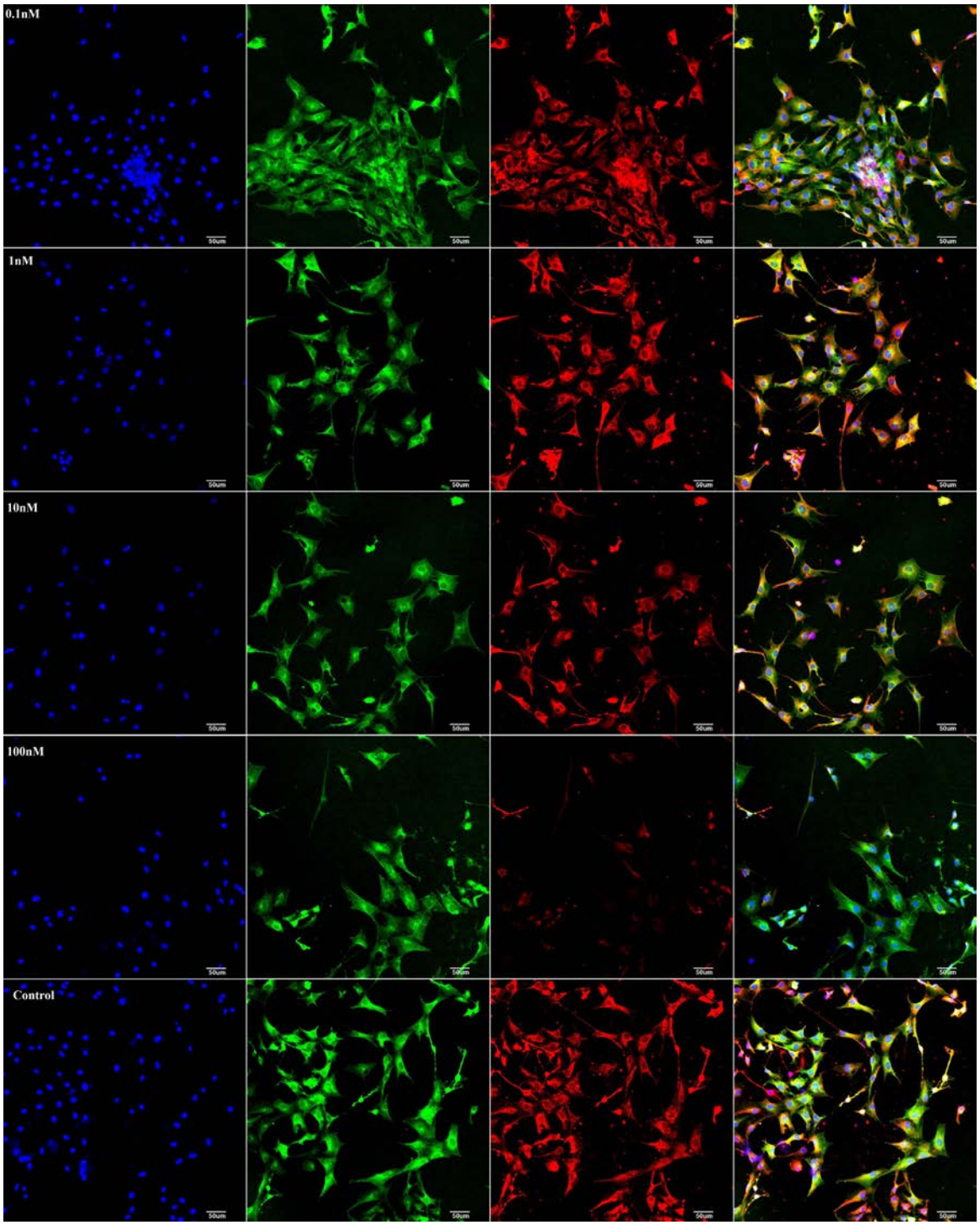


Figure 4.10: Effect of kisspeptin-10 treatment on Lyon RNSCs for 48 hrs. The kisspeptin treatment was not effective to decrease the astroglia number at all the four different doses.

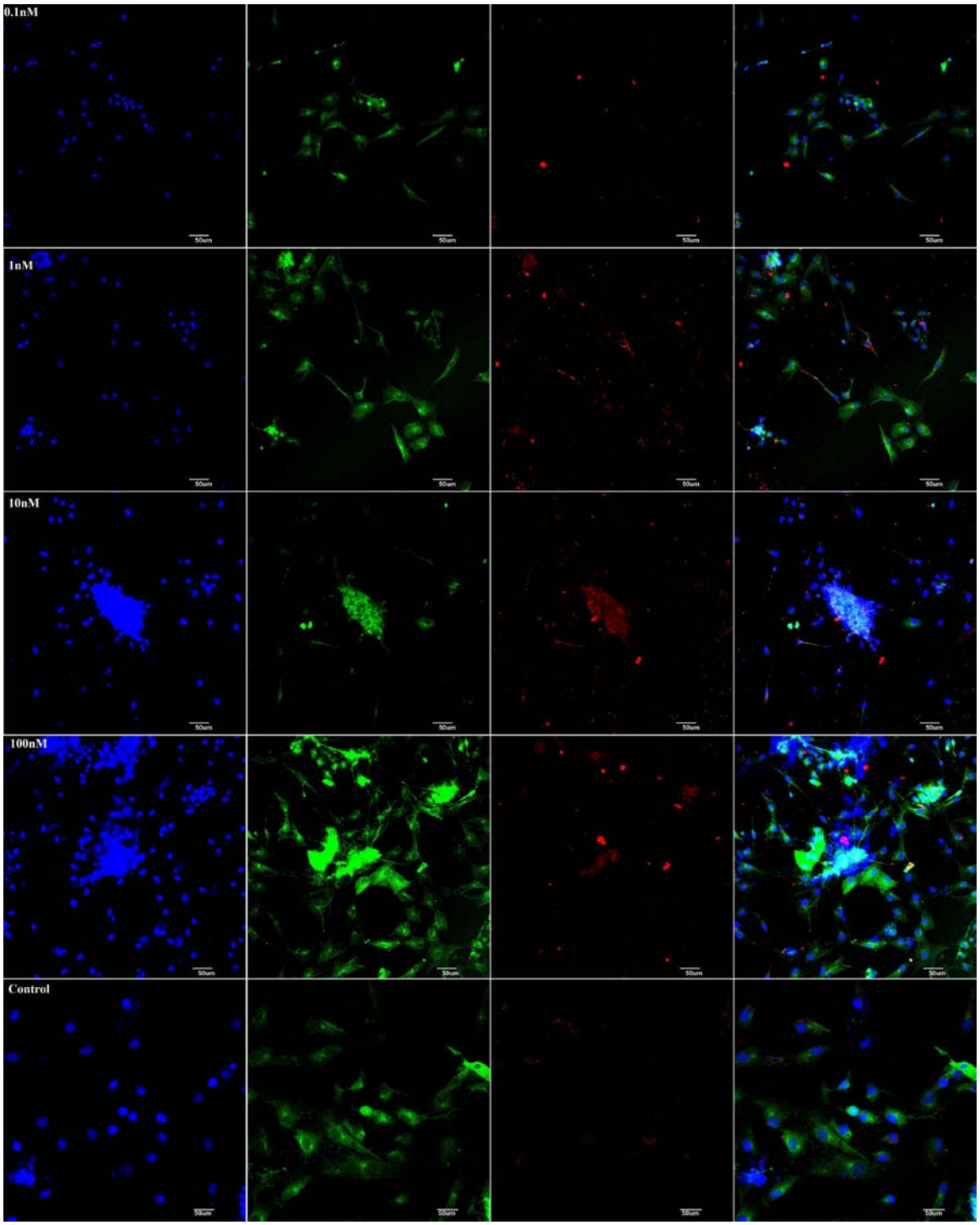


Figure 4.11: Effect of kisspeptin-10 treatment on Lyon RNSCs for 72 hrs. The kisspeptin treatment was effective to decrease the astroglia number at all the four different doses.

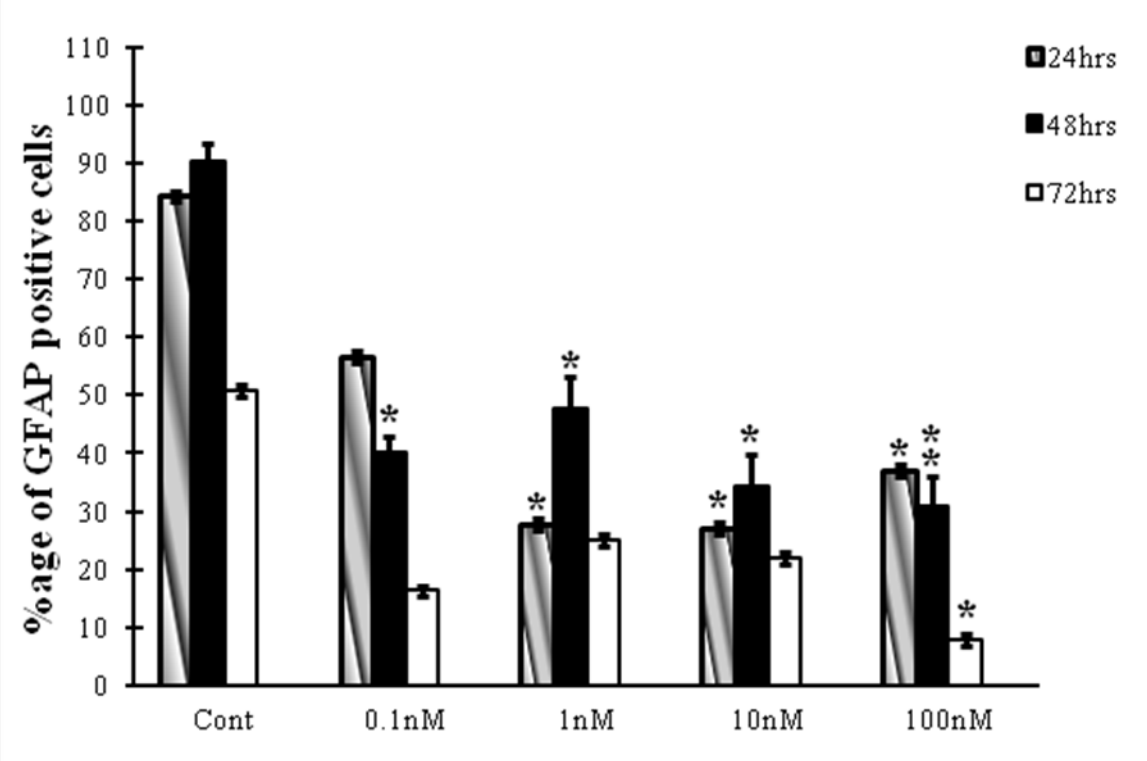


Figure 4.12: Kisspeptin-10 treatment for 24, 48 and 72 hrs decreased the astroglia expression in Lyon RNSCs Percentage of GFAP positive cells as compared with DAPI. All data presented as mean ± SEM (*p<0.05, **p<0.01 as compared with the control). All significance was determined by repeated measures two-way ANOVA followed by Bonferroni correction, $p < 0.05$.

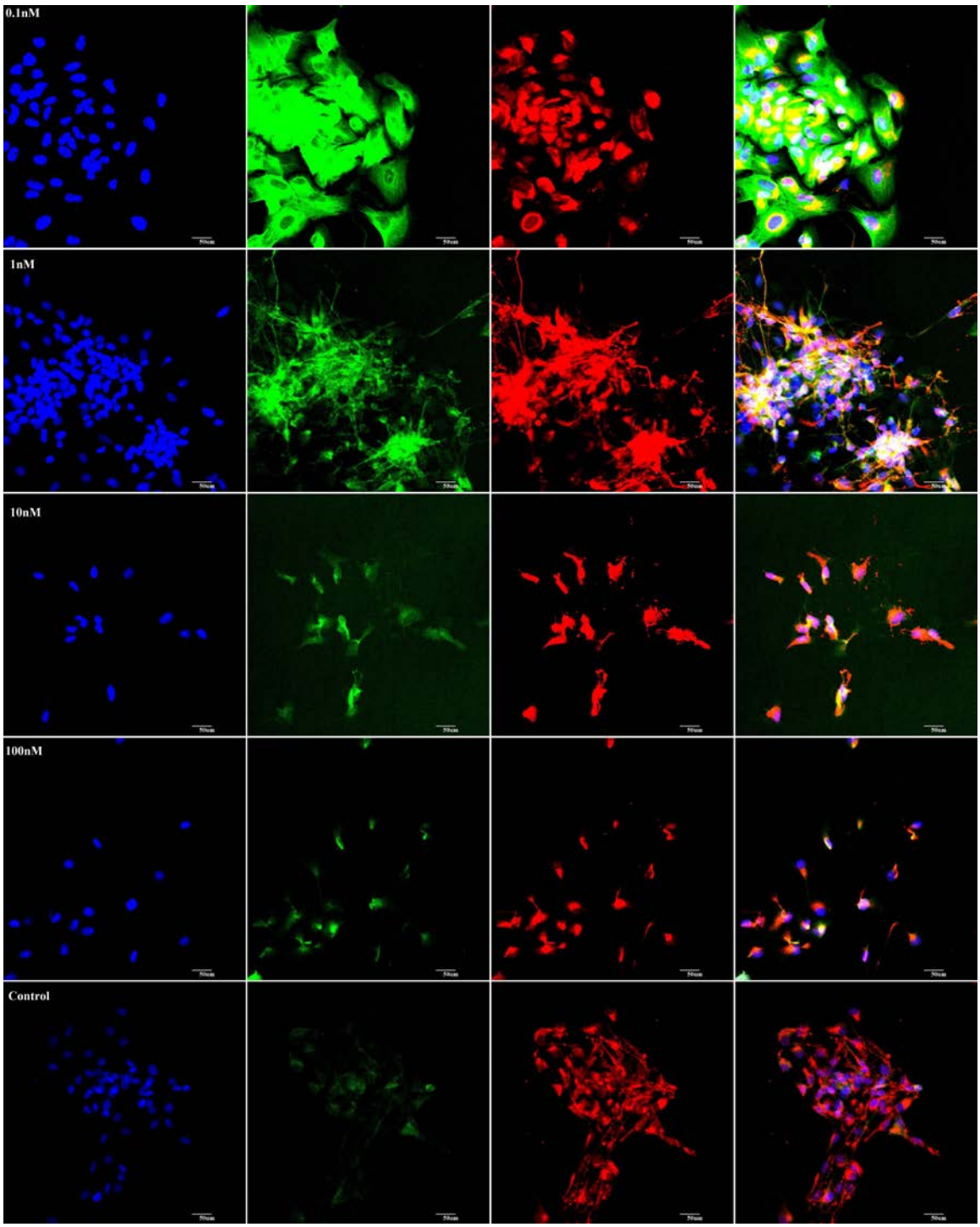


Figure 4.13: Effect of kisspeptin-10 treatment on Lyon RNSCs for 24 hrs. Column from left to right (DAPI, eGFP, nestin, Overlap).The kisspeptin-10 does not change the nestin expression in the Lyon RNCs

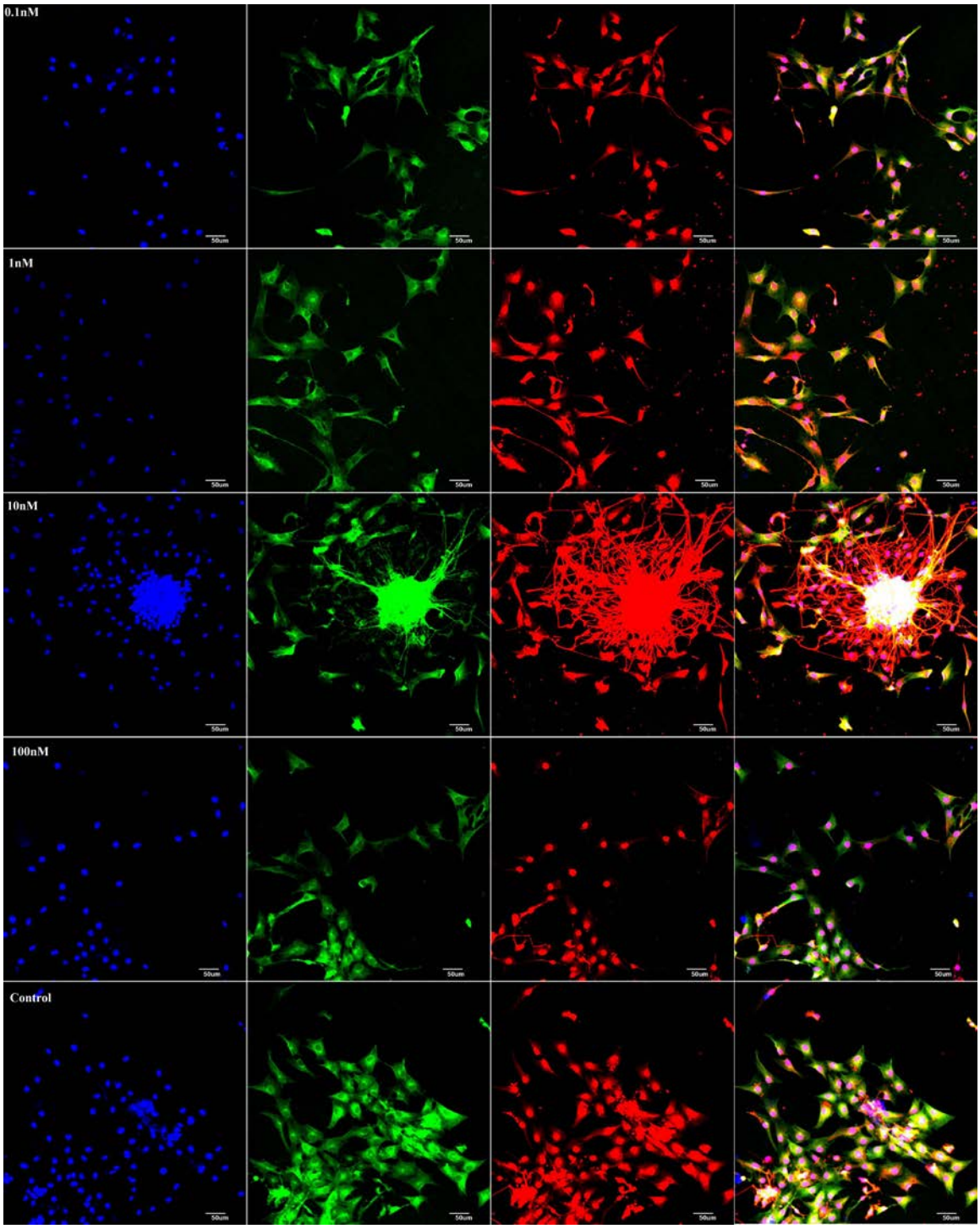


Figure 4.14: Effect of kisspeptin-10 treatment on Lyon RNSCs for 48 hrs The nestin expression was decreased 0.1 and 100 nM doses of kisspeptin-10.

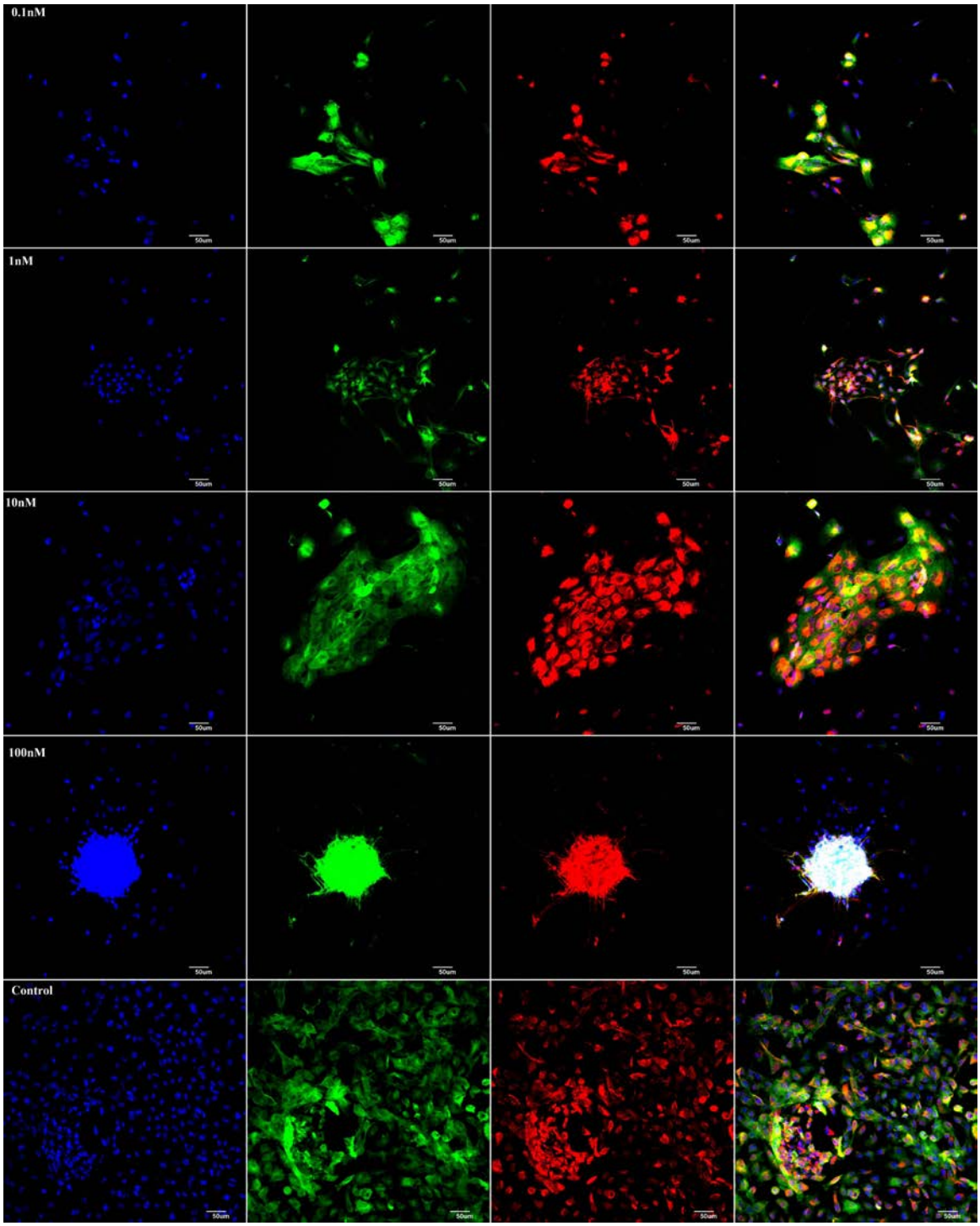


Figure 4.15: Effect of kisspeptin-10 treatment on Lyon RNSCs for 72 hrs Kisspeptin-10 treatment does not change the nestin expression as compared with the control.

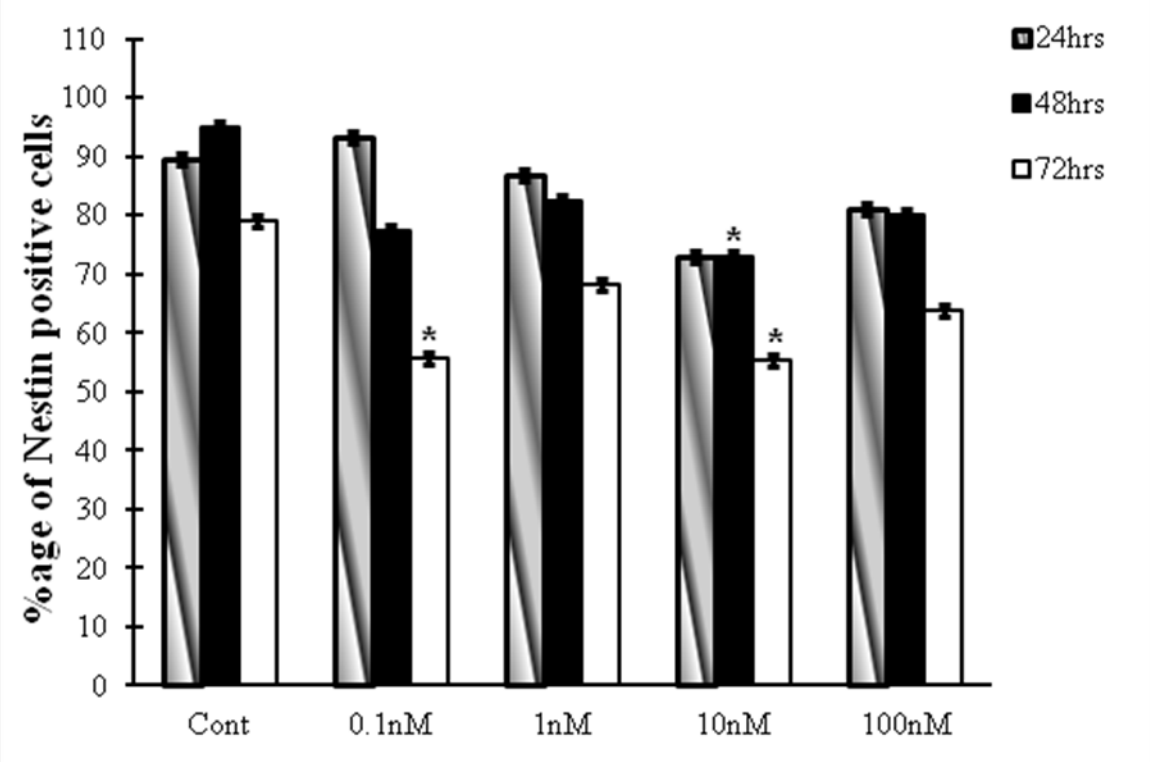


Figure 4.16: Percentage of nestin positive cells as compared with DAPI. All data presented as mean ± SEM (*$p<0.05$ compared with control). All significance was determined by repeated measures two-way ANOVA followed by Bonferroni correction, $p<0.05$.

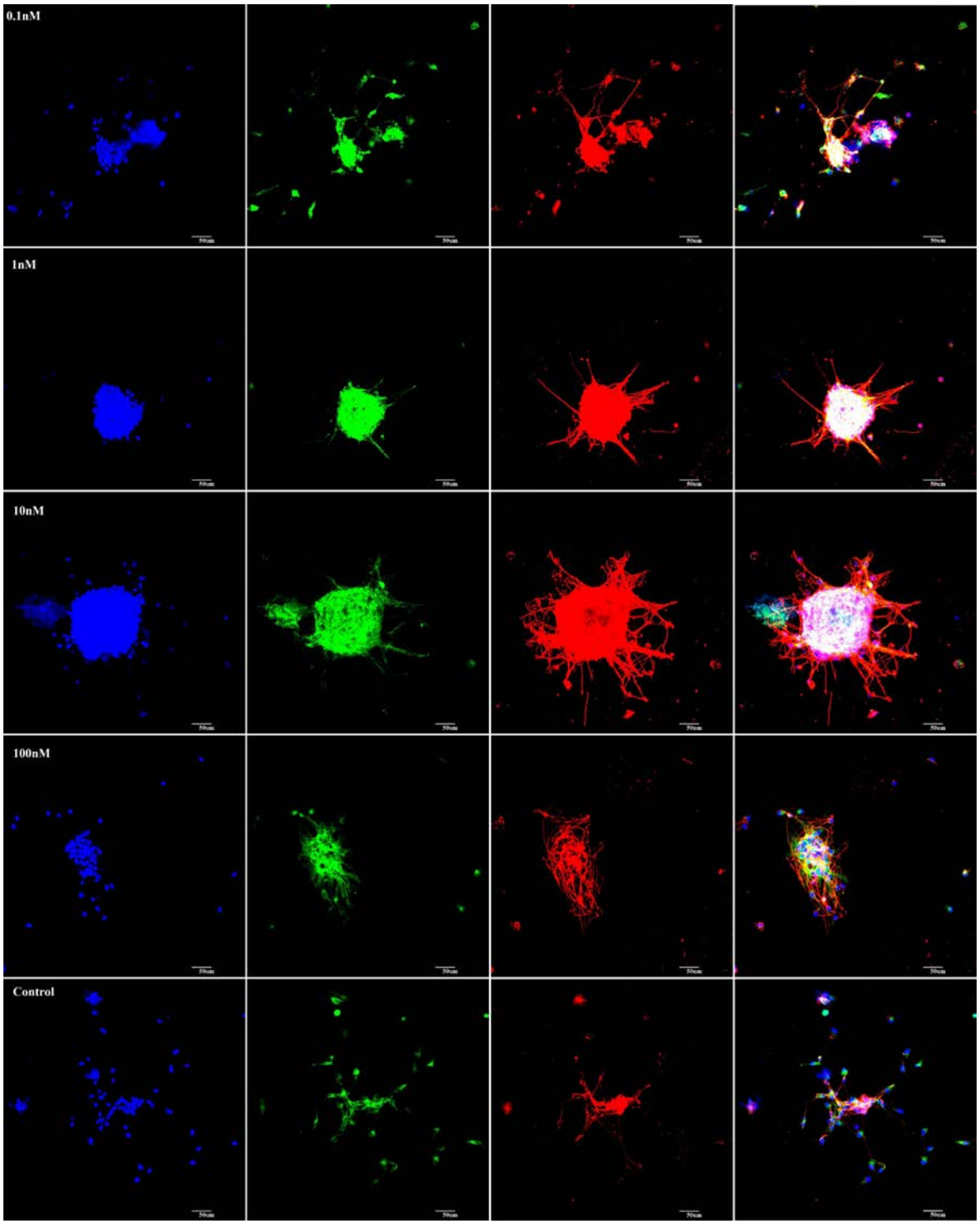


Figure 4.17: Effect of kisspeptin-10 treatment for 24 hrs on β-tubulin III expression in Lyon RNSCs. Column from left to right (DAPI, eGFP, β-tubulin III, Overlap). Kisspeptin-10 increased the expression of β-tubulin III (a neuronal marker).

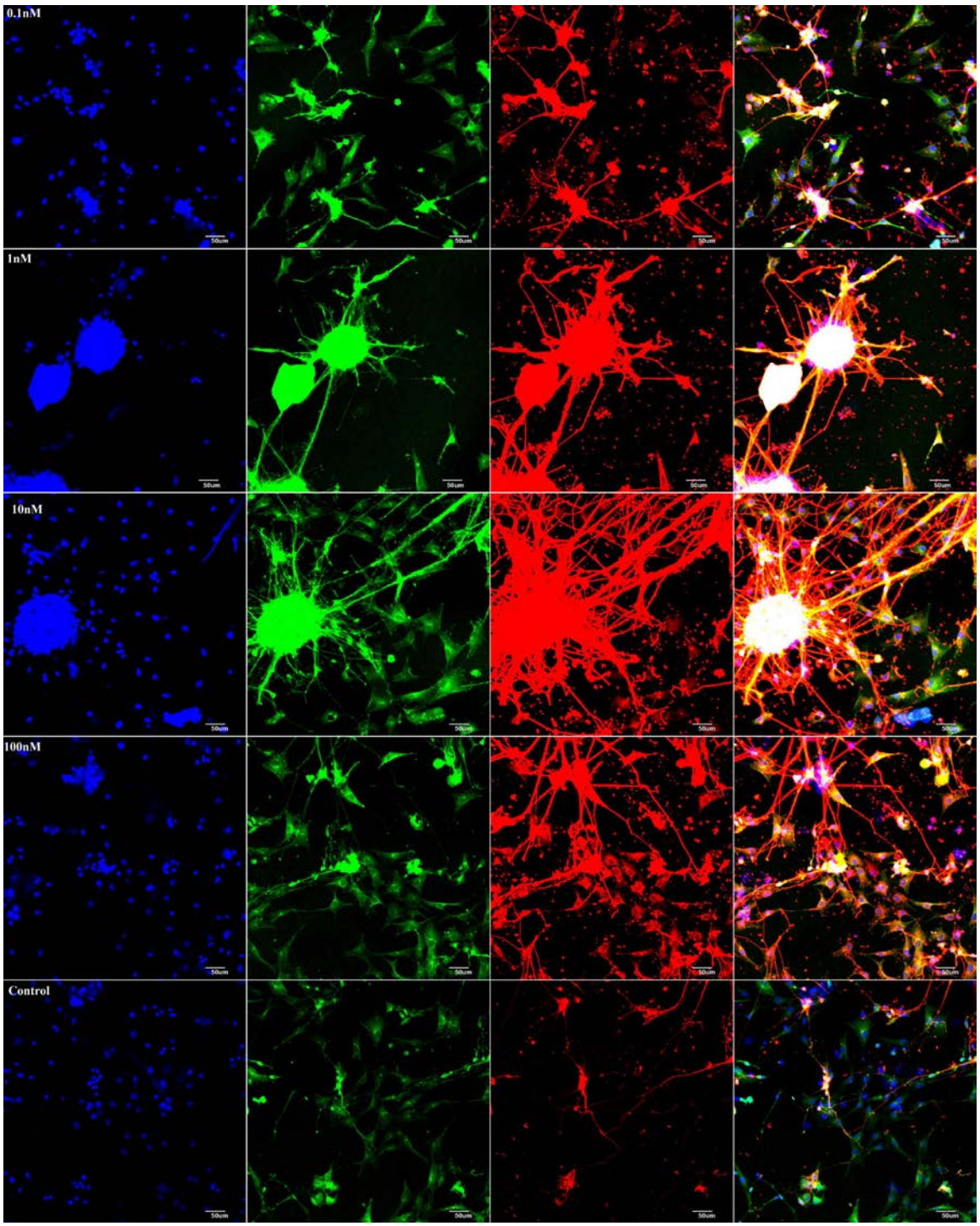


Figure 4.18: Effect of kisspeptin-10 treatment for 48 hrs onβ-tubulin III expression in Lyon RNSCs. An observeable increase in the expression appeared after kisspeptin-10 treatment. The dendritic extension and cell to cell interaction was also increased.

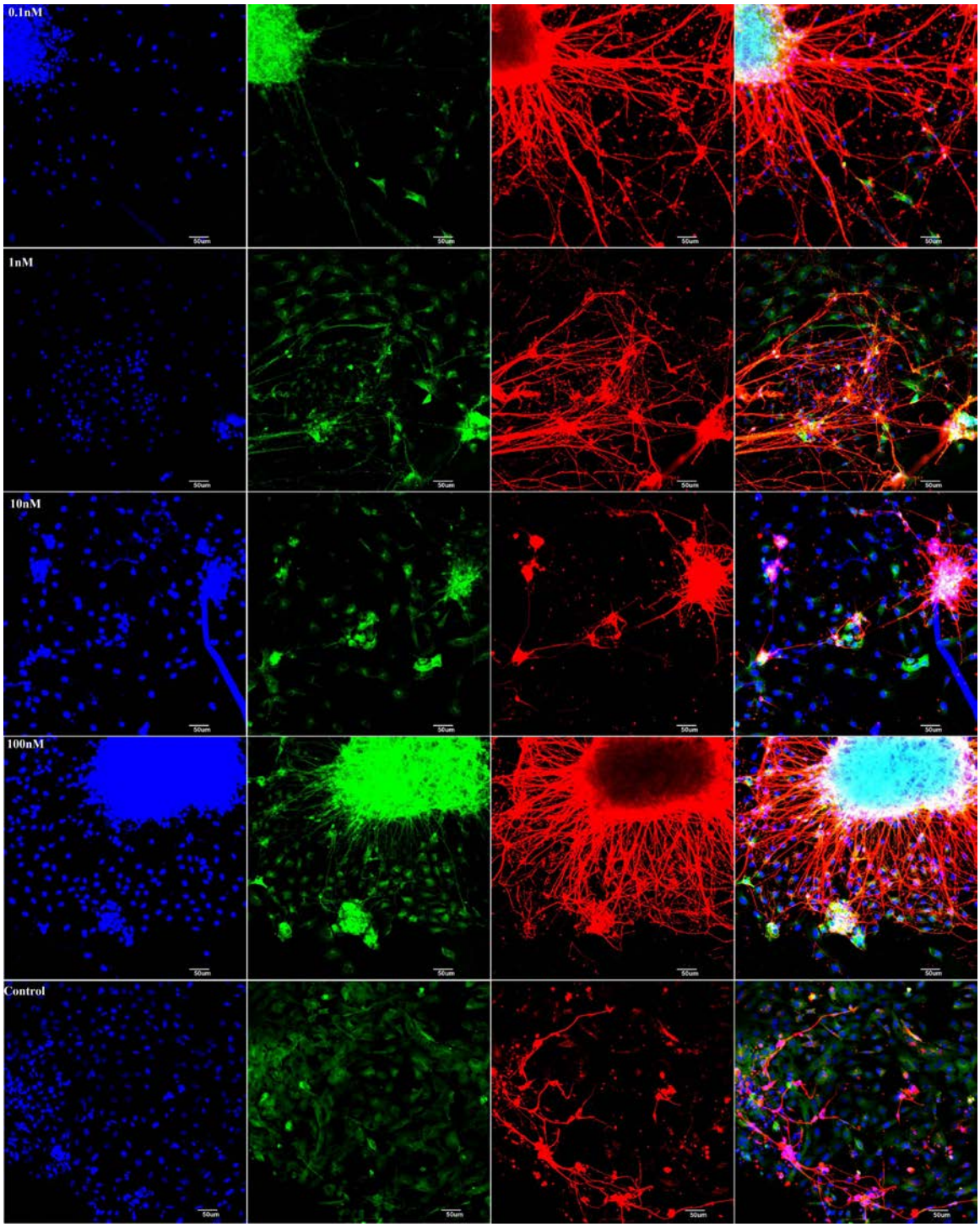


Figure 4.19: Effect of kisspeptin-10 treatment for 72 hrs on β-tubulin III expression in RNSCs. The expression was increased and the long dendritic extensions were observed both with time and dose dependently. More effective doses were 0.1and 100nM.

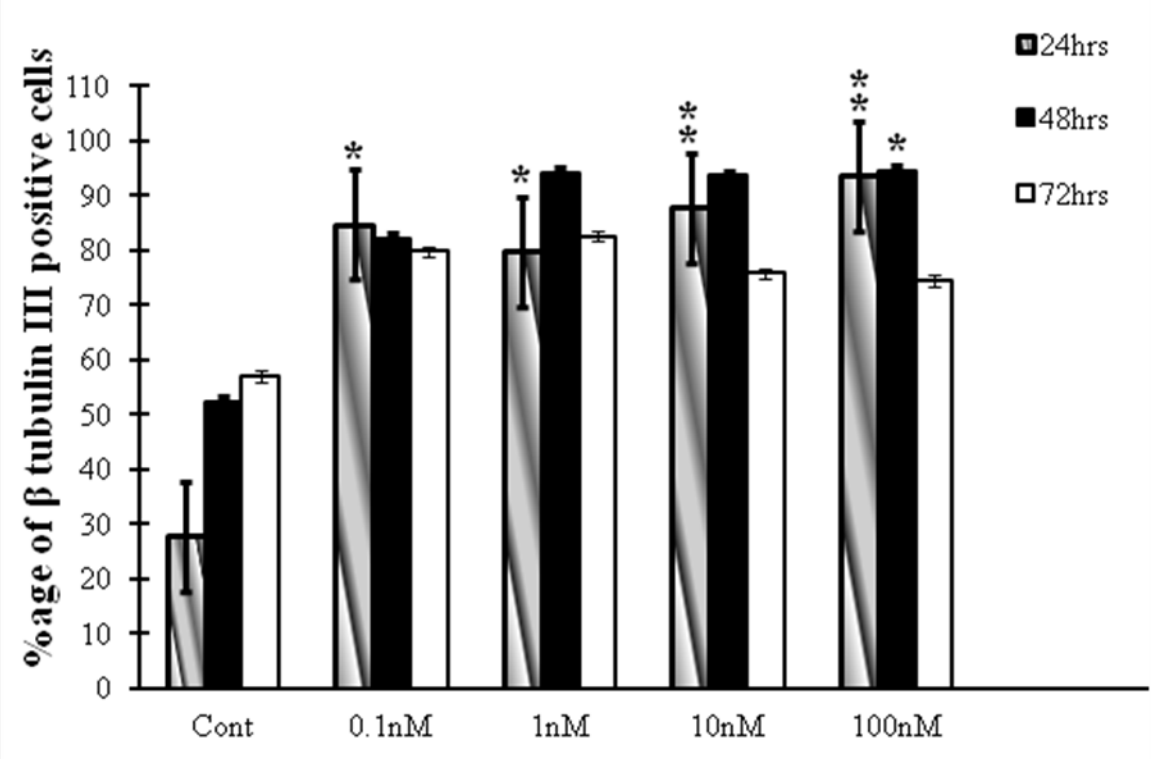


Figure 4.20: Percentage of β-tubulin III expression in RNSCs. β-tubulin III positive cells as compared with DAPI. All data presented as mean ± SEM β-tubulin III, *p < 0.01 for dose. All significance was determined by repeated measures two-way ANOVA followed by Bonferroni correction.. (*p<0.05, **p<0.01 vs control).

been reported to play major roles in reproduction and it is working upstream to the GnRH neurons (Maed et al., 2007), and immuno-cytochemistry analysis showed that majority of the neurons developed were GnRH neurons (Figure 4.21, 4.22, 4.23). After 24 hrs of the treatment we observed dense colonies of GnRH with the 0.1, 1 and 100nM treatment. After 48 hrs treatment with kisspeptin-10, the expression pattern of GnRH neurons remained same with as when done at lower doses (0.1, and 10nM treatments), but decreased with higher doses near 100 nM. Following 24 hrs of treatment, all the dose levels except the 100 nM of kissppetin-10 yielded an increased expression of GnRH with dense colonies and longer neuronal extension. Cell-to-cell interactions were similarly increased. A significant increase in the number of GnRH positive cells was likewise observed after 72 hrs of treatment at the three lower levels of kisspeptin-10 treatment (Figure 4.24).

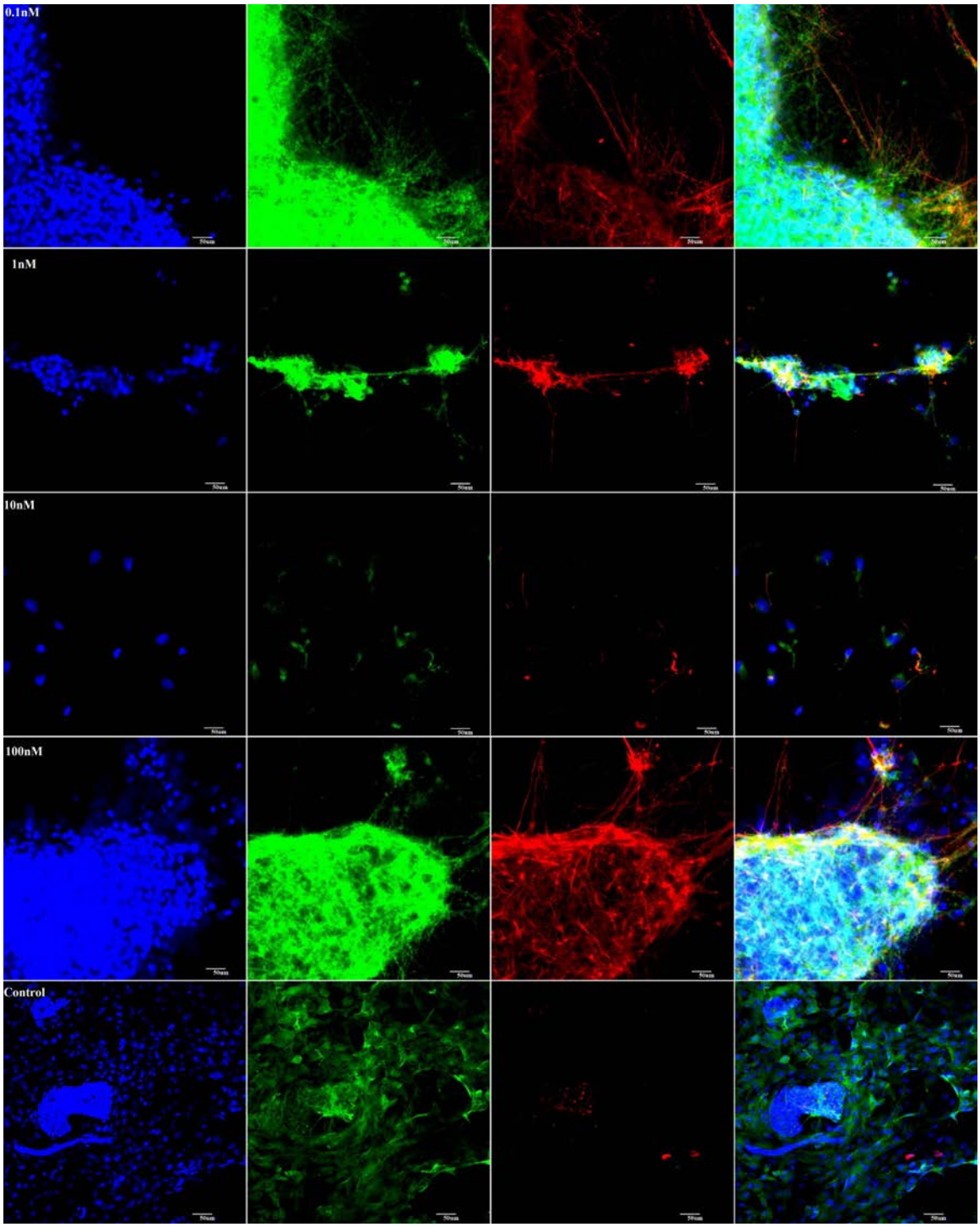


Figure 4.21: Effect of kisspeptin-10 treatment on Lyon RNSCs for 24 hrs. Column from left to right (DAPI, eGFP, GnRH, Overlap). Kisspeptin-10 24 exposure induced GnRH expression in RNSCs. The higher expression of GnRH was observed at 0.1 and 100nM doses of kisspeptin-10.

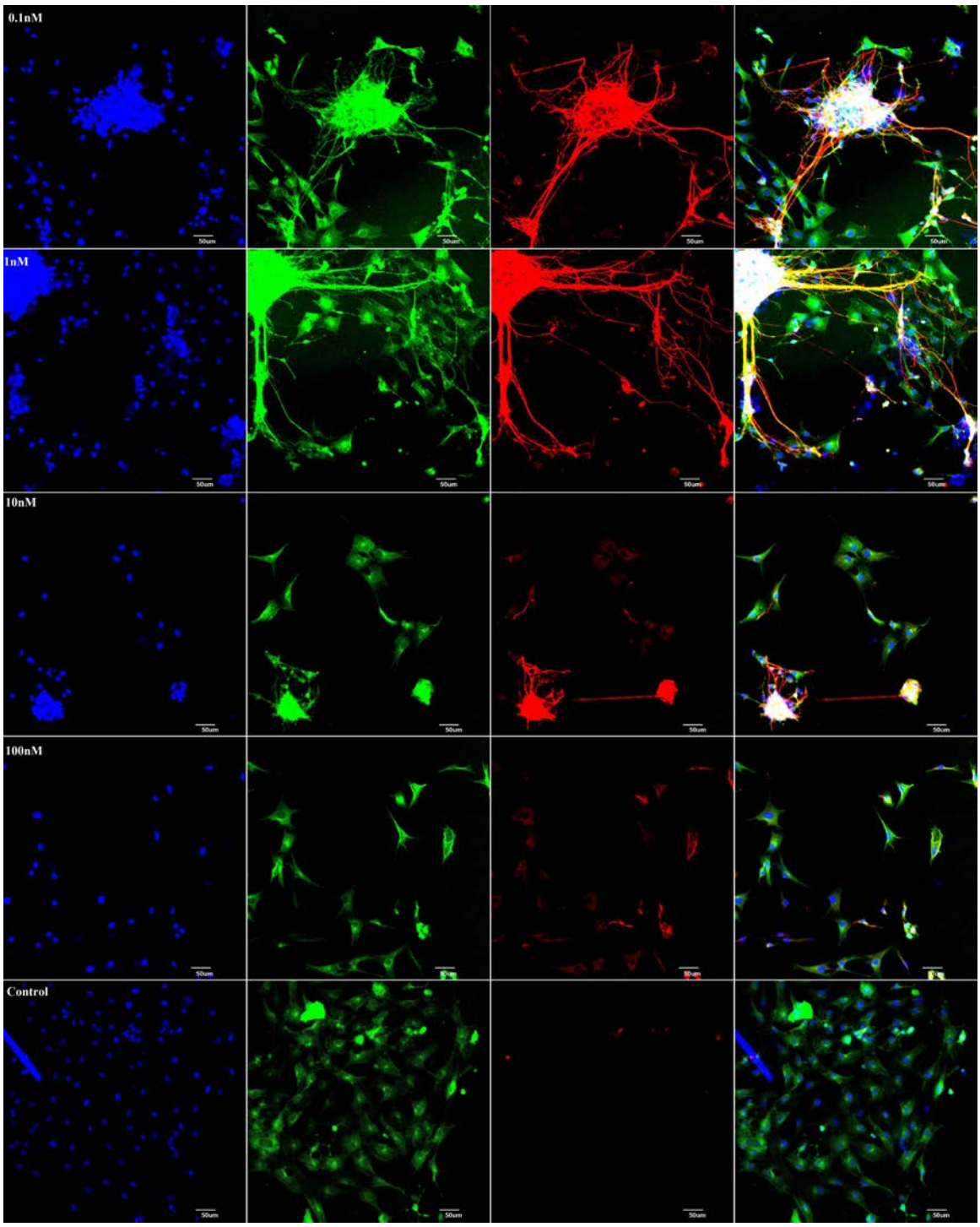


Figure 4.22: Effect of kisspeptin-10 treatment on Lyon RNSCs for 48 hrs. Kisspeptinexposure induced GnRH expression in RNSCs. The 0.1, 1 and 10nM doses of kisspeptin cased a marked increase in the GnRH expression and also the cell to cell interaction was also increased.

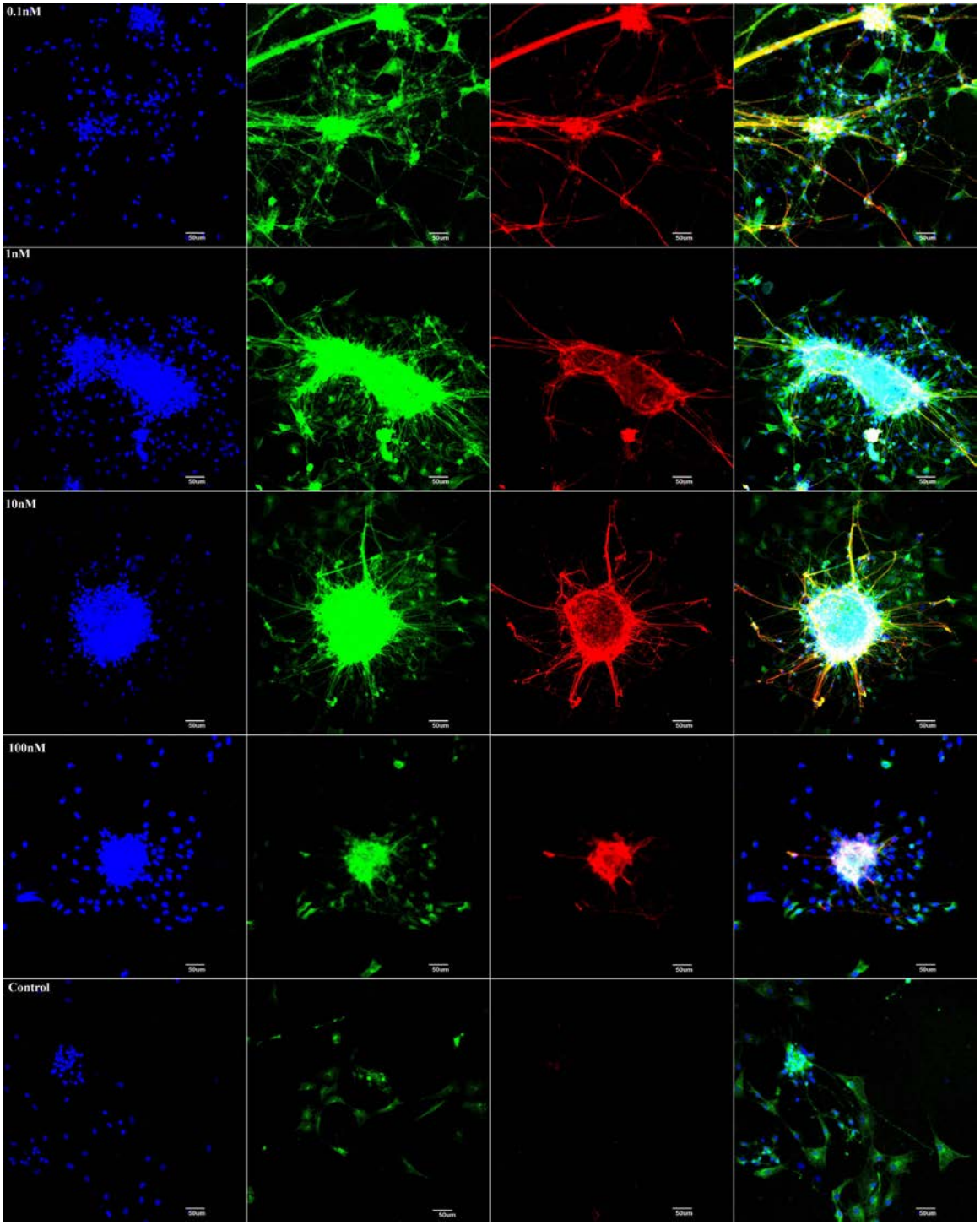


Figure 4.23: Effect of kisspeptin-10 treatment on Lyon RNSCs for 72 hrs.Kisspeptin-10 exposure induced GnRH expression in RNSCs. Both time and different doses of kisspeptin caused an observable increase in the GnRH expression.

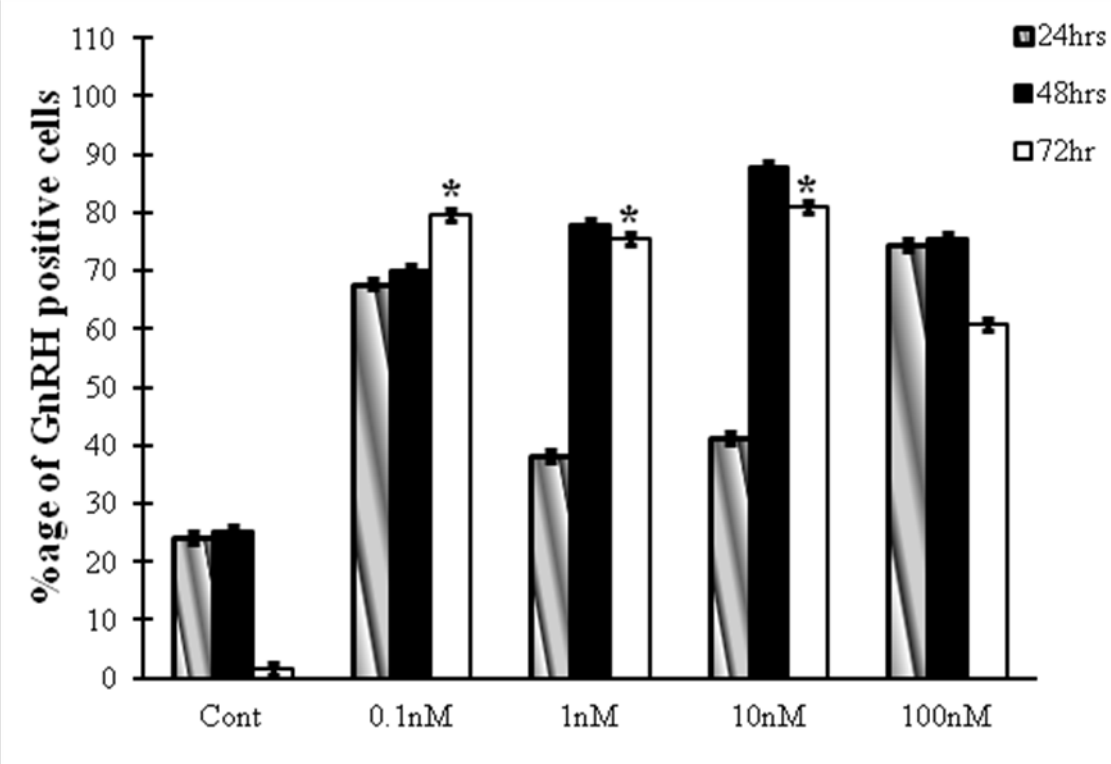


Figure 4.24: Percentage of GnRH positive cells as compared with DAPI. All data presented as mean ± SEM. $p<0.05$ for dose and non-significant for time. All significance was determined by repeated measures two-way ANOVA followed by Bonferroni correction,(* $p<0.05$ vs control).

4.5. Discussion

In this study, we analyzed the effect of kisspeptin-10 on monkey derived tau GFP-Lyon ES cells in regards to the proliferation and differentiation into specific mature neuronal lineages. kisspeptin-10 itself is a small peptide having a potential therapeutic value in treating reproductive dysfunction (Ratnasabpathay et al., 2013). Continuous human metastin administration cause desensitization of kisspeptin receptor which has therapeutic implications for a variety of different conditions, including IHH, restoration of infertility and selective, reversible suppression of the pituitary-gonadal axis to achieve suppression of gonadal steroids i.e., precocious puberty, endometriosis, uterine fibroids, and prostate cancer (Seminara et al., 2006). Previously these conditions were treated by GnRH and their analogs as there are relatively few treatment options for certain types of GnRH dysfunction, such as IHH and its variants. Better understanding of the effects of kisspeptin-10 signaling in Lyon ES stem cell growth may provide new avenues into drug research, reproductive dysfunction pathology and stem cell transplantation functionality. Similarly, understanding affect of kisspeptin-10 signaling on ESC may provide a framework for better understanding on ESC differentiation, which is a core problem in developing ES cell-based therapies. The present findings of kissppetin-10 effect on this particular ESC line derived from rhesus macaques may also bode well for furthering future human applications, given the close homology of the rhesus macaque.

We undertook the apoptotic assay, which showed that the apoptotic markers in the Lyon ES cells were not following kisspeptin-10 treatment (Huma et al., 2013). This is not entirely surprising, given our earlier findings as well as those of most other relevant studies which showed that the Kiss1/Kiss1r signaling was pro-apoptotic, as it suppressed Akt pathway (Navenot et al., 2005), activated RhoA-Rock/RhoA pathway and caused apoptosis in a breast cancer HEK-236 cell line (Navenot et al., 2009b). Likewise, Cho et al. (2009) showed that anti apoptotic inhibited TNFalpha induced Rho signaling, thereby inhibiting apoptosis. This finding implies that the role of kisspeptin-10 in apoptosis may be stage specific. Unfortunately, from the presently avaialble evidence it is not clear that whether kisspeptin inhibits TNF alpha induced

Rho signaling in the Lyon ES cell lines (thereby inhibiting apoptosis), but it is possible that Kiss1/Kiss1r act at stage specifically to modulate cell growth and differentiation in this cell line. Clearly, more detailed studies are needed to clarify this point and give a concrete description.

Kisspeptin-10 decreased Lyon ES cell number (proliferation) at different doses. This dose-dependent effect suggests that kisspeptin plays an anti-proliferative role in the growing Lyon ES cells. Kisspeptin-10 treatments also reduced the formation of rosettes in a dose dependent manner, implying that kisspeptin may induce differentiation of Lyon ES cells, because rosettes are well-known hallmark of stem cell differentiation into specific and more mature neuronal cells (Darmon et al., 1981). This is consistent with our observation where tested cells underwent a morphological change following kisspeptin-10 treatments. At the highest dose of kisspeptin-10 (100 nM), the cell bodies were larger in comparison to cells treated with other doses and neuronal fibers and/or extensions (Figure 4.4) were formed leading to increased cell-to-cell interactions. The subsequent immuno-cytochemistry revealed that these mature neurons and fibers were actually GnRH neurons, which are well known target of Kiss1/Kiss1r signaling (Mead et al., 2007).

As Lyon ES cells are pluripotent cell lines (Wianny et al., 2008) responsiveness to kisspeptin-10, suggests the cells may have an endogenous expression of Kiss1r (the kisspeptin receptor). Huma et al. (2013) suggested inhibitory effect of kisspeptin on cell proliferation.. In another study, inhibition was only witnessed in cells transfected with a kiss1r expressing plasmid (leading to higher kiss1r expression) (Ziegler et al., 2013). Although Zeigler et al. (2013) used endogenously expressed Kiss1r, kisspeptin treatment did not have an anti-proliferative effect on the cancerous cells which endogenously expressed the Kiss1r, suggesting that kisspeptin-10 anti-proliferation was dependent on high levels of Kiss1r expression. It is possible that Kiss1r receptor expression increased under the effect of kisspeptin-10 signaling as the number of GnRH cells increased with time. This is giving the evidence that under the effect of kisspeptin-10, Kiss1r expression might increase which alternatively increased the GnRH expression, a finding consistent with another study in which kisspeptin increased Kiss1r expression in frog testis (Chianese

et al., 2013). It is likely that the Lyon ES neuronal stem cells endogenously express Kiss1r receptors, which may increased under the effect of kisspeptin in our paradigm.Further validation and independent replication of our findings is needed to reach a more complete answer, as is more detailed and focus studies aimed at examining expression in affected cell lines.

Wide cellular differentiation potentials are proxy to the early rosettes stage and differentiation is either lost or decreased if the rate of proliferation increases (Jassell, 2000). Higher doses of kisspeptin-10 led to a lower rate of cell proliferation as well as a marked decrease number of rosette formation, suggesting that the cells had begun to differentiate in response to kisspeptin signals. Due to the parameters of our study, it is not clear if kisspeptin modulates the notch pathway and sonic hedgehog (SHH) signaling, which are both necessary to maintain the R-NSC stage, but a previous report found that in the absence of these pathways NSC decreased rosette numbers and formed more specific neuronal cell types (Doe, 2008; Huma et al., 2013) These findings are consistent with the observations of the present study, especially the morphological changes and ICC data in the Lyon ES cell line., This suggests that kisspeptin specifically modulates the formation of GnRH neurons during development. If this hypothesis is correct, then kisspeptin may be a potentially viable therapeutic for treating IHH and other related secondary HH disorders which manifest themselves due to GnRH dysfunction in the brain.

In present study, formation and development of the GnRH neurons resulting from application of kisspeptin peptides (kisspeptin-10) to the Lyon ES cell is a novel and potentially significant finding in a number of areas. In regards to our example of IHH, improper migration of the GnRH neurons and disability to secrete the GnRH is a major underlying cause.Our findings of kisspeptin-10’s effect on Lyon ES cells open doors to new possibilities for curing the infertility that corresponds with the hypothalamic pituitary level, which has previously proven resistant to more effective treatments. In theory, GnRH neurons can be transplanted safely and effectively, beginning first with the relevant non-human primate models prior to investigating more detailed human studies. Should this prove viable, it may give a clue as to the first steps in developing a transplantation method capable of fighting IHH related

infertility.However, our results are generally applicable to broader studies aimed at understanding how to better control cell signaling and develop more effectively, novel ES cell-based therapeutics.

Chapter 5: General Discussion

5.1. General Discussion

In the present research we studied the role of endogenous Kiss1/Kiss1r signaling (and also the KNDy kisspeptin, neurokinin B and dynorphin expression alteration) in the regulation of the higher primate hypothalamic pituitary gonadal axis and its modulation in seasonal breeding by pharmacological immunocytochemical and molecular techniques. We used three different approaches to investigate the critical role played by this signaling mechanism during adulthood and breeding and non-breeding seasons. Firstly, we peripherally administered P234 (kisspeptin receptor antagonist) and determined the effect on the basal testosterone and adiponectin secretion. It was believed that modulation in the activity of the HPG axis during breeding and non-breeding seasons is the result of a sequence of various changes in different morphological and physiological phenomena. It was previously known that plasma testosterone level and activity of different neuronal systems decreases during the non-breeding season. In the second study, therefore, we determined the effect of season on the up or down regulation of various genes involved in modulating the reproductive axis by using RT-qPCR. We also used immune-cytochemical techniques to analyze the protein expression of Kiss1, Kiss1r and GnRH expression during breeding and non breeding seasons. Thirdly, we used a therapeutic approach: we treated rhesus monkeys derived Lyon ES cells with different doses of kisspeptin. Kisspeptin treatment previously caused dendritic extensions of GnRH neurons in hypothalamic brain slices in vitro. Therefore, we designed this study to check whether kisspeptin treatment can induce specific neuronal differentiation in the pluripotent cell lines or not.

In the first study, the effect of peripheral administration of P234 in adult male rhesus monkeys on the plasma testosterone was investigated for the first time. The results indicated that the bolus systemic administration of P234 did not affect the basal testosterone release in the adult male rhesus monkeys. These results are consistent with the previous data which indicated that the basal LH release in the male rats (Roseweir et al., 2009; Pineda et al., 2010), mice and the reduction of pulsatile GnRH secretion in the adult female monkeys (Roseweir et al., 2009) was not changed

We observed the same pattern in the testosterone release. Testosterone is the end product released by the Leyding cells in response to LH (Saez, 1994).

It is very well known that kisspeptin plays critical/key role in reproductive neuro-endocrine activity (Gottsch et al., 2004; Navarro et al., 2004; Thompson et al., 2004; Dhillo et al., 2005; Shahab et al., 2005). Kisspeptin mainly acts at the hypothalamic level (Gottsch et al., 2004; Navarro et al., 2005; Shahab et al., 2005). Therefore, it is very logical to conclude that kisspeptin induced testosterone release most likely resulted from increased GnRH release. In our experiment peripheral administration of P234 did not fully block the kisspeptin dependent testosterone release. This may be due to two reasons. a) The P234 dose was not enough, and b) P234 does not cross the BBB.

The possibility that the dose was not enough to effectively block the Kiss1r is less likely. We calculated the dose based upon the information available with Rosweir et al. (2009) on monkeys and Pineda et al. (2010) on female rats. The evidence that the same dose of P234 effectively blocked the Kiss1r present on the adipose tissue as, it decreased adiponectin levels in the present study clearly suggests ruling out this possibility.The second possibility as to why we did not get significant result is that P234 alone does not cross the BBB. There is no completely established evidence which shows that P234 crosses the BBB. Systemic administration of P234-penitratin (penitratin: a cationic cell-penetrating peptide) can inhibit the kisspeptin induced LH and FSH release (Pineda et al., 2010). However, it was not reported whether the same effects were obtained by the systemic administration of P234 lacking penitratin. In our study, we injected P234 lacking penitratin and did not get the significant decrease in the basal testosterone release. From this we can say that P234 does not cross the BBB and the result which we obtained is either due action of P234 at the ME level or at the testis. As far as the ME is concerned, Kiss1r has been reported on the GnRH fibers located in the ME region outside the BBB (Inoue et al., 2008). Similarly the GnRH fibers originating in the monkey hypothalamus that project outside the BBB have been shown to be contacted by the kisspeptin fibers in the ME (Ramaswamy et al., 2008). It can be concluded that P234 blocked the Kiss1r on the GnRH neurons at the ME level. However, inhibition of the Kiss1r in ME was probably not very strong

or the contribution of the ME Kiss1r was minimal to ultimately regulating the testosterone secretion.

Regarding the direct testicular actions of kisspeptin, in our study the kisspeptin induced testosterone release was found modestly reduced by the systemic administration of P234. However, there was not a complete inhibition of testosterone release. Recently, Kiss1 and Kiss1r expression has been reported in the monkey testis (Tariq et al., 2013), which gives an idea of autocrine/paracrine action of the kisspeptin. So, we can possibly assume that the decrease in the kisspeptin-induced testosterone release could have been due to the peripheral action of P234 on the testicular tissue to block the Kiss1r in the testis. Also, our laboratory observation in monkeys indicated that kisspeptin is capable of enhancing stimulated testosterone release by Leydig cells *in viv;o* but has no effect on basal testosterone secretion following GnRH receptor antagonist pretreatment (Irfan et al., 2008). In another study the direct action of kisspeptin on monkey testis was checked, 120 minute treatment increased the testosterone release *in vitro* (Tariq and Shahab, unpublished data). From all these we can have an idea that it is possible that P234 blocked the peripheral Kiss1r present at the testicular tissue and restricted the testosterone release.

At the time when we decided to conduct this experiment physiological data were available on expression studies. The reason of conducting a study was to check whether the endogenous tone has any effect on the physiology or not. Data which we obtained gave us a clear idea that endogenous kisspeptin tone has a very clear physiological effect on the regulation of the HPG axis. From all this observation, even if P-234 did not cross the BBB, it could have reached the ME. We tend to conclude that the Kiss1r in the ME are not enough that their blockage does not fully block the testosterone release. It must be controlled centrally at the hypothalamic level.

The role of kisspeptins has been reported in the seasonal regulation of the HPG axis in various species, like, Libyan jird (Boufermes et al., 2014), hamsters (Revel et al., 2006a; Greives et al., 2007) and sheep (Caraty et al., 2007; Smith et al., 2007). The Kiss1/Kiss1r system, which influences GnRH secretion avidly, is regulated by both melatonin and the feedback action of gonadal steroid hormones (Revel et al., 2006b;). Accordingly, Kiss1/Kiss1r system may play a key role in

regulating reproduction by seasons (Huang and Chu, 2011). The kisspeptin signaling has previously been reported to play a role in conveying photoperiodic and environmental clues to increase the reproductive activity during the breeding seasons in hamster and sheep (Ansel et al., 2010; Revel et al., 2006a; reviewed by Clarke et al., 2013). The second objective of the present thesis was to check the effect of seasons on the kisspeptinergic signaling by assessing qualitative and quantitative expression of the Kiss1and Kiss1r changes during the breeding and non breeding season in adult male rhesus monkeys. This study involved the use of 3 different techniques, a).Hormonal analysis: Testosterone, metastin like ir, b).Semi-quantitative mRNA expression analysis: RT-qPCR, and C).Qualitative analysis of protein expression: ICC

The peripheral testosterone release increased during the breeding season. A clear increase in testis weight during the breeding season was also observed. All these findings are in line with the previous studies conducted in different animals regarding seasonal reproduction (Pelletier et al., 1982; Revel et al., 2006a; Chiver et al., 2014). These parameters demonstrate the seasonal activation of the HPG axis in rhesus monkeys . More interestingly, we observed an increase in the metastin like ir in the CSF during the breeding season. This was a novel finding as this was suggestive of an increased central release of kisspeptin during breeding season. It has not been reported before in any species. The peripheral kisspeptin levels on the other hand were not in the detection range of the RIA used in the present study.

We also observed changes in the protein and mRNA expression of Kiss1, Kiss1r and GnRH in the MBH during the breeding and non breeding season in the adult male rhesus monkeys. Both protein and mRNA expression of Kiss1 and Kiss1r increased during the breeding season. In case of GnRH we observed increased mRNA expression during the breeding season while no observable change in the protein expression was observed. The observed increase in Kiss1 mRNA and protein expression during the breeding season in monkeys is supported by the previous studies on different species, viz., Syrian hamster and sheep, where Kiss1 mRNA increased during the breeding season (Revel et al., 2006a; Smith et al., 2007; Wagner et al., 2008) but contradictory to the observations in the Siberian hamster (Greives et

al., 2007; Mason et al., 2007). A notable increase in the Kiss1r mRNA was also observed in the MBH of the adult male rhesus monkey during breeding season. This observation was contradictory to the results obtained from the experiments performed in the sheep (Smith et al., 2007; Li et al., 2012). This difference in observation could possibly be due to species variations and/or due to different technical approaches used. Increase in the mRNA and protein expression of Kiss1/Kiss1r and GnRH mRNA expression were associated with the increased metastin like ir in CSF during the breeding season. Together, these observations show a remarkable increase in the kisspeptinergic signaling during the breeding season in adult male rhesus macaques.

We also quantified NKB, pDYN, NK3R and KOR mRNA expression in the MBH of the adult male rhesus monkeys in breeding and non-breeding seasons. No observable change was observed. This information is contradictory to what was observed in the other species, like, ram where pDYN decreased during the breeding season (Scott et al., 2008). NKB, DYN and Kiss1 expression increased in the ARC but decreased in the dorso-medial hypothalamus during the short day (Bartzen-Sprauer et al., 2014) in Syrian hamsters. This indicates that NKB and DYN expression is photoperiod dependent in seasonal rodents and sheep but not in rhesus monkeys. It is likely that these genes in rhesus monkeys are not regulated by the photoperiod. Findings of this study provide an important clue towards the seasonal switch of the HPG-axis in adult male rhesus monkeys. A possible mechanism for the transition between breeding and non-breeding seasons can be inferred from these observations. Onset of breeding season in adult male rhesus monkeys leads to an increase in Kiss1 and Kiss1r mRNA expression in the ARC, causing an increase in the central kisspeptin signaling. However, what environmental/ neural/ neuroendocrine signals stimulate seasonal increase in kisspeptinergic signaling still remains to be elucidated. Among the seasonal species the kisspeptin expression appears different because kisspeptin synthesis is regulated by many seasonally regulated factors, like, leptin and sex steroids (Smith et al., 2008; Wagner et al., 2008; Quennell et al., 2009). These seasonal changes might be due to the increased negative feedback effect as the inhibitory effect of long term estrogen treatment on kiss1 MRNA and kisspeptin expression on ARC is greater during the non-breeding season (Smith et al., 2008). Various hypothalamic neurons governing release of pituitary

gonadotrophs express melatonin receptors (Roy et al., 2001; Dubocovich and Markowska, 2005). Pineal gland, in response to photoperiodic signals, secretes melatonin in both long day and short day animals (Challet, 2007). Many animals use variations in the melatonin secretion as seasonal clock (Lincoln et al., 2003). Melatonin acts at the pre-mammilary area of the posterior hypothalamus and the pars tuberalis of the pituitary plays role as mediator of seasonal changes (Malpaux et al., 2001). The alteration in the period of secretion of melatonin, thus serves as a biological indicator for the organization of day-length-dependent cyclic functions, such as, reproduction, actions, coat growth and camouflage coloring in seasonal animals (Arendt and Skene, 2005). By which pathway melatonin is acting on kisspeptinergic signaling is unclear until now. Kispepetin cells in the ewe do not express melatonin receptors (Li et al., 2011). Similarly, in Syrian hamsters melatonin receptors do not overlap with the kiss1 expressing neurons in the ARC (Maywood and Hastings, 1995; Hanon et al., 2008). Melatonin secretion is suppressed by long days in seasonally breeding animals (Wright, 2010). Short-day melatonin patterns enhance puberty in rhesus monkeys and long day melatonin patterns delay pubertal events in rhesus monkeys (Wilson and Gordon, 1989). Melatonin administration induces early onset of puberty in ewes (Arendt et al., 1983). So there must be some inter-neuronal network, which conveys these photopheriodic signals to kisspeptinergic system. The first possibility is that, RFRP neurons may work upstream with the kisspeptinergic system as shown in rat (Rizwan et al., 2009) and mouse (Poling et al., 2013). The RFRP3 infusion increases the kiss1 levels in the ARC of Syrian hamster (Ancel et al., 2012). So, possibly these may be the neurons, which convey the photoperiodic signals to kisspeptinergic systems (Simonneaux et al., 2013) (Figure 3.16). The second possibility is that melatonin is directly acting on the pituitary as the pituitary cells do express the melatonin receptor (Hazlerigg, 2001). The gonadotrophins of anterior pituitary express the melatonin receptors (Johnston et al., 2003; Balik et al., 2004). Melatonin most probably might be playing a role in regulating the negative feedback mechanism conveying signals back to the hypothalamus as reported in Syrian hamster and ewe (Tamarkin, 1976; Goodman et al., 1982; Karsch et al., 1993). KISS-1 cells receive and assimilate signals from various origins, including sex hormones that feedback on KiSS-1 neurons to down regulates the KiSS-1 expression, body energy stores, and possibly other signals (e.g., ambiant temperature, stress, and pheromones).

We suggest the activity of the KiSS-1 neurons is influenced by photoperiod, via the melatonin signal, either directly or via intermediate targets and controlling kisspeptin expression and release (Figure 3.16).

Thirdly, Energy stores may also control the melatonin signaling (Figure 3.16). Leptin is secreted at higher quantities at night both in rodents and humans when the melatonin levels are higher (Ahima et al., 1998; Sinha et al., 1996). Melatonin administration in rats increases leptin concentration (Baltaci and Mogulkoc, 2007; Song and Chen, 2009). Kiss1 neurons express the leptin receptor gene (Cravo et al., 2011). Lack of leptin was associated with suppression of Kiss1 mRNA expression in male mice and central administration of leptin rescued Kiss1 mRNA expression (Smith et al., 2006). In islet and CRI-D2 cells, leptin increased the KiSS-1 mRNA expression (Sagheb et al., 2014). The GABAergic and Dopaminergic cells express melatonin receptor proteins (Wiechmann and Wirsig-Wiechmann, 2001). Melatonin has been observed to act directly on GABAa receptors (Coloma and Niles, 1988). In pre pubertal rhesus monkeys GABA antagonist induced kisspeptin secretion (Kurian et al., 2012). This could be a possible pathway that melatonin is controlling/ conveying the photoperiodic signals to control seasonal breeding in rhesus monkeys by leptin-GABAergic pathway (Figure 3.16, 5.1).

We have proposed the possible pathways which could be playing a role in conveying melatonin signals. As, the melatonin secretion is increased during the breeding season, which stimulates the leptin release, leptin in turn stimulates kisspeptin. So, it might control the kisspeptin release by positively stimulating the kisspeptin release. From this information we suggest that signals which controls these seasonal changes in kisspeptin expression may include photoperiod acting via melatonin (Revel et al., 2006b; Chan et al., 2009) (Figure 3.16, 5.1).

It is very clear from the evidences of these studies that kisspeptinergic signaling is very critical for fertility and maintaining the reproductive system active. The signaling was increased at the onset of the breeding season in rhesus monkeys and decreased during nonbreeding season.

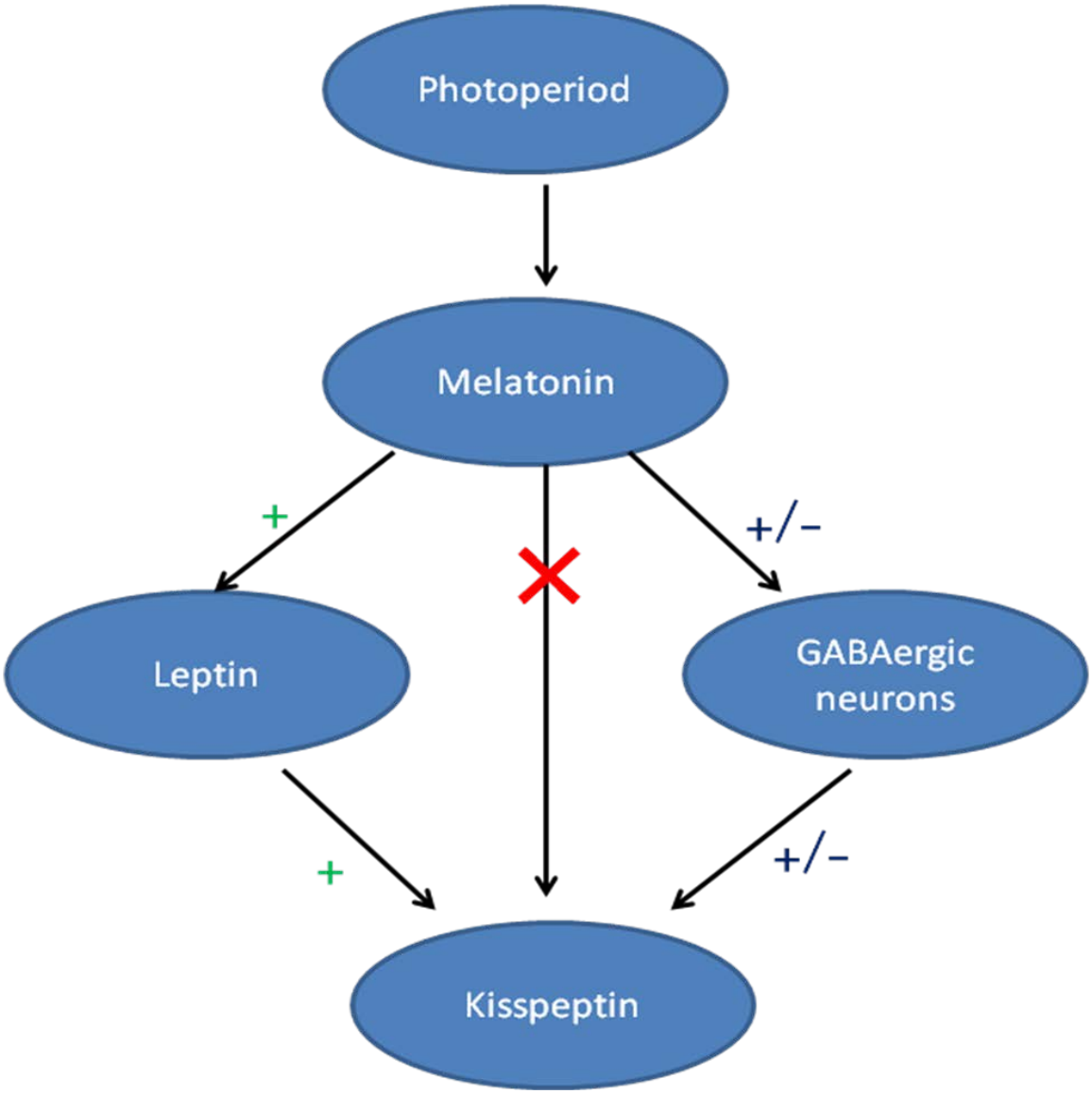


Figure 5.1: Diagrammatic presentation of proposed leptin-GABAergic pathway conveying photoperiodic signals.

From this we can suggest that kisspeptin can be used as a therapeutic agent to cure infertility. Kisspeptin-10 has also been reported to have the potential therapeutic value in treating reproductive dysfunctions (Ratnasabapathay and Dhillo, 2013). Previous studies of continuous administration of human metastin administration indicated that it caused desensitization of kisspeptin receptor which may have a therapeutic implication for a variety of conditions like selective, reversible suppression of the pituitary gonadal axis to achieve suppression of gonadal steroids (Seminara et al., 2006). Constant iv infusion of kisspeptin is observed to restore the pulsatile release of LH. From all the studies the role of kisspeptinergic signaling has been evaluated. It is very clear that Kiss1/Kiss1r play a very critical and central role in the regulation of HPG-axis. The third objective of our study was to check the effect of kisspeptin-10 at the stem cell level. Embryonic stem cells are pluri-potent and have potential to differentiate into almost all types of cells (Wianny et al., 2008). We selected monkey derived tau GFP-Lyon ES cells (Wianny et al., 2008). We used kisspeptin-10 as a tool to investigate the role in the differentiation of ESCs into specific cell types. This can provide a framework for better understanding how signaling affects ESCs differentiation, which is a core problem developing ES cell-based therapies. In this study, we used different techniques to investigate the effect of kisspeptin-10 on apoptosis, proliferation and differentiation of Lyon-ES cells.

Kisspeptin-10 did not induce apoptosis in the Lyon-ES cells; this observation appears to be in line with previous studies (Huma et al., 2013). In another study kisspeptin behaved as anti-apoptotic by inhibiting the TNF alpha induced Rho signaling, thereby inhibits apoptosis (Cho et al., 2009). Some of the studies are contradictory to our observation. Kisspeptin suppressed the Akt pathway and showed a pro-apoptotic role (Navenot et al., 2009a). In another study KP appeared to be apoptotic by activating RhoA-Rock/RhoA pathway in a breast cancer HEK-236 cell line (Navenot et al., 2009b). These observations give us an idea that kisspeptin acts as apoptotic or anti-apoptotic factors and such actions are possibly cell type or stage specific. Unfortunately, we did not study pathways kisspeptin signaling used to act as apoptotic/anti apoptotic.

The number of cells decreased in a dose dependent manner following the kisspeptin-10 treatment in the growing Lyon-ES cells. This observation is in line with the previous study done in R366.4 cells where the number of cells decreased with kisspeptin treatment (Huma et al., 2013). Kisspeptin treatment also reduced the number of rosettes in a dose dependent manner; giving an indication that kisspeptin might play a role in differentiation of the Lyon-ES cells into specific cell types because rosettes are well known to be the hallmark of differentiation of cells into the specific, more mature neuronal cells (Darmon et al., 1981). This concept was consistent with our observation that the tested cells underwent a morphological change following kisspeptin treatment. At higher dose of kisspeptin-10 the morphological change was very prominent, having larger cell bodies, neuronal fibers and increased cell to cell interaction. But, which type of cell were these was not clear. Later, the immunocytochemical observations revealed these morphologically differentiated cells were actually GnRH neuronal cells, which are well known targets of kiss1/kiss1r signaling (Mead et al., 2007).

In this study kisspeptin-10 treatment lowered the proliferation rate of the cells kisspeptin-10 has also been reported to inhibit cell proliferation in R366.4 cells (Huma et al., 2013). The responsiveness of Lyon-ES cells to kisspeptin-10 suggests these cells might express Kiss1r endogenously and these are pluri-potent cell lines (Wianny et al., 2008). In another study, this inhibition was only witnessed in cells transfected with a Kiss1r expressing plasmid (leading to higher kiss1r expression) (Ziegler et al., 2013). Although the cancerous cells Zeigler et al. (2013) used endogenously expressed Kiss1r, kisspeptin treatment did not have an anti-proliferative effect, suggesting that kisspeptin-10 anti-proliferation was dependent on high levels of Kiss1r expression. It is possible that in our study the Kiss1r expression was increased under the effect of kisspeptin-10 as the number of GnRH cells was also increased under kisspeptin effect and with time. This clearly suggests that kisspeptin treatment increased the Kiss1r expression which alternately increased the GnRH expression. This finding is consistent with another study, which reported an increase in Kiss1r expression in the frog's testis following kisspeptin treatment (Chianese et al., 2013). It is likely that the resulting Lyon-ES neuronal stem cells endogenously express kiss1r, which may have increased under the effect of kisspeptin treatment.

Further validation and independent replication of our findings is needed to reach a complete answer, as is more detailed and focus studies aimed at examining the expression in affected cell lines.

Cellular differentiation potentials are proxy to the early rosettes stage and differentiation is either lost or decreased if the rate of proliferation increases (Jessell, 2000). The higher doses of kisspeptin-10 in the present experiment caused a decrease in the cell proliferation as well as a marked decrease in the number of rosette formation, suggesting that the cells had begun to differentiate in response to kisspeptin signals. However, it was not clear which specific pathway kisspeptin utilizes: notch pathway or Sonic Hedgehog (SHH) signaling, which are both necessary to maintain the R-NSC stage. A previous report found that in the absence of these pathways, neuronal stem cells decreased rosette numbers and formed more specific neuronal cell types (Doe et al., 2008; Huma et al., 2013). These findings are consistent with the observations of the present study, especially the morphological changes and ICC data in the Lyon-ES cell line, all of which suggest that kisspeptin specifically modulates the formation of GnRH neurons during development. Accordingly kisspeptin may be a potentially viable therapeutic for treating IHH and other related secondary HH disorders which manifest themselves due to GnRH dysfunction in the brain (Figure 5.2).

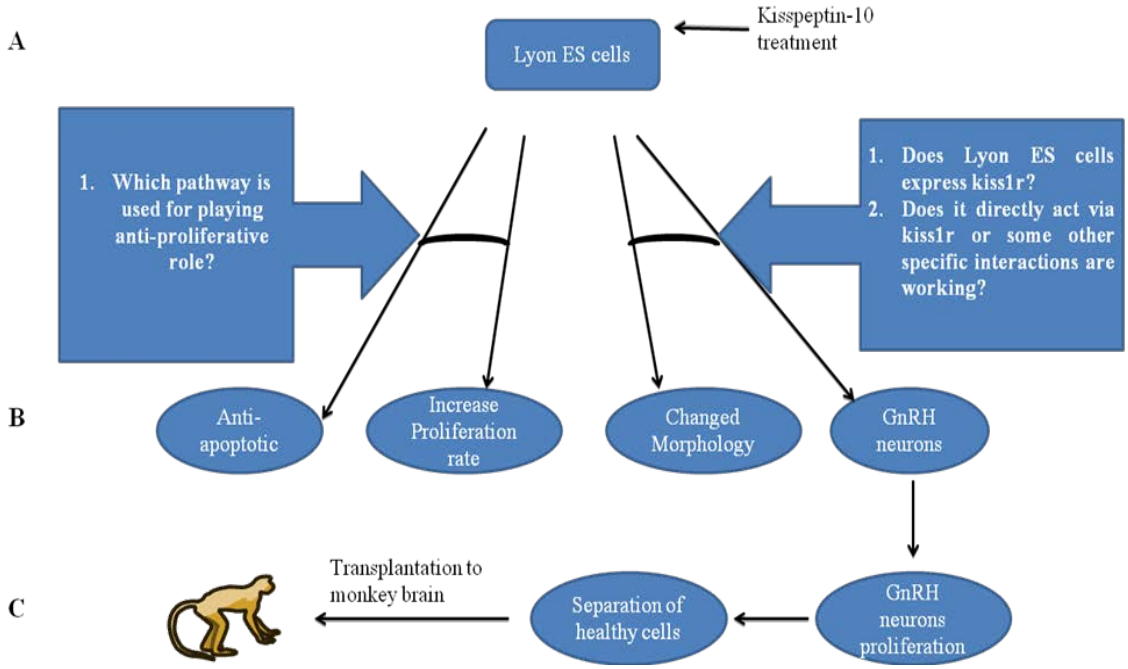


Figure 5.2: Diagrammatic presentation of possible future research and application of the Lyon ES cells.

5.2. Conclusions and Future Perspectives

Present studies give a number of ideas to suggest multi-directional future research . The P234 was the effective Kiss1r antagonist as it blocked peripheral kiss1r and we observed decreased adiponectin release following P234 treatment. Simple P234 did not cross the BBB and needs the penitratin to be mixed for crossing BBB. It has blocked the Kiss1r at the ME level. Further, we need to compare the effect of peripheral administration of P234 and P234-penetrating mixed to further check the effectiveness of the P234 in monkeys.

Seasons appear to play a key role in the rhesus reproduction. This seasonal reproduction in adult male rhesus monkeys is regulated by kisspeptinergic signaling. Semi quantitative differences in the MBH expression of the kisspeptin mRNA and protein during the breeding and the non breeding seasons in adult male rhesus monkeys were demonstrated. The expression of hypothalamic Kiss1, Kiss1r and their close contacts with GnRH fibers were found to be higher during the breeding season. Further, our findings establish the season related changes in the expression of Kiss1r on GnRH neurons for the first time. Based upon these findings, we postulate that kisspeptin acts as an important arbitrator of the various environmental cues on the reproductive axis. It acts as an excitatory factor to enhance the GnRH pulse generating activity during breeding season and a decrease in kisspeptin signaling leads to commencement of the non-breeding season in higher primates. Now, what are the inter-neurons, which are conveying the melatonin signals to kisspeptin neurons still need to be studied in detail to get a clear picture.

Thirdly, from the stem cell study, we can conclude that the formation and development of the GnRH neurons resulting from the application of kisspeptin-10 to the Lyon-ES cells is a novel and potentially significant finding. Specifically, in subjective case of IHH, the improper migration of GnRH neurons and disability to secrete GnRH are known as major underlying factors, but there are at present no viable treatment options. Our findings of kisspeptin’s effect on Lyon-ES cells open the doors to new possibilities for treating such disorders and its co-morbidities, such as the infertility that corresponds with the hypothalamic pituitary level, which has

previously proven resistant to more effective treatments. In theory, if our work could be extended to safe transplantation of GnRH neurons into relevant non-human primate models, we would have a foundation for developing treatments for disorders such as IHH related infertility (Figure 5.2).

Our results are applicable to the broader question of understanding how to control cell signaling, which is the first major hurdle to overcome in developing more effective, novel ES cell-based therapeutics.

Overall, from the present study, we may assume that kisspeptin infusion increased kisspeptin release, which in response stimulated the testosterone release thus triggering reproductive axis for reproductive potential while the effect is being abolished by systemic infusion of P234. Reproduction is an energy dependent process regulated by different hormones released by adipose tissue including adiponectin. Kisspeptin signaling reduced following the systemic infusion of P234 and so do the kisspeptin dependent testosterone release was decreased. During breeding season the kisspeptinergic signaling increased, resulting in the up regulation of the HPG axis. This was further confirmed by the Lyon ES experiment in which kisspeptin treatment increased the GnRH like neuronal expression. Despite our findings, the actual mechanism/pathways involved are still not very clear (Figure 5.3).Based on our findings the present study, a plan for future studies is proposed in Figure 5.4.

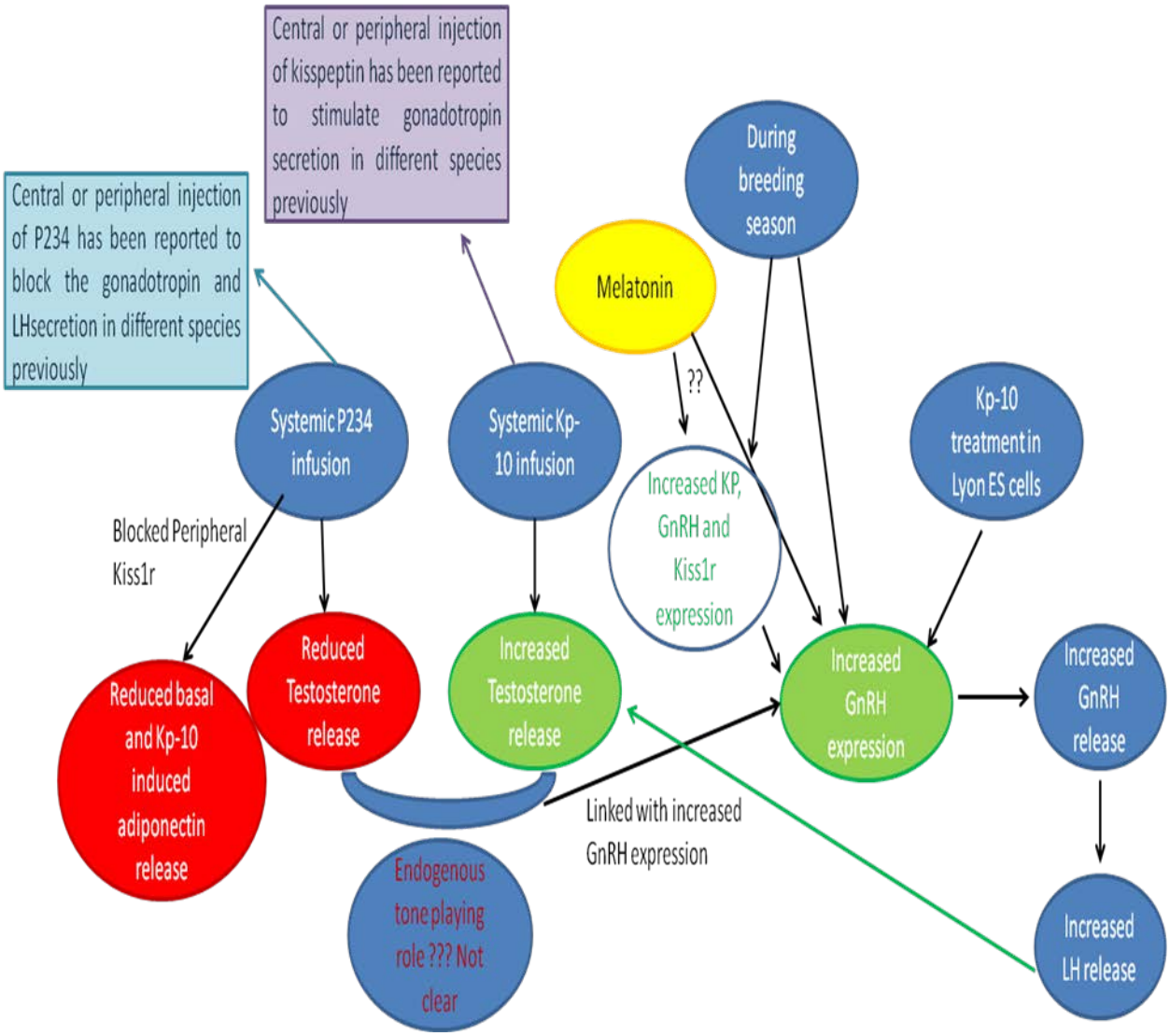


Figure 5.3: Overall conclusion of this thesis.

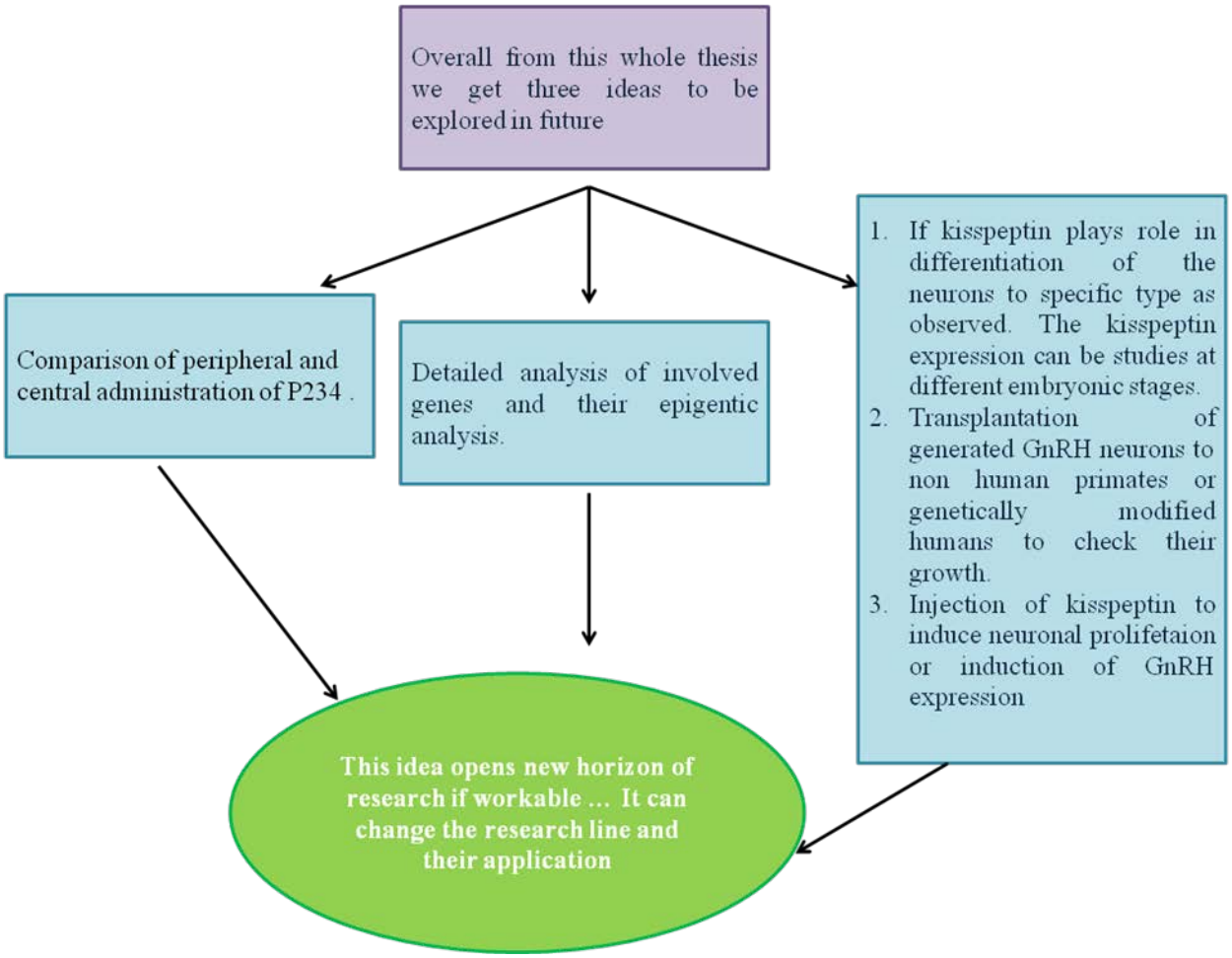


Figure 5.4: Diagrammatic presentation of future overall future perspectives.

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