Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

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2023

STUDY OF INTERACTION BETWEEN NPY AND KISSPEPTIN NEURONAL SYSTEM IN THE HYPOTHALAMUS DURING PUBERTAL DEVELOPMENT IN THE MALE RHESUS MONKEY (*Macaca mulatta*)

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

BY

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QUAID-I-AZAM UNIVERSITY

ISLAMABAD, PAKISTAN

2023

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled "Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)" was conducted by **Ms. Riffat Bano** under the supervision of **Prof. Dr. Muhammad Shahab**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Zoology of Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of Endocrinology.

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Charms strike the sights, but merit wins the soul

DEDICATED TO

MY LOVING AND CARING

MOTHER: KHURSHIED BANO

FATHER: MUHAMMAD DILAWAR KHAN

AND

MY MOST RESPECTABLE, HONORABLE,

HUMBLE AND CARING

SUPERVISOR: PROFESSOR DR. MUHAMMAD SHAHAB

Definitely both deserve a - reath of rau - el while I am going to get feather in my cap. Diligent efforts and unprecedented support of my humble supervisor and impulsive sharing of my beloved parents paved my way to success and one my soul.

In the name of Allah, The Most Gracious, The Most Merciful.

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ACKNOWLEDGEMENTS

All the praises and appreciations are for omnipotent *Allah,* the most merciful and generous that knows better the hidden truths of the universe. First and foremost, I would like to thank Allah, the eternal source of knowledge, Who gave me strength and enabled me to undertake and execute this research task. It is His mercy that He bestowed mankind with the courage, potential and ability to make worthy contributions to the already existent and continuously expanding sea of knowledge. I pay my deepest respect to *Holy Prophet Muhammad (Peace Be Upon Him)* who declared it an obligatory duty of everyone to seek and acquire knowledge. His influential teachings have severed as a source of strength and inspiration to all of us in each aspect of life.

First and foremost, I offer my sincerest gratitude to my supervisor, Dr. Muhammad Shahab, who has supported me throughout my study with his patience and knowledge whilst allowing me the room to learn. I was extraordinarily fortunate in having him as my Professor in Quaid-i-Azam University during my Master and M.Phil. degree as I could never have embarked and started all of this research journey without his prior teachings. I am thankful to him for giving me extraordinary experiences throughout the work. I am much indebted to him for his valuable advice in discussion, and for his constant motivation and believing in me to surpass this challenge. Furthermore, I humbly acknowledge him for his precious time to guide but foremost thanks to him for his patience to answer some of my unintelligent questions. His truly scientist intuition has made him as a constant oasis of ideas and passion in science, which exceptionally inspire and enrich my growth as a student, as researcher and as scientist want to be. I am indebted to him more than he knows. His ideas and concepts have had a remarkable influence on my entire career in the field of reproductive neuroendocrinology research. I learnt from him “Perfection is not attainable, but if we chase perfection, we can catch excellence.”

I would also like to thank Dean, Faculty of Biological Sciences Dr. Sarwat Jahan, and Chairperson of the Zoology Department Dr. Amina Zuberi for their cooperation and efficient administrative services. I also want to thank my M.Sc. teachers Dr. Shahzad

Mufti (Late), Dr. Samina Jalali, Dr. Irfan Zia Qureshi, and Dr. Riffat Naseem Malik who always supported and encouraged me.

I remain indebted to all my school, college, and university teachers for providing me the means to learn and for their guidance, love, and care.

I feel highly privileged in taking opportunity to express my deep sense of gratitude to Professor Richard Palmiter, Professor of Biochemistry Investigator, Howard Hughes Medical Institute (HHMI) at the University of Washington, Seattle for giving me opportunity to work in his laboratory. His wide knowledge and his logical way of thinking have been of great value for me. I am thankful for his kind and empathetic nature. My warm and sincere thanks especially go to Dr. Stephanie L. Padilla for her nice company and guidance during my six months stay at the University of Washington.

How can I forget the love, care, and friendly company of sweetest Shehla Baji and her mother (late) during my stay in USA on International Research Support Initiative Program (IRSIP). I want to thank my sister for her sweet company and life lasting memories. I really felt that I am the part of her family. I am highly indebted for your hospitality. Thanks also extended to Sohail Bhai (Brother of Shehla Baji) for his valuable company and sincere advice during my stay in USA. I am thankful to him for his enlightenment discussions. I would also want to thank my dear friend Shama Usman and her family living in Seattle for their joyful company. I am thankful to her for her hospitality and time during which I could visit different places in Seattle. Thanks, also extended to everyone in USA who took care of me during my stay. I cannot forget the sincere warmth and welcoming behavior of US people.

I would like to thank Higher Education Commission for sponsoring my trip to one of the prestigious labs of USA under International Research Support Initiative Program (IRSIP).

I would like to thank Brigadier. Dr, Hafeez ud Din (Commandant) and Colonel Dr. Dawood Ahmad (Head of Immunology Department) CMH, Rawalpindi for giving me opportunity to do sectioning of brain tissues on cryostat and to do fluorescent microscopy in their prestigious institute.

I would also like to acknowledge the cooperative attitude shown by the entire Faculty of Zoology Department whenever I approached them for their endless services and advice. I wish to extend my warmest thanks to all those who have helped me with my work in the Faculty of Biological Sciences, Quaid-i-Azam University.

In my daily work I have been blessed with a friendly and cheerful group of junior students and collective and individual acknowledgments are also owed to my juniors' colleagues who all were with me during my PhD. It is very hard to write names of all juniors so for that I make a humble apology but believe me I will always pray for you. Thank you Dr. Ajmal (my junior lab fellow) for supporting me in my hard times. Special thanks extended to my junior fellow and a sister to me Dr. Sadia Batool for her presence somehow perpetually refreshed, helpful, and memorable. Sadia I am in debit for your love and care. I am thankful to all my friends specially Dr. Ayesha Ishtiaq (Department of Biochemistry, QAU), Dr. Anham Zafar and Sadia (Department of Chemistry, QAU), and Dr. Farwa Naqvi (Department of Zoology, QAU) for their countless prayers, love, and support.

It is a pleasure to convey my gratitude to all my friends, colleagues, students, juniors, and seniors who prayed for me during my journey of Ph.D., in my humble acknowledgment.

I wish to express sincere thanks to my dear friends and seniors Dr. Saima Jabeen, Dr. Noureen Akhtar, Dr. Sumbal, Dr. Shazia Shams (Zoology Department), Dr. Ammara (IBGE Islamabad), Dr. Noshaba, Dr. Maleeha, (Microbiology Department) Dr. Munnaza (Biotechnology Department), Dr. Hina Salahuddin, (Plant Sciences Department), Dr. Marium Anees (Biochemistry Department), Dr. Saira, Dr. Shumaila, Dr. Saba (Chemistry Department), Dr. Sara, Dr. Sania (Biochemistry Department) Rubina Api (Lecturer Computer Sciences Department), and Zeeshan Kashif Bhai (Lecturer Punjab College, Rawalpindi) for their prayers, continued encouragement and moral support.

A very special and sweet thanks with deepest sense of gratitude to my closest friend Dr. Aalia Batool for her valuable love, care, support, and time to listen to me whenever I needed her. I can never forget her valuable advice and love she poured on me. Her presence in my life and her persistent encouragement gave me constant courage to face every

challenge with valor and to come off with flying colors. Words are short to pay my sincere gratitude for her.

I want to thank my roommates Miss Fatima Safna (BS student of Microbiology Department from Sri Lanka at Quaid-i-Azam University, Islamabad), and Kulsoom (Ph.D student of Pharmacy Department at Quaid-i-Azam University, Islamabad) during my first ever stay in hostel at Quaid-i-Azam University, Islamabad. They were very cooperative, courteous, and friendly.

I offer my gratitude to Muhammad Anwar, Muhammad Sajid (Lab Assistants Reproductive Neuroendocrinology) and Dr. Dilpat Rai (veterinarian) and Manzoor Bhai (Animal caretaker) Department of Zoology, QAU. for their cooperation during my research work in dissections and blood sampling. I wish to express sincere thanks to Mr. Naeem Masih (Office administrator Department of Zoology) and lab assistants from different laboratories who had given me so much respect.

I also want to thank my dear students who always gave me a lot of respect, honor, and appreciation for my role as teacher.

Where would I be without my family? My parents deserve special mention for their inseparable support and prayers. My Father, Muhammad Dilawar Khan, in the first place is the person who put the fundament my learning character, showing me the joy of intellectual pursuit ever since I was a child. My Mother Khurshied Bano is the one who sincerely raised me with her caring and gentle love. Without the prayers of my sweet mother, I am nothing. I would like to thank my parents for their motivation and trust in my decisions. Thank you for your unconditional support with my studies. Thank you for giving me a chance to prove and improve myself through all walks of my life. I would like to pay my gratitude and thanks to my loving and dear brothers Nadir, Anwar, Babar, Salman and Usman and my sisters Naheed, Nighat, and Nuzhat, Thanks also extended for my brother in laws Aamir Bhai, Saif Bhai and Anwar Bhai and sisters in law Nabeela Bhabi and Sana Bhabi for their prayers, support, and encouragement that made me very special. I want to say thanks to all my family and cousins who supported me.

Last but not the least, I owe great thanks to Eishah, Tayyab, Wania, Abdullah, Ayesha, Maham, Hania, Ali, and Siddiq (my nephew and nieces) as they always revitalized and recharged me with their love, innocence, and youthful company whenever I was down and out.

Thank you everyone for believing in me.

Finally, I would like to thank everybody who remembered me in their prayers and raised their hands for me as well as I want to express my apology if I forgot to mention anyone.

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

RIFFAT BANO

LIST OF ABBREVIATIONS

AgRP	Agouti-related peptide
am	Ante meridiem
ANOVA	Analysis of variance
Antg	Antagonist
ARC	Arcuate
AUC	Area under the curve
AVPV	Anteroventral periventricular nucleus
BNST	Bed Nucleus of the Stria Terminalis
bp	Base pair
BSA	Bovine serum albumin
BW	Body weight
ºC	Centigrade
Ca^{2+}	Calcium divalent ion
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
CNS	Central nervous system
CSF	Cerebrospinal fluid
Ct	Cycle threshold
C-terminal	Carboxy terminal
DAG	Diacylglycerol
DBB	Diagonal band of broca
dbl	Double labelled
DEPC	Diethylpyrocarbonate
DMH	Dorsomedial hypothalamus
DMSO	Dimethyl sulfoxide
DYN	Dynorphin
EIA	Enzyme immune assay
ELISA	Enzyme linked immunosorbent assay
ERα	Estrogen receptor alpha

ERK	Extracellularly regulated kinase
4PL	Four parameters logistic
FSH	Follicle–stimulating hormone
GABA	Gamma aminobutyric acid
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GnIH	Gonadotropin inhibitory hormone
GnRH	Gonadotropin– releasing hormone
GPCRs	G-protein-coupled receptors
GPR54	G-protein-coupled receptor 54
GTP	Guanosine-5'-triphosphate
HPG axis	Hypothalamic-pituitary-gonadal axis
hr	Hour
HRP	Horse reddish peroxidase
icv	Intracerebroventricular injection
ip	intraperitoneal
IGF-1	Insulin like growth factor 1
IgG	Immunoglobulin G
IHH	Idiopathic hypogonadotropic hypogonadism
im	intramuscular
IP3	Inositol-1, 4, 5-triphosphate
IR	Immunoreactive
IU	International unit
iv	Intravenous
kg	Kilogram
Kir	Potassium inward rectifying channel
KP-10	Kisspeptin peptide 10
KISS1	Kisspeptin gene (human and non-human primates)
Kiss1	Kisspeptin gene (non-primate)

KISS1	Kisspeptin protein (human and non-human primates)
Kiss1	Kisspeptin protein (non-primate)
KISS-1	K (Hershey 'Kisses'), iss (interim nomenclature for suppressor sequence)1
KISS1R	Kisspeptin receptor (human and non-human primates)
Kiss1r	Kisspeptin receptor (non-primate)
KISS1R	Kisspeptin receptor gene (human and non-human primates)
Kiss1r	Kisspeptin receptor gene (non-primate)
KNDY	Kisspeptin/ neurokinin B/ dynorphin
KP	Kisspeptin
L	Length
LH	Luteinizing hormone
µg	Microgram
µl	Microliter
MAPK	Mitogen-activated protein kinase
MBH	Mediobasal hypothalamus
ME	Median eminence
mg	Milligram
min	Minutes
MKRN3	Makorin ring finger protein 3
ml	milliliter
mm	millimeter
mRNA	Messenger ribonucleic acid
n	Number of animals
NaCl	Sodium chloride
NDS	Normal donkey serum
NGS	Normal goat serum
NIH	National institute of health
NKB	Neurokinin B
NKB3R	Neurokinin B receptor

NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y gene (human and non-human primates)
Npy	Neuropeptide Y gene (non-primate)
NPY	Neuropeptide Y protein (human and non-human primates)
Npy	Neuropeptide Y protein (non-primate)
NPY1R	Neuropeptide Y 1 receptor gene (human and non-human primates)
NPY1R	Neuropeptide Y 1 receptor protein (human and non-human primates)
Npy1r	Neuropeptide Y 1 receptor gene (non-primate)
Npy1r	Neuropeptide Y 1 receptor protein (non-primate)
ng	nanogram
OD	Optical density
OVX	Ovariectomized
%	Percent
p	Short arm of chromosome or probability
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	Potential of hydrogen
PIP2	Phosphatidyl inositol bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
pm	post meridiem
POA	Preoptic area
PP	Pancreatic peptide
PVH	Paraventricular hypothalamus
PYY	Peptide YY
q	Long arm of chromosome
RF-amides	Arginine-phenylalanine-amides
RFRP	RF amide related peptide

RNA	Ribonucleic acid
rpm	Revolutions per minute
RT–PCR	reverse–transcription polymerase chain reaction
RT-qPCR	Real-time quantitative PCR
sec	second
SEM	Standard error of mean
STAT3	Signal transducer and activator of transcription 3
TMB	3, 3', 5, 5'- tetramethylbenzidine
TRPC	Transient receptor potential cation channel
US	United States
µM	Micromolar
µm	Micrometer
V	Volume
veh	Vehicle
VMH	Ventromedial hypothalamus
W	Width

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Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

GENERAL ABSTRACT

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Introduction: The neuroendocrine mechanism underlying intermittent pattern of GnRH secretion by gonadotropin-releasing hormone (GnRH) pulse generator activity during postnatal development mimicking sinusoidal wave nature, the crest of which experience at infantile and adult stage characterizing mini and true puberty intervene by a trough at juvenile stage in which pulse generator brought into check resembling hypogonadotropic state, still needs to be meticulously defined. Particularly, the quest to find neurobiological central restrain that keeps GnRH pulse generator activity at nadir during juvenile period is still an intriguing mystery. Before the advent of kisspeptinocentric era, the role of neuropeptide Y (NPY) has been contemplated as potent neuroinhibitory factor in restricting GnRH/LH release in various species including non-human higher primates. Furthermore, owing to the heterogeneity of NPY receptors, pharmacological intervention with various antagonists partially supports NPY inhibition of GnRH/LH release via NPY 1 receptor (NPY1R). Since the discovery of kisspeptin different physiological, pharmacological, clinical, and genetic approaches have been implemented to decipher the prime importance of kisspeptin as component of the GnRH pulse generator to trigger pubertal onset in primate and non-primate mammals. Albeit kisspeptin has been shown to be an integral component of the GnRH pulse generator but not the trigger for pubertal onset. Close proximity between the two neuropeptides and NPY's direct regulation of kisspeptin neurons reaffirm the significance of these two neuropeptides in regulating reproductive axis activity. These observations harken back to the concept that diminishing activity of GnRH pulse generator before pubertal onset may be attributed by NPY which may act as a central brake for kisspeptin neurons. At the time of puberty, the attenuation of NPY brake may cause instigation of hypothalamic-pituitary-gonadal (HPG) axis activity via kisspeptin dependent pathway. The current investigations were designed to examine the interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey by performing three experiments. First experiment was to see correlative fluctuations in the relative mRNA expression of key neuropeptides (*NPY* and *KISS1)* and their receptors from hypothalamus encompassing mediobasal area. Second experiment was concerned about assessment of developmental variations in NPY

and NPY1R expression and their interaction with kisspeptin neurons during pubertal development. Third study designed to delineate the role of NPY1R in mediating the putative inhibitory action of NPY on the HPG axis after intravenous (*iv*) administration of highly selective NPY1R antagonist (BIBO 3304) in GnRH primed juvenile male rhesus monkey.

Materials and Methods: Fifteen intact male rhesus monkeys (*Macaca mulatta*) were used to conduct first two experiments. These animals were assigned into four different developmental groups [infant, (4-7 months), n=3], [juvenile, (10-14 months), n=4], [pre-pubertal, (21-24 months), n=4], [adult, (7-10 years), n=4] based upon physical (body weight and testicular volume), morphometric parameters (epithelial height and seminiferous tubule diameter) of testicular histology, and hormonal (testosterone) data. Hemi-hypothalamic blocks from each animal, snapped frozen in liquid-nitrogen then stored at -80 º C until processed for RNA extraction, were used to study differential expression of *KISS1* and *NPY* along with *KISS1R* and *NPY1R* through real-time quantitative PCR (RT-qPCR) by using gene specific primers. Other hemi-hypothalamic blocks from all fifteen animals after fixation (4% PFA) and dehydration (20% and 30% sucrose) were processed for cryosectioning to obtain 20 µm thick sections. These sections were used to assess morphological interactions and variations in NPY and NPY1R expression on kisspeptin neurons during pubertal progression through double label fluorescent immunocytochemistry using specific antibodies against kisspeptin, NPY and NPY1R. In addition, four GnRH primed juvenile intact male rhesus monkeys (*Macaca mulatta*) 10-14 months old were used to decipher the effect of intravenous (*iv*) administration of NPY1R specific antagonist BIBO 3304 on the HPG axis activity indirectly via testosterone measurement as a surrogate of LH. Sequential blood samples (~ 0.5-0.7 ml) at 30 min interval, three samples at -60, -30, 0 min before and nine samples from 30 min up to 270 min after vehicle or NPY1R antagonist (BIBO 3304) administration were withdrawn. Three *iv* boli (1ml/animal) at 0, 60 and 120 min of vehicle or BIBO 3304 were administered. Plasma was harvested and used to measure plasma testosterone concentration by commercially available human testosterone ELISA kit.

Results: Significant uprise in both mRNA of *KISS1* and its receptor *(KISS1R)* ($p<0.05$-0.01) was noted in adult animals in comparison to pre-pubertal animals. Contrasting to kisspeptin and its receptor, *NPY* and its receptor *NPY1R* showed significant decrease ($p<0.05$) in mRNA expression in adult group with respect to preceding age group. Inverse correlative age-associated fluctuations across pubertal development stages were also evident in pre-pubertal group with respect to infant animals. As pre-pubertal group manifested significantly higher ($p<0.05$) relative expression of *NPY* mRNA and insignificant ($p>0.05$) decrease in *KISS1* relative mRNA. The same pattern of expression was also observed for *NPY1R* and *KISS1R* mRNA. Immunofluorescent data revealed significantly augmented ($p<0.001$ and $p<0.05$) percentage interaction of kisspeptin neurons with NPY in juvenile and pre-pubertal monkeys as compared to infant monkeys. Moreover, significant decrease ($p<0.01$ and $p<0.05$) percentage of dual labelled kisspeptin and NPY was evident in adult and pre-pubertal group in comparison to juvenile group. Percentage of kisspeptin neurons with NPY1R expression showed significant increase ($p<0.01$ and $p<0.001$) in juvenile and pre-pubertal animals in comparison to infant. In contrast, the percentage of dual label kisspeptin neurons with NPY1R showed significant decrease ($p<0.001$ and $p<0.01$) in adult group as compared to pre-pubertal and juvenile group, respectively. Intravenous (*iv*) administration of NPY1R specific antagonist showed that neither treatment nor time has a significant effect on testosterone secretion. Moreover, no significant difference in mean testosterone levels and area under the curve (AUC) for testosterone between vehicle or antagonist administered animals was noted. No significant difference in mean pre- and post- BIBO 3304 or vehicle testosterone secretion was also observed.

Conclusions: In summary gene and protein expression analyses demonstrated that suppression of the kisspeptin neurons may be attributed due to enhanced activity of NPY neurons. Increased expression of NPY and NPY1R and decreased expression of kisspeptin and kisspeptin receptor during juvenile/pre-pubertal stage may be interpreted that enhanced NPY signaling may downregulate GnRH pulse generator activity by diminishing KISS1-KISS1R signaling. Developmentally increased expression of NPY1R on kisspeptin neurons during pre-pubertal/juvenile stage is also suggestive of NPY mediated inhibition

of kisspeptin neurons via NPY1R. Thus, enhanced NPY signaling may act as upstream central neurobiological brake for dormancy of kisspeptin neurons keeping GnRH pulse generator in check during pre-pubertal period, while alleviation of NPY brake may likely to occasion kisspeptin dependent resurgence in the HPG axis activity at the onset of puberty. However, non-exclusive involvement of NPY1R in mediating the putative inhibitory action of NPY on the HPG axis during the juvenile period as evident from the pharmacological data is suggestive to reappraise the role of NPY via NPY1R signaling in occasioning pre-pubertal suppression of GnRH pulse generator in rhesus monkey. The results of the present data corroborated the notion that suppression of the HPG axis during pre-pubertal stage after transient stimulation in infant stage may be contributed, at least in part, by NPY mediated suppression of kisspeptinergic drive to GnRH neurons. However, an extensive exploration about the functionality of NPY1R in mediating NPY based inhibition of GnRH release during the juvenile period remains to be substantiated by different experimental paradigms to unwind twists and turns in the pubertal development story. In addition, it is pertinent to propose that NPY mediated inhibition of the kisspeptin neurons may involve different receptor subtype other than NPY1R. Furthermore, NPY may act in conjunction with some other neurobiological component, and the collaborative message of these may be translated to repress GnRH pulse generator activity before pubertal onset. The nature of this component that regulates NPY-kisspeptin-GnRH pathway still needs to be defined explicitly. Nonetheless, current findings amplify the role of NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in non-human higher primates. These findings hitherto may be helpful to open new avenues in the development of novel clinical, pharmacological, and therapeutic strategies to cater fertility problems in humans.

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

GENERAL INTRODUCTION

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One of the most important and fundamental characteristics of species is to reproduce. Reproduction is the most complex phenomenon which ensures species progression. In higher mammals, the time at which first reproductive potential is attained is termed as puberty (Terasawa and Fernandez, 2001). The intricate process of puberty is essential for the initiation and maintenance of reproductive phenomena in mammals. Maturation of the reproductive neuroendocrine strata, comprised of hypothalamus, pituitary, and gonad, constitute hypothalamus-pituitary-gonadal (HPG) axis, is the prerequisite for this fascinating event to set up. Among primates, the reproductive axis is controlled by decapeptide gonadotropin– releasing hormone (GnRH), which is secreted in a pulsatile fashion (Conn and Crowley, 1994). The decapeptide GnRH released in pulsatile manner at median eminence (ME) that directs pituitary gonadotrophs to release gonadotropins, "luteinizing hormone (LH)" and "follicle–stimulating hormone (FSH)". These gonadotropins reach the distant target sites (testes and ovaries) to trigger steroidogenesis and gametogenesis, ensuring the reproductive capability to be achieved (Terasawa and Garcia, 2020).

Major intrinsic and extrinsic determinants controlling timing and subsequent reproductive potential encompass body fat content, dietary regime, strenuous situations, circadian rhythms, and steroid milieu. All these elements harmonize to impact on neuropeptides and neuromodulator at the hypothalamic level to regulate GnRH pulse generator activity hence play a pivotal role in initiation of puberty (Ebling, 2005). GnRH neurons form the final common pathway for the central control of reproduction/puberty, thus understanding the factors that control GnRH neurons is of fundamental importance. Different neurotransmitters and neuromodulators both stimulatory [glutamate, dopamine, norepinephrine, serotonin, galanin, and kisspeptin] and inhibitory [opioids peptides, gamma aminobutyric acid (GABA), gonadotropin inhibitory hormone (GnIH), and neuropeptide Y (NPY)] are involved in regulating GnRH release (Terasawa and Fernandez, 2001; Ebling, 2005).

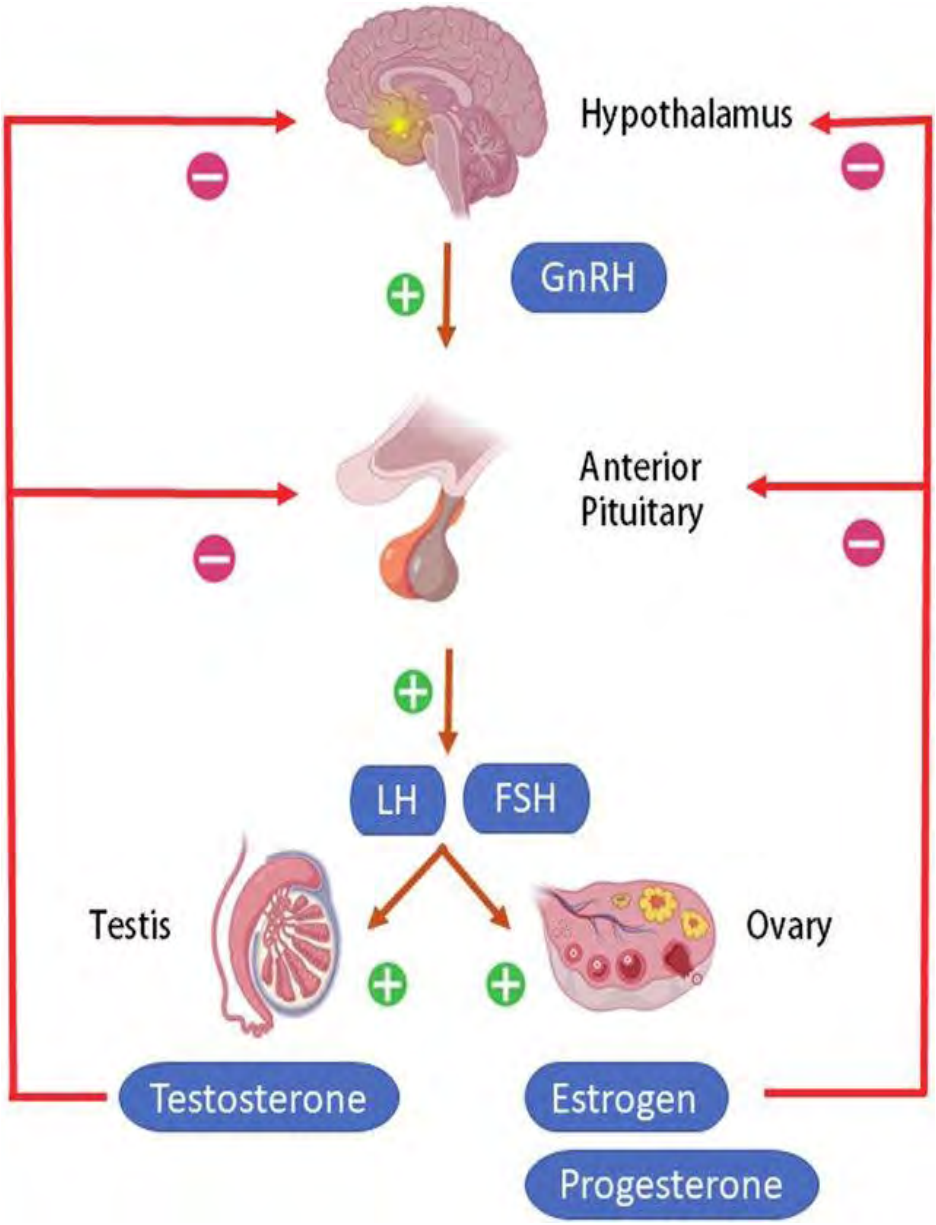

Figure i. Schematic presentation of HPG axis. (Plant and Steiner, 2022).

Puberty is an intricate neurophysiological process that ensures an individual to become capable of reproducing. Pubertal development is also characterized by morphological, developmental, physiological, and hormonal maturation of genital organs with secondary sexual characters. Developmental maturation of the gonads leads to the process of gametogenesis (spermatogenesis in males and ovulation in females) and steroidogenesis (production of testosterone and estrogen in male and female). Both these integral processes of pubertal development are dictated by gonadotropins (LH and FSH) of the anterior pituitary. The synthesis and secretion of these gonadotropins are, in turn, driven by inevitable rhythmic discharge of hypothalamic hormone, GnRH which is transferred to pituitary gonadotrophs by means of hypophysial portal circulation (Plant and Shahab, 2002).

Puberty is manifested endocrinologically by the heightened release of gonadotropin releasing hormone (GnRH) from the hypothalamus by which pituitary and gonads respond accordingly. Although GnRH secretion is dampened during the juvenile phase however the pituitary-gonadal component of the axis is still capable of responding. This has been shown in prepubertal male and female macaques where intravenous and median eminence infusion of N-Methyl-D, L-Aspartate (NMDA) caused robust discharge of LH (Gay and Plant, 1987; Plant *et al*., 1989; Terasawa, 2022). These observations showed that puberty onset is a hypothalamic/neurobiological event and GnRH neurons preserve their neurosecretory capability for hypophysiotropic drive even during juvenile period as in adults. It is interesting to note that pubertal onset is not the first occasion during postnatal developmental progression when GnRH secretion is heightened, in fact enhanced GnRH secretion is also well experienced during infancy (Plant, 2001; Terasawa, 2022). In both male and female monkeys augmented activity of the hypothalamic-pituitary-gonadal unit in terms of gonadotropins and testosterone release has been documented during infantile period just like in adults. Despite the higher levels of gonadotropins and testosterone, passive gametogenic activity of testes and ovaries in infantile animals has been described (Plant, 2015). Above mentioned information shows that increased hypothalamic-pituitary drive is of short life and gonads are not fully mature to respond to this drive as seen by the non-responsiveness of the Sertoli cells to FSH and androgen stimulation (Majumdar *et al*.,

2012). However, proliferation of spermatogonia type A and Sertoli cells takes place in infantile testes but the presence of type B spermatogonia which is a distinctive indicator of spermatogenesis is not observed (Simorangkir *et al*., 2003; Simorangkir *et al*., 2012). Interestingly, it has been demonstrated that during the juvenile phase when the gonadotropins levels become nadir, undifferentiated spermatogonia type A (both dark and pale) together with Sertoli cells continue to proliferate at this developmental stage (Marshall and Plant, 1996; Simorangkir *et al*., 2005). Infrequent differentiating spermatogonia type B are also observed in juvenile testes of highly evolved primates (Plant *et al*., 2005). In this regard, initiation of spermatogenesis in juvenile monkeys has been achieved after intermittent infusion of gonadotropins (Ramaswamy *et al*., 2000; Ramaswamy *et al*., 2017). It shows that potential of the gonads to respond to hypothalamic-pituitary drive is maximally attained during the prepubertal period. Nevertheless, at this stage GnRH secretion has been restrained by an unidentified neurobiological factor and therefore the quiescence of the prepubertal gonad is maintained.

The triggering agent required to initiate pubertal process emanates from the central nervous system. This triggering stimulus acts as a neural oscillator residing within hypothalamic area of forebrain. This oscillator is termed as GnRH pulse generator because of its ability to generate intermittent pattern of gonadotropin releasing hormone (GnRH) secretion. GnRH helps to relayed triggering agent information to the downstream targets i.e., pituitary and gonads. Although GnRH pulse generator secretory pattern seems to be fully functional by the postnatal period, sexual maturity is unable to be achieved until the age of 3 to 4 years (Plant, 2019; Terasawa, 2022).

The scattered network of GnRH neurons is believed to function in a synchronized manner for pulsatile release of GnRH to trigger gonadotropins secretion from pituitary gonadotrophs (Terasawa, 2022). Pubertal development in higher primates is governed by characteristic pattern of GnRH pulse generator activity from infancy to adulthood. An upsurge in GnRH pulse generator activity is evident during infancy and in adulthood. After brief stimulation of GnRH pulse generator during infancy, it is brought into dormant state during juvenile period before it is reevoked at the time of puberty thus, opting for on-off-on mode characterizing infant (mini puberty), juvenile (hypogonadotropic state), and

pubertal state (adolescent puberty), respectively. Therefore, pulsatile GnRH release at the end of juvenile period is the neuroendocrine hallmark for the onset of puberty. The ebb and flow pattern of GnRH pulse generator during pubertal development from infancy to adulthood is quite intriguing. Especially, neuroendocrine mechanism involved central hiatus of GnRH pulse generator during juvenile phase is still ambiguous (Terasawa, 2019; Witchel and Plant, 2021).

Two different hypotheses have been devised to explain the physiological mechanism involved for pubertal onset. The first hypothesis entitled "gonadstat" hypothesis or "differential sensitivity to ovarian steroids" while the second hypothesis is known as "central inhibition of GnRH release".

With respect to preceding concept, higher brain centers concerning gonadotropins secretion becomes non-responsive to steroids at the age of sexual maturity. At that time the onset of puberty occurs. The said concept is congruent to the observations in rodents but not pertinent to primates. A characteristic feature of the succeeding hypothesis involves quiescent mode of GnRH release during juvenile state is due to activity of central neurobiological inhibitor and gonadal steroids feedback has no role in dictating pubertal onset. According to gonadstat hypothesis pre-pubertal suppression of gonadotropins discharge is due to increased sensitivity of hypothalamic GnRH pulse generator to gonadal steroids. However, such notion cannot be accounted for primates. This is because neonatal orchidectomy does not prevent pre-pubertal hiatus of gonadotropins both LH and FSH secretion. This observation seems to imply that pre-pubertal suppression of HPG axis is attributed by nongonadal factor (Plant and Zorub, 1982). Therefore, new concept of approach is to decipher nature of the switch which causes hibernating mode of GnRH pulse generation during juvenile stage (Terasawa and Fernandez, 2001; Plant and Shahab, 2002).

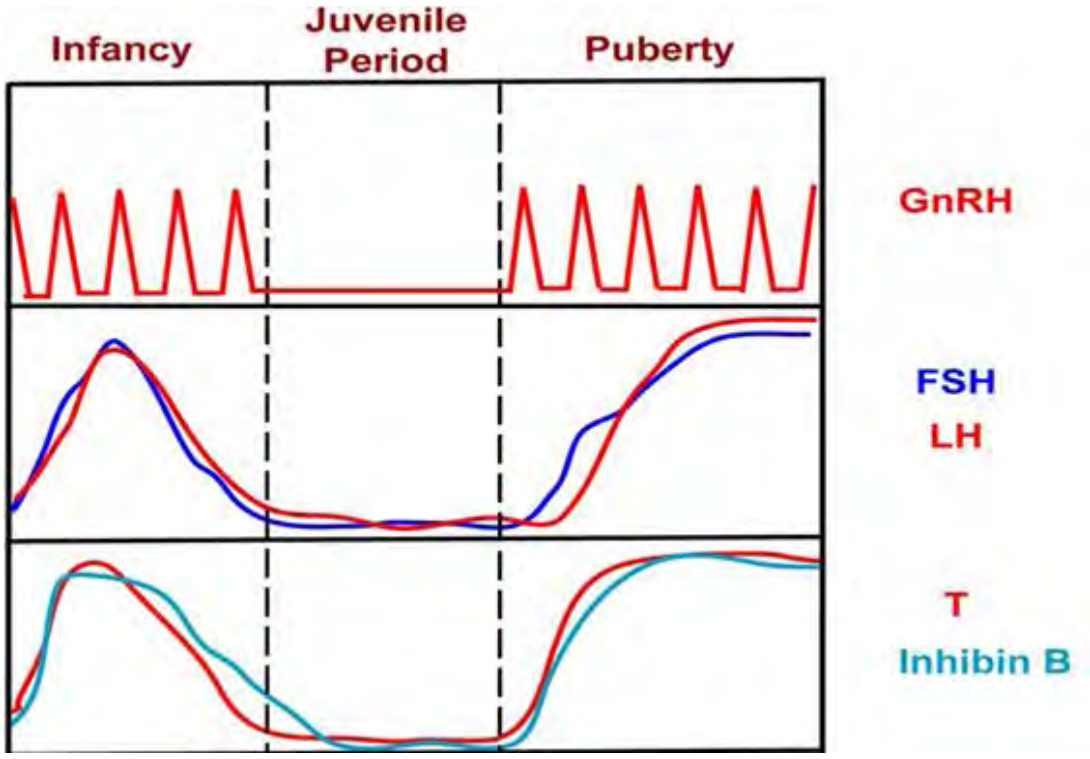

Figure ii. Schematic presentation indicating fluctuations in the levels of GnRH, pituitary, and gonadal hormones during developmental events (infancy, juvenile, and pubertal stage). (Bhattacharya *et al*., 2019).

Among various neuropeptides kisspeptin (stimulatory) and NPY (inhibitory) are the most noteworthy candidates implicated to enkindle pubertal onset.

From the last two decades, kisspeptin (KP) has emerged as the most potent neuropeptide that plays a vital role to instigate resurgence in pulsatile GnRH secretion culminating in pubertal onset (Terasawa, 2022). KISS-1 was isolated by Lee and colleagues in 1996 in human melanoma as metastasis suppressor gene. KISS-1 encodes a prepro 145 amino acid product, which upon post-translational modification leads to several peptides known as – kisspeptins. Long arm of chromosome 1 harbor KISS-1 gene containing 4 exons and it is also regulated by the elements on chromosome 6 (Lee *et al*., 1996; West *et al.,* 1998).

The largest family of receptors known as G-protein-coupled receptors (GPCRs) is mainly involved in transducing the message of a particular ligand. In this family many receptors have been identified without known ligand designating them as orphan receptors (Sakellakis, 2022). The receptor for kisspeptin was also an orphan receptor and was referred GPR54. In 1999, KISS1R, previously known as GPR54 spanning seven trans membrane domains, was first isolated from rat brain (Lee *et al*., 1996). The receptor belongs to rhodopsin family of receptors (Kotani *et al*., 2001). Receptor protein comprises 398 amino acids in humans while 396 amino acids are beaded in rat/mouse, respectively (Muir *et al*., 2001). Localization of human KISS1R gene was mapped on chromosome 19 (19p13.3) containing 5 exons with 4 introns (Ohtaki *et al*., 2001). Indeed, KP-54 (metastin) is shown to be a potent ligand for GPR54 (Kotani *et al*., 2001). Human kisspeptin receptor showed 85% homology with rat kisspeptin receptor while 54% homology was observed with mice receptor (Kotani *et al*., 2001; Stafford *et al*., 2002).

Arginine phenylalanine (RF) amide peptide family is characterized by arginine-phenylalanine amide structure on carboxy terminal which is a characteristic feature of these peptides and KP is one of the members of RF amide family harboring RF amide motif at carboxy terminal (Dockray, 2004).

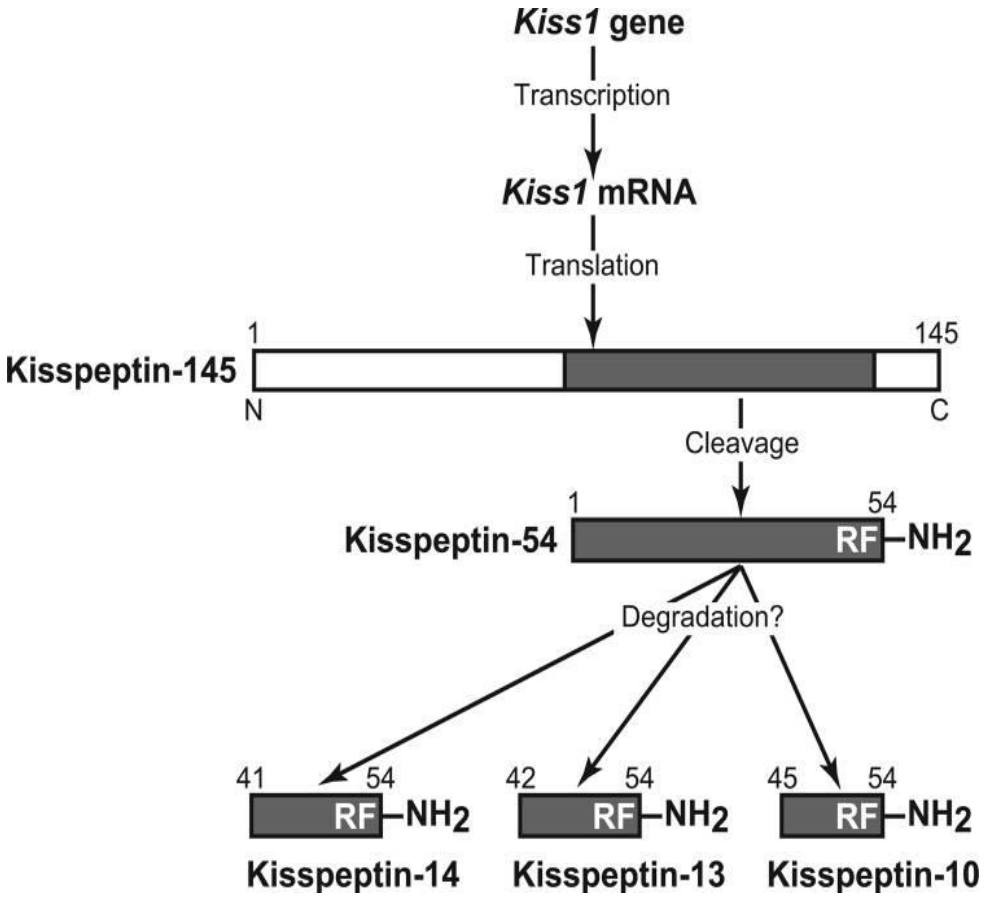

Figure iii. Transcription and translation of KISS1 gene leads to 145 prepropeptide. Post-translational splicing leads to the production of different biologically active products. (Oakley *et al*., 2009).

145 amino acids long prepro precursor of KP undergoes posttranslational splicing resulting in different biologically active products which are recognized according to the number of amino acids. As a result of splicing 145 amino acids long peptide cleaved into four (kisspeptin 54, kisspeptin 10, kisspeptin 14, and kisspeptin 13) biologically active shorter products (Kotani *et al*., 2001). The largest product is KP-54 containing all other shorter peptides. Kotani *et al*., 2001 named KP as kisspeptin-54, Muir *et at*., 2001 as KiSS-1 (68-121) and Ohtaki *et al*., 2001 as metastin. Differing at their amino termini all these post-translational products are pharmacologically active but have common amidated RF-carboxy terminus indicating that the C-terminal sequence is mandatory for receptor binding and for its downstream activation (Ohtaki *et al*., 2001).

Kisspeptin transmits its message via GPR54 or KISS1R, Gq subfamily of G-proteins pathway, which results in downstream calcium (Ca^{2+}) mobilization (Kotani *et al*., 2001; Stafford *et al*., 2002) as depicted in Figure ii. Kisspeptin interaction with KISS1R leads to the activation of phospholipase C (PLC) leading to conversion of phosphatidyl inositol bisphosphate (PIP2) into inositol-1, 4, 5-triphosphate (IP3). Furthermore, IP3 causes augmentation in intracellular Ca^{2+} to modulate permeability of ion channel and depolarization of cells. In addition, activation of protein kinase C (PKC) via diacylglycerol (DAG), activation of arachidonic acid release with mitogen-activated protein kinase (MAPK) pathways, and phosphorylation of extracellular signal-related kinase 1/2 (ERK1/2) and p38 MAP kinases are also involved in intracellular signaling pathways activated by the KISS1R (Kotani *et al*., 2001; Castano *et al*., 2009). In addition, inhibition of potassium inward rectifying channel (Kir) via activation of transient receptor potential cation channels (TRPC) and involvement of β arrestin 1 and 2 has also been shown to mediate KISS1R signaling. Thus, kisspeptin through KISS1R activates multiple MAPKs resulting in release of Ca^{2+} which contributes multiple functioning of kisspeptin like hormone secretion, neuroendocrine functions, antimetastatic, and antiproliferative effects (Xie *et al*., 2022).

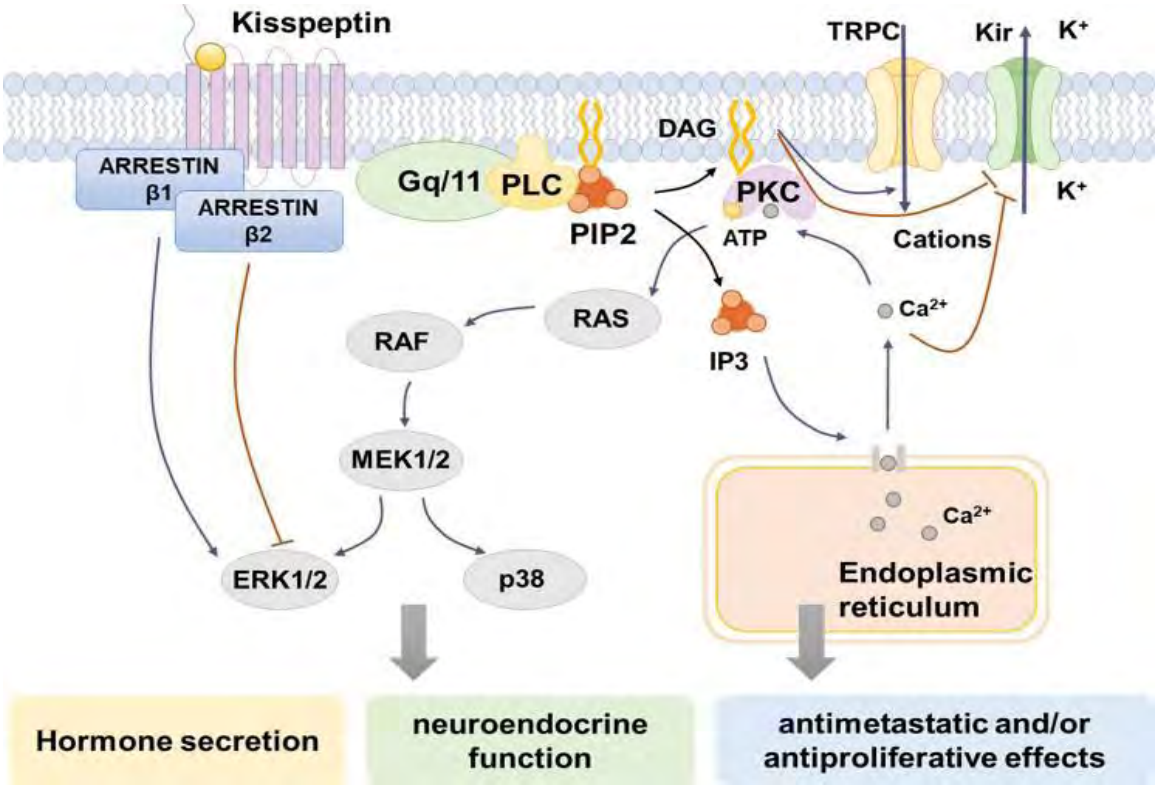

Figure iv. A schematic representation of the mechanism of action of kisspeptin. Kisspeptin interaction with KISS1R causes phospholipase C (PLC) activation leading to production of intermediatory molecules (IP3 and DAG). Furthermore, IP3 causes intracellular Ca^{2+} release to modulate permeability of ion channel and depolarization of cells. In addition, activation of protein kinase C (PKC) via diacylglycerol (DAG), inhibition of potassium inward rectifying channel (Kir) via activation of transient receptor potential cation channels (TRPC) and involvement of β arrestin 1 and 2 causes phosphorylation of mitogen-activated protein kinases (MAPKs) pathways to induce different physiological effects (Xie *et al*., 2022).

Topographical distribution of KISS1 and KISS1R has been extensively studied in various animal models like rats (Irwig *et al.,* 2004; Brailoiu *et al.,* 2005), hamster (Mason *et al*., 2007), guinea pig (Tomikawa *et al*., 2010), goat (Okamura *et al*., 2017), mice (Clarkson *et al.,* 2009), sheep (Estrada *et al*., 2006; Smith *et al*., 2009), monkey (Shahab *et al.,* 2005; Rometo *et al*., 2007), and humans (Hrabovszky *et al*., 2010). Expression of peptide and its receptor is documented in entire central nervous system (Muir *et al*., 2001). Majority areas of the brain showed clear evidence for the presence of kisspeptin via hybridization immunochemistry. Prominent domains where abundance of expression was observed include spinal cord, medulla and pons, amygdala, hypothalamus, midbrain, nucleus accumbens, thalamus, bed nucleus of stria terminalis, cerebral cortex, caudate putamen, parabrachial nucleus, periaqueductal gray, locus coeruleus, and raphe nuclei (Irwig *et al*., 2004). The majority of the kisspeptin neuronal populations chiefly reside within hypothalamus concerning neuroendocrine control. Arcuate (ARC) nucleus (equivalent to infundibular nucleus in humans) and anteroventral periventricular (AVPV) are two prominent sites where abundant expression of kisspeptin has been observed in most of the species including humans. Less prevalent areas of kisspeptin neurons include dorsomedial (DMH) [mice (Clarkson *et al*., 2009; True *et al*., 2011; Hanchate *et al*., 2012), guinea pig (Bosch *et al*., 2012), sheep (Smith *et al*., 2009)], ventromedial hypothalamus (VMH) [rat and mice (Kinoshita *et al*., 2005; Adachi *et al*., 2007; Desroziers *et al*., 2010; Dungan and Elias, 2012), sheep (Goodman *et al*., 2007)], medial amygdala [mice (Clarkson and Herbison, 2006; Clarkson *et al*., 2009), rat (Kinoshita *et al*., 2005; Adachi *et al*., 2007)], and bed nucleus of stria terminalis (BNST) [monkey (Rometo *et al*., 2007; Ramaswamy *et al*., 2010), mice (Clarkson *et al*., 2009; Hanchate *et al*., 2012), rat (Kinoshita *et al*., 2005; Adachi *et al*., 2007; Desroziers *et al*., 2010 ; Dungan and Elias 2012)]. Surprisingly, kisspeptin expression has also been noted in ME/Infundibular stalk in monkeys and humans (Goodman and Lehman, 2012). Minor expression has also been reported in areas like neocortex, insular cortex, piriform cortex, lateral septum (Lehman *et al*., 2013).

Likewise, KISS1R/GPR54 is expressed in most of the brain areas like pons, midbrain, thalamus, hypothalamus, hippocampus, amygdala, cerebellum, frontal cortex, periaqueductal gray, spinal cord, and striatum (Muir *et al*., 2001).

With respect to hypothalamic expression of receptor distinct expression in different nuclei of the hypothalamus was noted.

KISS1R has also been consistently observed in two hypothalamic areas; ARC and POA/AVPV, in all species which were examined. Representative animal from each specie includes sheep (Smith *et al.,* 2009), mice (Herbison *et al*., 2010), rhesus monkey (Shahab *et al.,* 2005), rat (Lee *et al*., 1999), and in highest representative primates i.e., humans (Muir *et al*., 2001). By utilizing in situ hybridization approach, mRNA expression for the receptor was noted in many eminent nuclei. Following these three nuclei/areas i.e., dorsomedial nuclei, anterior and ventromedial hypothalamic area, and preoptic areas, are chiefly reported to express GPR54. Moreover, diagonal band of Broca (DBB), anterior and lateral hypothalamus along with medial preoptic areas in rats have shown abundant expression (Irwig *et al*., 2004). In humans and rats, KISS1R also present in the hippocampus, locus coeruleus, and medial amygdala (Lehman *et al*., 2013).

Particularly, in rhesus monkey, kisspeptin neurons have been identified in POA (including the AVPV) and in the ARC/ME and medial basal hypothalamus (MBH) (Ramaswamy *et al*., 2008; Vargas Trujillo *et al.,* 2017). Above-cited hypothalamic nuclei were also found to have similar expression pattern for kisspeptin receptor in monkeys (Shibata *et al*., 2007).

In 2003, idiopathic hypogonadotropic hypogonadism (IHH) condition was noticed in persons exhibited GPR54 mutation by two different working groups from US and France (Seminara *et al*., 2003; de Roux *et al*., 2003), paved the path for indispensable role of kisspeptin as potent regulator of the HPG axis for pubertal initiation. After that gene knock out model for Kiss1 or Gpr54 has been recapitulated in rodents (Lapatto *et al*., 2007; d'Anglemont de Tassigny *et al*., 2007; Uenoyama *et al*., 2015) also revealed hypogonadotropic hypogonadism condition as observed in humans. Interestingly, it has been revealed that GPR54 mutations are not the only cause for hypogonadotropic hypogonadism condition in humans, in addition, inactivating mutations of KISS1 gene also lead to manifestation of similar condition (Topaloglu *et al*., 2012). Conversely, activating mutations of GPR54 caused precocious puberty (Teles *et al*., 2008). All these observations

suggest that kisspeptin-GPR54 signaling is the key regulator for activation of gonadotropic axis leads to pubertal onset in mammals.

A huge pile of studies has unmasked that central and peripheral injection of kisspeptin invigorate reproductive hormones secretion particularly gonadotropins in many animal models. Different researchers documented this observation in mice (Gottsch *et al*., 2004), sheep (Caraty *et al*., 2007), rats (Navarro *et al*., 2004a; Navarro *et al.,* 2005a, Navarro *et al.,* 2005b), pig (Lents, 2019), goat (Scott *et al*., 2018), cows (Dobson *et al.,* 2020), humans (Dhillo *et al*., 2005) and monkey (Plant *et al*., 2006a, Plant *et al*., 2006b). These findings foster the idea that kisspeptin is critical in regulating HPG axis activity and hence the reproductive functions.

Kisspeptin can act directly on hypothalamic GnRH neurons. This property of direct regulation of GnRH neurons by kisspeptin can be attributed owing to the presence of GPR54 on these neurons. The first study that demonstrated the localization of GPR54 on GnRH neurons was conducted in freshwater fish (Parhar *et al.,* 2004). It shows the potent stimulatory effect of kisspeptin on the reproductive axis through GnRH release. This notion has also been corroborated by the elevated expression of *c-fos* (an early gene marker) after kisspeptin administration (Han *et al*., 2005).

The notion that gonadotropins response to kisspeptin is regulated by GnRH is further implied by the abolished stimulatory effect of kisspeptin on LH/GnRH release after administration of GnRH antagonist-acyline (Shahab *et al*., 2005). Interestingly, specific deletion of GPR54 in GnRH neurons of mice leads to infertility (Kirilov *et al*., 2013). This observation suggests that activation of GPR54 is necessary to trigger GnRH secretion to induce fertility. The concept of direct regulation of GnRH secretion has also been affirmed by observing intimate association between two neuronal systems (kisspeptin and GnRH) in goats and rats (Matsuyama *et al*., 2011; Uenoyama *et al*., 2011).

Different hypothalamic nuclei behave differently after gonadectomy and sex steroid replacement. Arcuate nucleus has been shown to regulate negative feedback effect of sex steroids on kisspeptin neurons, however, AVPV is involved in positive feedback regulation

of kisspeptin neurons following steroid introduction (Smith *et al.,* 2005; Smith *et al.,* 2007). Same pattern of expression of kisspeptin neurons in both nuclei was observed in syrian hamster (Ansel *et al*., 2010). In case of monkeys, this feedback regulation of sex steroids has only been pertained to arcuate nucleus (Rometo *et al*., 2007). Particularly, AVPV kisspeptin neurons are sexually dimorphic (Wang and Moenter, 2020). These neurons are critical in inducing preovulatory LH surge (Kauffman *et al*., 2007). Nearly three quarter of AVPV kisspeptin neurons are shown to express ERα (Kumar *et al*., 2015). Therefore, it was demonstrated that heightened activity of kisspeptin neurons residing within AVPV indicated by increased expression of early gene marker (*c-fos*) and increased firing potentials during estrogen feedback is attributed via ERα (Wang *et al*., 2016). Despite of sexually dimorphic behavior of AVPV kisspeptin neurons, ARC kisspeptin neurons are not sexually dimorphic (Clarkson and Herbison, 2006).

Since reproduction is energy demanding process that is why, it has been suggested that kisspeptin detects body energy status to modulate reproductive capability and pubertal initiation by conveying metabolic information to GnRH pituitary axis (Castellano *et al*., 2005; Wahab *et al*., 2010). Decreased expression of kisspeptin peptide and its receptor was noted in rodents and non-human primates after short-term fasting (Luque *et al*., 2007; Wahab *et al*., 2011). Impact of under-nutrition causes abnormality in reproductive functions, but KP has the capacity to reinstate the defective function of gonadotrophic axis under such situation (Castellano *et al*., 2005; Castellano *et al*., 2009; Navarro *et al*., 2020). Different peripheral nutritional signals mediate metabolic regulation of kisspeptin neurons like leptin (Smith *et al*., 2006), IGF-1 (Hiney *et al*., 2009) and ghrelin (Forbes *et al.,* 2009) regulate Kiss1 and GPR54 mRNA expression within hypothalamic areas. Leptin involves positive regulation of kisspeptin expression (Smith *et al*., 2006). Decreased kisspeptin expression was observed in leptin knock out mice while leptin administration improved expression profile of kisspeptin (Smith *et al*., 2006). Leptin directly regulates kisspeptin neurons by means of leptin receptors which are present on approximately 40% of kisspeptin neuronal population (Smith *et al*., 2006; Cravo *et al*., 2011). In guinea pig ARC kisspeptin neurons are stimulated via leptin (Qiu *et al*., 2011). The stimulatory effect of ghrelin on kisspeptin expression has also been reported (Forbes *et al*., 2009).

Nevertheless, two reports challenged the direct action of leptin on kisspeptin neurons. In the first study, it has been observed that leptin failed to induce Signal transducer and activator of transcription 3 (STAT3) phosphorylation within kisspeptin neurons (Quennell *et al*., 2011). The second study in mice showed that targeted deletion of leptin receptor from kisspeptin neurons failed to disturb normal puberty onset (Donato *et al*., 2011). Thus, non-exclusive involvement of leptin receptor mediating pubertal onset in rodents via kisspeptin neurons raises the notion that some neurobiological factor is involved in diminishing kisspeptin neuronal activity before pubertal onset, and it might be direct target for leptin signaling also.

Although kisspeptin has been shown to be imperative in regulating GnRH secretion but the regulation of kisspeptin neurons themselves has not been attributed until the idea of KNDy neurons put forth by Goodman *et al*. 2007. It is worthy to mention that arcuate kisspeptin neurons have been shown to co-express triple neuropeptides, i.e., kisspeptin, neurokinin B (NKB) and dynorphin (DYN) thus designated the term KNDy neurons (Lehman *et al*., 2010). These KNDy neurons were conceptualized to act as a GnRH pulse generator (Goodman *et al*., 2013). The co-expression of these neuropeptides has been documented to be 100% in rats (Murakawa *et al*., 2016), mice (Navarro *et al*., 2009), goat (Wakabayashi *et al*., 2010), sheep (Goodman *et al*., 2007), and in humans (Hrabovszky *et al*., 2012). However, in rhesus macaques only fraction of cohort of arcuate kisspeptin neurons are documented to co-express these three neuropeptides (Ramasawmy *et al*., 2010; True *et al*., 2017). Therefore, in non-human primates these neurons are considered as components of the pulse generator. As far as physiological role of these neuropeptides is concerned, it is evident that neurokinin B and kisspeptin plays a stimulatory role, however, dynorphin is considered as inhibitory in controlling GnRH release (Goodman *et al*., 2013). The concept of autoregulation of KNDy neurons is conceived owing to the presence of NKB3R and dynorphin receptors on KNDy neurons itself (Goodman *et al*., 2013). Thus, stimulatory, and inhibitory action of NKB and dynorphin respectively decides the kisspeptin release as the output of these cells (Goodman *et al*., 2014).

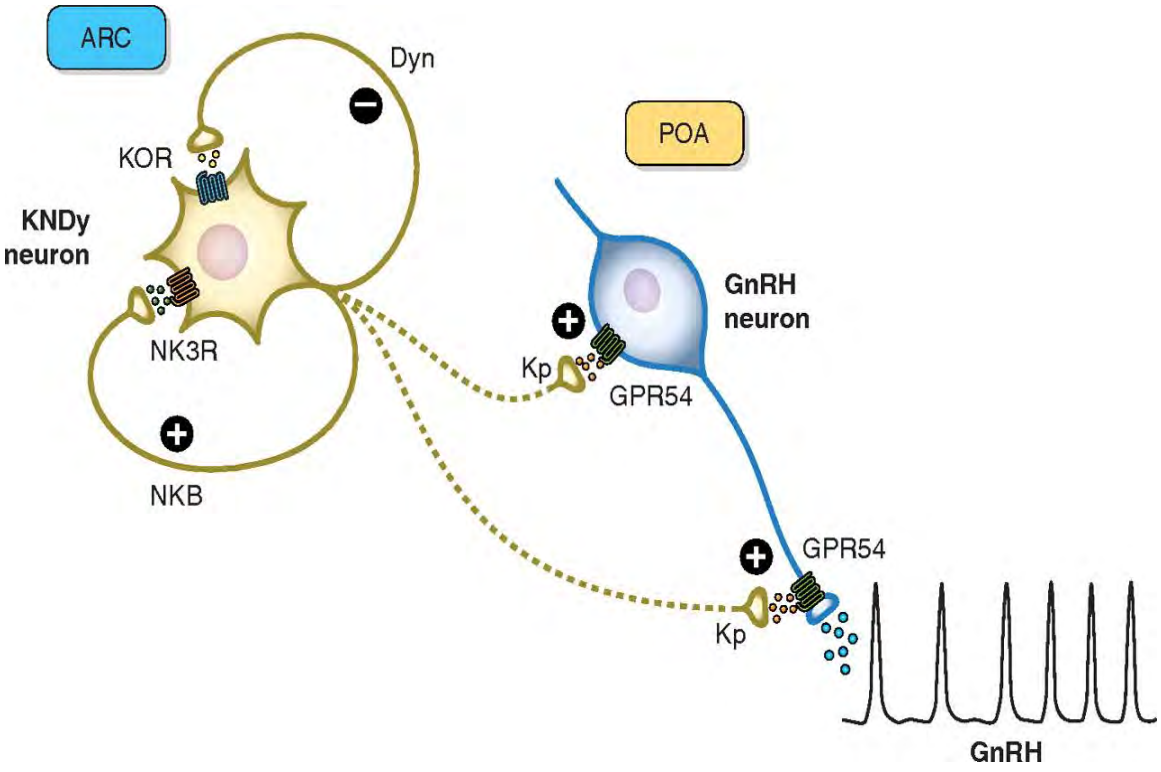

Figure v. Schematic illustration of a model for the synchoronized discharge of arcuate kisspeptin/neurokinin B/dynorphin A (KNDy) neurons and subsequent GnRH secretion. (Goodman *et al.,* 2013).

Parenthetically, these neurons are devoid of kisspeptin receptors and therefore this autoregulation of two peptides to dictate kisspeptin release provides potential explanation for GnRH pulse generator (Herbison, 2018).

The stimulatory effect of neurokinin B signaling in pubertal onset has been shown by Topaloglu *et al*. 2009. Surprisingly mutation in neurokinin B (TAC3) and its receptor (TAC3R) gene caused hypogonadotropic hypogonadism state as observed in case of kisspeptin and its receptor mutation. After that, systematic series of studies in non-human higher primates have been conducted to clarify the role of NKB signaling in controlling the HPG axis (Garcia *et al*., 2019). Albeit the neurokinin B signaling seems to play an important role in neurobiology of puberty, notably, no difference of response in pre-pubertal and pubertal male monkeys against neurokinin B agonist (senktide) to release GnRH or kisspeptin was observed. However, both sexes of rhesus macaques showed dose dependent response after kisspeptin administration. In addition, more pronounced GnRH response was observed in pubertal monkeys after kisspeptin administration in comparison to pre-pubertal animals (Terasawa and Garcia, 2020). Comparison of above-mentioned findings related to neurokinin B and kisspeptin in monkeys can be interpreted that kisspeptin plays a major role in controlling the HPG axis activity during pubertal development.

The link between brain, pituitary and gonads is tightly orchestrated. The interplay between different components of the HPG-axis ensures pubertal initiation and sustainment of reproductive activity (Plant, 2015). Elevated hypothalamic expression of kisspeptin and its receptor during pubertal progression was evident in rodents (Navarro *et al*., 2004a). A similar expression profile was also evident in female monkeys (Shahab *et al.,* 2005). Unlike female monkeys, only kisspeptin was shown to be raised in male pubertal monkeys (Shahab *et al.,* 2005). Both peripheral and central administration of kisspeptin caused vigorous release of gonadotropins in different developmental stages (Navarro *et al*., 2005a). Central administration of KP showed advancement in pubertal signs like vaginal opening in female rats pointing towards earlier arousal of gonadotrophic axis (Navarro *et al*., 2004b). Similarly, central and peripheral administration of KP increased LH levels in juvenile male rhesus monkey (Shahab *et al*., 2005). Elevated secretion of metastin within

median eminence in pubertal female monkeys also authenticate the role of kisspeptin as potent secretagogue of GnRH release (Keen *et al*., 2008). The positive effect of metastin on gonadotropins secretion in human males after systemic infusion was also noticed (Dhillo *et al*., 2005). Furthermore, higher basal levels of kisspeptin have been manifested in boys 14.5±0.5 years and 15.6±0.2 years corresponding to two Tanner stages i.e., Stage IV and V (Bano *et al.,* 2009). In addition, elevated projections from kisspeptin neurons to GnRH neurons are also observed in lambs after puberty as compared to prepubertal period (Nestor *et al*., 2012). Interestingly, intravenous kisspeptin administration caused elevation in LH and testosterone secretion at Tanner stage V in boys. However, this effect was not observable during preceding Tanner stages suggesting that pituitary gonadotrophs are not fully responsive to GnRH at these stages and sensitization of kisspeptin receptor may be achieved during later pubertal stages (Nabi *et al.,* 2018).

Almost a decade ago, top hierarchical position of kisspeptin neurons in controlling GnRH neuronal activity and downstream targets of the HPG axis has been unveiled after the discovery of kisspeptin receptor antagonist (p234) (Roseweir *et al*., 2009). A systematic comprehensive series of experiments had been conducted in different animals to observe the effectiveness of the antagonist. It has been shown that the antagonist p234 potently inhibits the GnRH neuronal activity as seen by deceased firing rate in mouse. In rodents (rats and mice) *icv* administration of the analog effectively inhibited the kisspeptin-induced release of luteinizing hormone (LH) (Roseweir *et al*., 2009). In addition, systemic (*ip*) administration of p234-penetratin significantly reduced gonadotropins secretion following either central or peripheral KP administration (Pineda *et al*., 2010). These results indicate that kisspeptin is a potent regulator in controlling the HPG axis activity. The antagonist was also able to reduce pulsatile GnRH secretion in female pubertal monkeys (Roseweir *et al*., 2009). Central infusion of p234 to pubertal females delayed vaginal opening (Pineda *et al*., 2010). This shows an integral role of kisspeptin in pubertal onset. Furthermore, post-castration rise in LH in rat, mice, and sheep was blocked by p234 suggesting that kisspeptin neurons may act as mediator for the negative feedback of the sex steroids in mammals (Roseweir *et al*., 2009). Likewise, chronic *icv* infusion of p234 in adult female rats also restrained the preovulatory surge of gonadotropins (Pineda *et al*., 2010).

A plethora of studies have demonstrated that KISS1-KISS1R signaling plays a major role in triggering pubertal onset and subsequently in the regulation of the hypothalamic pituitary gonadal axis during adulthood. Cascade of events leading to initiation of puberty through kisspeptin-GPR54 signaling are evident in many studies. In this respect very elegant information is articulated on levels of kisspeptin along developmental phases. In female macaques low kisspeptin levels are reported in pre-pubertal stage (Kurian *et al.,* 2012) and it was also noted that KP-54 release is synchronous with GnRH release (Guerriero *et al.,* 2012). In male rhesus macaques during infant stage content of kisspeptin in MBH is comparable to adult male monkeys however, a decrease in content is observed in juvenile animals with low levels of GnRH depicted by LH (Ramaswamy *et al.,* 2013). Interestingly, the same study also reported robust pattern of expression of GnRH fibers in both infantile and juvenile stage in median eminence (Ramaswamy *et al.,* 2013). Moreover, no variation in close association between kisspeptin immunoreactive fibers with GnRH cells and no difference in the number of close appositions of kisspeptin per GnRH cells was observed in pre-pubertal and ovariectomized (OVX) adult female monkeys (True *et al*., 2017) suggesting the presence of a neurobiological brake operating during juvenile phase that restrains kisspeptin drive to GnRH neurons.

Before the dawn of kisspeptinocentric period, NPY has been regarded as one of the cardinal factors regulating the HPG axis activity during the pubertal development. Therefore, it is imperative to consider various spokes of the HPG axis umbrella to better define underlying neurobiological mechanism for GnRH pulse generator activity during pubertal event.

In 1982, Tatemoto isolated a novel peptide with C-terminal tyrosine amide from the porcine brain extracts named neuropeptide Y (NPY). Primary structure of NPY showed that it has five tyrosine residues conferring its name as NPY consisting of 36 amino acids. NPY and the two other peptides i.e., pancreatic peptide (PP) and peptide YY (PYY) are also members of the same neuropeptide family (Lundberg *et al*., 1982). NPY is located on chromosome 7p15.3, on chromosome 4q24 and on chromosome 3 in human, rat, and in rhesus macaque respectively, each with 4 exons and 3 introns (Bahar and Sweeney, 2008). NPY is one of the most conserved neuropeptides in evolutionary ladder (Larhammar *et al*., 1993).

The prepro-NPY gene consists of 551 base pairs (bp) in its coding region, that ultimately encodes 36 amino acids linear peptide after processing of 97 amino acids prepropeptide. The prepro product of NPY gene consisting of 97 amino acids further contains signal peptide, NPY product, and carboxy terminal peptide, each segment is of 28, 36, and 30 amino acids long, respectively (Tatemoto, 1982; Minth *et al*., 1984). Human NPY has 98% homology with macaque while 94% with rodents, while macaque and rodent also have 94% similarity (Allen, 1990).

A plethora of subtypes of G-protein coupled receptors belonging to class A rhodopsin family are documented for the biological activity of NPY (Blomqvist and Herzog, 1997; Gehlert, 1998). Five isotypes of receptors; Y1 (Larhammar *et al.,* 1992), Y2 (Rose *et al.,* 1995), Y4 (Bard *et al.,* 1995), Y5 (Gerald *et al.,* 1996) and y6 (Weinberg *et al.,* 1996) have been cloned in mammals. Among these, four of the receptors Y1, Y2, Y4, and Y5 are functionally active in humans while y6 is not functional in primates due to an inactivating mutation therefore its biological relevance remains to be clarified (Larhammar *et al*., 1992; Michel *et al*., 1998; Pedrazzini *et al*., 2003). The subtypes found in mammals possibly originated by means of gene duplication prior to the origin of vertebrates (Larhammar *et al*., 2001). Y1, Y2 and Y5 have been localized to the same chromosomal segment in human, while Y4 and y6 have been mapped to separate chromosomes. All these subtypes have been cloned in mouse, human (Michel *et al*., 1998), pig (Wraith *et al*., 2000) and guinea pig (Sharma *et al*., 1998; Lundell *et al*., 2001). Each receptor has preference affinity for a particular ligand within NPY-PYY family of peptide (Magni, 2003). NPY Y1 receptor (NPY1R) and NPY Y5 receptor (NPY5R) prefer to bind with NPY and PYY, NPY Y2 receptor (NPY2R) has higher affinity for PYY, while NPY Y4 receptor (NPY4R) has preferentially binds to PP (Tatemoto, 1982). Pharmacological studies also suggested the existence of another receptor isotype designated as Y3 and it is controversial putative NPY preferring receptor (Gehlert, 1998; Lee and Miller, 1998).

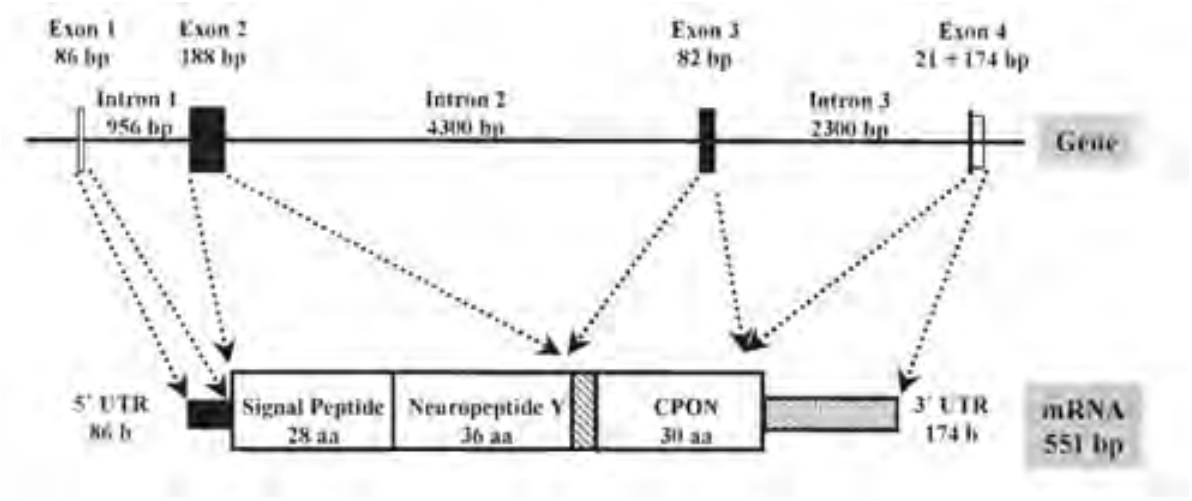

Figure vi. Schematic representation of human NPY gene and mRNA as indicated by Cerdá-Reverter and Larhammar, 2000.

Among these receptor subtypes, NPY1R and NPY5R are most important to carry out different physiological functions (Brothers and Wahlestedt, 2010). Particularly, NPY1R has been implicated in regulating neuroendocrine reproductive axis activity (Kalra *et al*., 1992; Raposinho *et al*., 1999) and feeding circuit (Gerald *et al*., 1996; Kanatani *et al*., 1996).

NPY1R was the first receptor to be cloned in rodents and then it was subsequently cloned in different animals including humans (Herzog *et al*., 1992; Larhammar *et al*., 1992). Like NPY peptide, its receptor, NPY1R is also highly conserved throughout evolutionary strata from non-mammalian to mammalian species (Krause *et al*., 1992; Larhammar *et al*., 2001). In mammals 92-95% homology has been observed in NPY1R gene. Human NPY1R has 98% homology with macaque while 94% with rodents, while macaque and rodents also have 94% similarity. The cytogenetic localization of NPY1R is different for each specie. Such as NPY1R gene is located on chromosome 4q32.2 in humans, on chromosome 5 in rhesus monkeys, and on chromosome 16p14 in rodents. NPY1R gene consists of 4 exons with two introns and the posttranslational product in humans is 384 long while in macaques and rodents, it gives 383 and 382 amino acids long product respectively (blast.ncbi.nlm.nih.gov; Larhammar *et al*., 1992).

Different techniques like immunohistochemistry, in situ hybridization and reverse–transcription polymerase chain reaction (RT–PCR) have been employed to observe NPY1R expression in central and peripheral tissues (Matsuda *et al*., 2002). A wide distribution pattern of NPY1R expression has been documented in the central nervous system (Kopp *et al*., 2002). In the brain NPY1R is abundantly expressed in hypothalamus, cerebral cortex, neocortex, hippocampus, amygdala, thalamus, and bed nucleus of the stria terminalis (Caberlotto *et al.,* 1997; Kopp *et al*., 2002). Particularly, different hypothalamic nuclei such as arcuate nucleus, paraventricular nucleus, dorsomedial nucleus, the medial preoptic area, and ventromedial nuclei are also positively stained for NPY1R expression. Different peripheral structures like blood vessels (Cabrele and Beck-Sickinger, 2000), adipose tissue (Hausman *et al*., 2008), colon, kidney, the gastrointestinal duct, adrenal gland, heart, the reproductive system (Wharton *et al*., 1993) have also shown NPY1R immunoreactivity (Matsuda *et al*., 2002). Due to wide distribution pattern of NPY1R, it is

involved in multiple physiological functions like heart rate, anxiety (Balasubramaniam, 2002), vasoconstriction of blood vessels (Cabrele and Beck-Sickinger, 2000), food intake (Kanatani *et al*., 2000), and bone homeostasis (Sousa *et al*., 2012).

NPY exerts its effect through a class of G proteins coupled receptors spanning seven transmembrane domains which upon activation leads to inhibitory pathways. Therefore, the common mechanism of action which NPY employed is via the pertussis toxin sensitive GTP binding protein Gi/Go that inhibits adenylate cyclase, down regulate cAMP and blocking membrane bound calcium channels (Herzog *et al*., 1992). Upon activation enhanced potassium conductance also caused membrane hyperpolarization (Sun *et al*., 1998; Sun and Miller, 1999). In addition, it can induce the phosphorylation of extracellularly regulated kinase (ERK) thereby stimulates the mitogen-activated protein kinase (MAPK) pathways (Nie and Selbie, 1998).

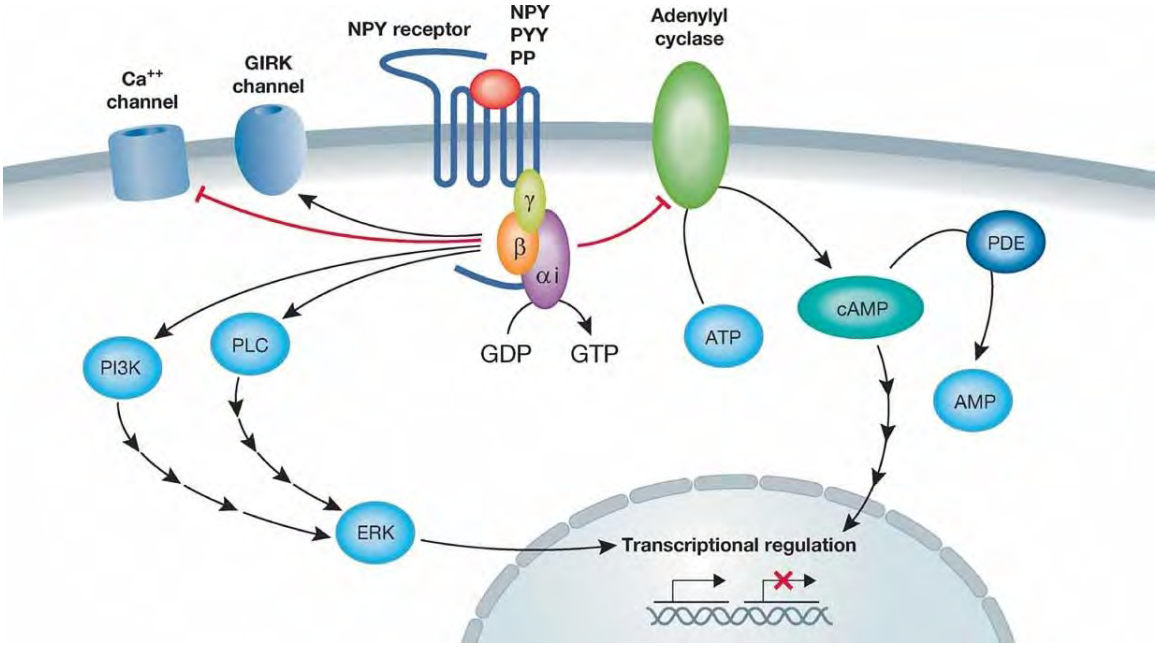

Figure vii. Schematic representation of intracellular signaling transduction events for NPY receptors. (Brothers and Wahlestedt, 2010).

NPY is abundantly expressed throughout the body structures (Zukowska *et al*., 2003). Particularly, NPY is conspicuously expressed in central and peripheral nervous system (Everitt *et al*., 1984). In central nervous system (CNS), immunoreactive distribution of NPY cell bodies are found in different areas like amygdala, cerebral cortex, hippocampus, hypothalamus, brain stem, thalamus, nucleus of solitary tract, nucleus accumbens and cerebellum structures (Allen *et al*., 1983; Chronwall *et al*., 1985; Danger *et al*., 1990; Cerdá-Reverter and Larhammar, 2000). In addition, immunoreactive fibers are also traced in the locus coeruleus, ventral tegmental area, and nucleus raphe pallidus (Allen *et al*., 1983; Chronwall *et al*., 1985). Due to scattered distribution of NPY, both intra and extra hypothalamic sources of NPY have been purposed. These sources send their projections to different hypothalamic nuclei as well to regulate neuroendocrine functions (Chronwall *et al*., 1985; Morris, 1989; Pelletier, 1990). NPY expression in different peripheral tissues like retina, intestine, spleen, bone marrow, smooth muscle, and thymus has also been observed (Medina *et al*., 2000; Choi *et al*., 2019). The tissues like spleen, vas deferens, blood vessels which are strongly innervated by sympathetic nerves are shown to be positive for NPY (Lundberg *et al*., 1983).

Abundant expression of NPY neurons has also been observed in different hypothalamic nuclei. The most prominent expression was noted in arcuate nuclei, dorsomedial hypothalamic nuclei, medial preoptic area, paraventricular nucleus, suprachiasmatic nucleus, and in median eminence (Chronwall *et al*., 1985). Moreover, extensive expression of NPY fibers and neuro terminals are present throughout the hypothalamus. Particularly, NPY fibers finely innervate in the medial preoptic, supraoptic nucleus, paraventricular nucleus, preoptic nucleus, arcuate nuclei, and median eminence (Chronwall *et al*., 1985; Sahu *et al*., 1988a; Ciofi *et al*., 1991; Contijoch *et al.,* 1993; Chaillou *et al.,* 2002).

Owing to the wide spectrum of NPY expression, it acts as a pleiotropic factor regulating multiple physiological functions. NPY has been physiologically implicated to regulate feeding behavior, blood pressure, thermoregulation, vasoconstriction, circadian rhythms, addiction, memory retention, anxiety, stem cell differentiation, immune system regulation, and neuroendocrine axes (Zukowska *et al*., 2003; Eva *et al.,* 2006; Brothers and Wahlestedt, 2010).

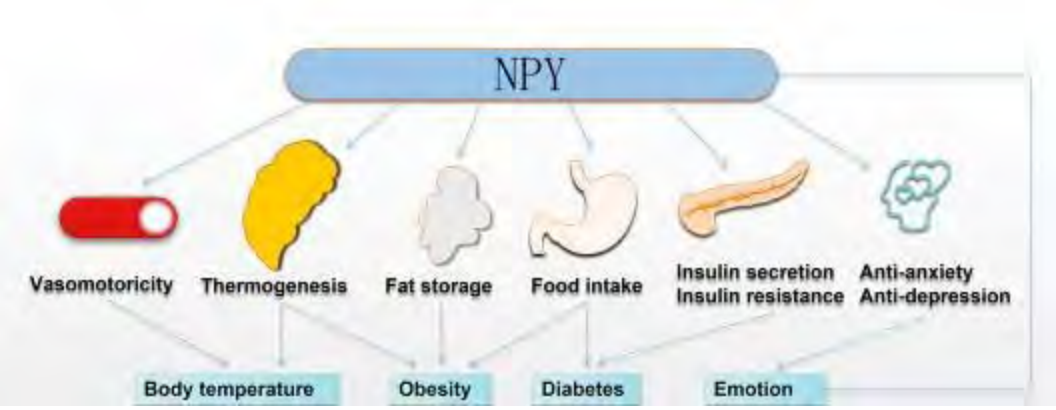

Figure viii. Representative structures showing multitasking physiological activities of NPY (Chen *et al*., 2020).

From all these physiological activities, reproductive neuroendocrinologist mainly focused on feeding and reproductive effects of NPY because of its abundant expression in different hypothalamic nuclei known as first order higher centers of feeding and reproduction.

Under the negative energy situation such as fasting caused increased hypothalamic expression of NPY in rodents and higher primates which was potently reversed after refeeding (Kalra *et al*., 1991; Sahu *et al.,* 1988b; Grove *et al.,* 2003). Similarly, selective ablation of NPY neurons from adult mice caused inhibition of feeding (Gropp *et al*., 2005; Luquet *et al*., 2005), suggesting NPY neurons are indispensable for feeding activities (Kohno and Yada, 2012). This NPY feeding response was shown to be blunted in NPY1R knock out mice (Kanatani *et al*., 2000). The data indicate NPY mediates feeding response via NPY1R (Pedrazzini *et al*., 1998).

NPY neurons have been shown to express leptin (Mercer *et al*., 1996) and insulin (Maejima *et al*., 2011) receptors. Therefore, these metabolic factors may play a critical role in transmitting energy status of the body and have negative impact on NPY regulation (Takahashi and Cone, 2005; Mayer and Belsham, 2009). Ample evidence indicated that NPY gene expression increased in response to diminished inhibitory feedback of circulating insulin or leptin (Ahima *et al*., 2000; Loh *et al*., 2017). As the energy status linked to the energy demanding process i.e., reproduction, hence enhanced NPY mRNA expression in leptin deficient mice leads to perturb activity of the reproductive axis culminating in delayed pubertal onset (Erickson *et al*., 1996; Pralong *et al*., 2002). These effects can be reversed by ablation of either NPY or NPY1R (Pralong *et al*., 2002; Sainsbury *et al*., 2002; Pralong, 2010; Sheffer-Babila *et al*., 2013). Recently, congenital deletion of leptin receptors on NPY/AgRP neurons showed mild impact on the reproductive axis (Egan *et al*., 2017). Similarly, negative regulation of NPY neurons by insulin has also been documented (Schwartz *et al*., 1992; Maejima *et al*., 2011). All these observations suggest that NPY dependent HPG axis activity may be regulated by peripheral metabolic factors. However, NPY regulation of reproductive axis is very intricate. Numerous studies have been done to clarify how NPY is indulged to control the HPG axis activity particularly to address pubertal timings in various animal models including higher primates.

It is interesting to note that both GnRH and NPY neurons are originated from the olfactory placode and migrate into the brain during embryogenesis (Hilal *et al*., 1996). NPY is recognized as a key neuropeptide involved in the control of the HPG axis. This has been attributed due to neuroanatomical association and co-localization between NPY and GnRH neurons. It has shown that NPY neurons appose to GnRH neuronal cell bodies in arcuate nucleus (Li *et al*., 1999; Dudas *et al*., 2000; Turi *et al*., 2003). Specifically, this direct morpho structural association between NPY fibers and GnRH neurons was evident in rat (Li *et al*., 1999; Campbell *et al*., 2001), mouse (Ward *et al*., 2009), sheep (Norgren and Lehman, 1989; Tillet *et al*., 1989) and monkey (Plant and Shahab, 2002). Moreover, NPY neurons also projected to presynaptic terminals of GnRH neurons in the median eminence (Li *et al*., 1999). Furthermore, co-localization of NPY1R has also been evident onto GnRH neurons through immunocytochemical technique (Li *et al*., 1999). NPY1R was also manifested in GnRH neurons of adult mice (Roa and Herbison, 2012) and GnRH subpopulation of nasal explant of mice (Klenke *et al*., 2010). These neuroanatomical arrangements between NPY and GnRH neurons suggest the commanding role of NPY to regulate gonadotropins secretion through direct modulation of GnRH neurons.

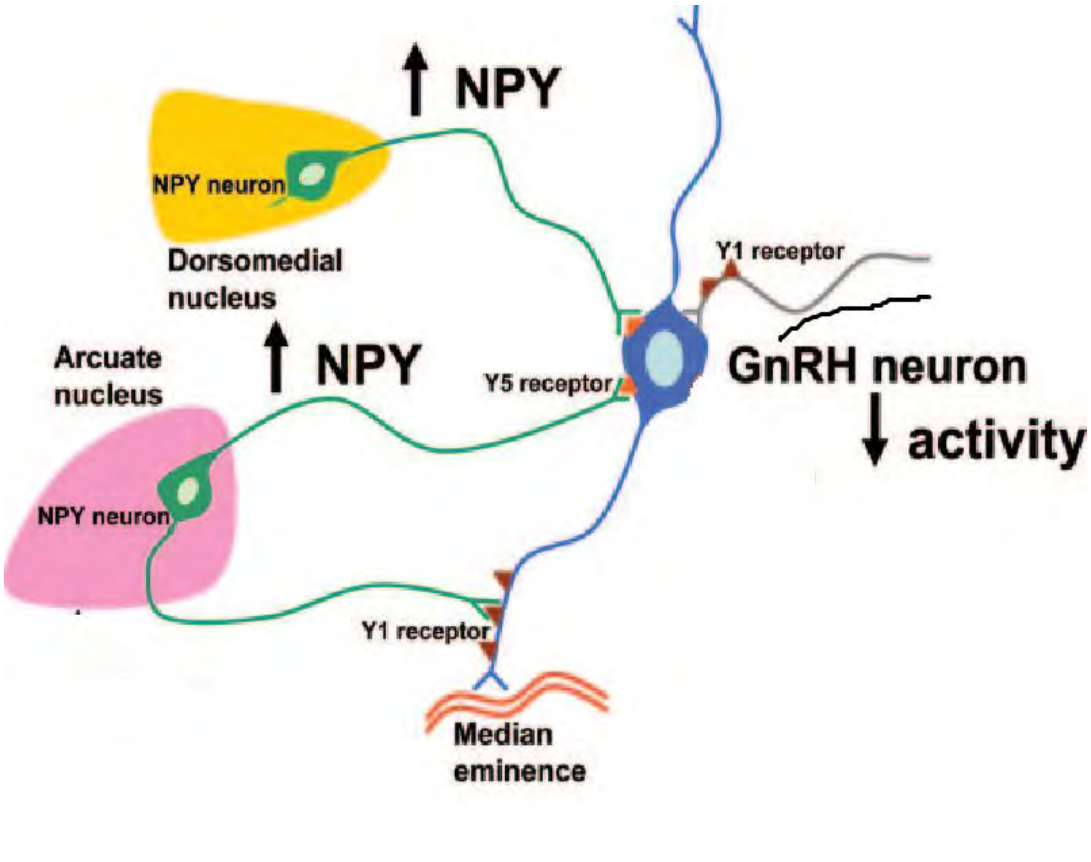

Figure ix. NPY regulation of GnRH neuronal activity. (Xu *et al*., 2009).

Although, NPY has been documented as pivotal central modulator of the HPG axis, but the effect of NPY on gonadotropic axis is vague and depends upon the species, metabolic and hormonal profile, pattern, and the route of administration (Kalra and Crowley, 1992; Wojcik-Gladysz and Polkowska, 2006). Therefore, dual role of NPY (both stimulatory and inhibitory) on the HPG axis has been demonstrated.

NPY based GnRH and LH secretion has been shown to exhibit marked differences depending upon sex, specie, site, and steroid milieu (McDonald, 1990; Kalra and Crowley, 1992; Barker-Gibb *et al*., 1995; Manfredi-Lozano *et al*., 2018). Both in-vivo and ex-vivo studies have demonstrated NPY based stimulation of GnRH secretion (McDonald, 1990; Sutton *et al*., 1998; Advis *et al*., 2003). For instance, in rodents NPY stimulated LH secretion in gonad intact or steroid-replaced animals near LH surge (Karla and Crowley, 1984; Crowley and Kalra, 1987; Sahu *et al.,* 1989). Khorram *et al*. 1987 also documented positive effect of NPY on hypothalamic GnRH secretion in steroid-replaced ovariectomized rabbits. In non-human higher primates, mediobasal hypothalamic infusion of NPY caused stimulatory effect on GnRH release in monkeys (Woller and Terasawa, 1991; Woller *et al*., 1992a; Woller and Terasawa, 1992b; Gore *et al*., 1993). Passive immunoneutralization of NPY also diminished GnRH pulsatility indicating correspondence between NPY and LH pulses in gonadectomized animals (Woller *et al*., 1992a; Kalra and Kalra, 1983). The positive association between NPY and GnRH was evident by observing enhanced NPY mRNA levels before preovulatory LH surge (Sahu *et al*., 1995). It is well established that NPY dependent GnRH release is mediated via NPY1R because NPY1R antagonist BIBP3226 injection halted the stimulatory effects of NPY on GnRH/LH release (Leupen *et al*., 1997). Similarly, uprise in GnRH gene expression in GT1-7 neuronal population has been documented upon NPY treatment which was inhibited by the application of NPY1R antagonist (Dhillon *et al*., 2009).

Apart from the well-documented stimulatory effects of NPY on GnRH/LH secretion, NPY also has an inhibitory action on GnRH release and hence on the reproductive axis. NPY has been shown to be notorious for its inhibitory action on GnRH/LH release in rodents (Kalra and Kalra, 1983), lagmomorph (Khorram *et al*., 1987), ovine (McShane *et al*., 1992; Barker-Gibb *et al*., 1995; Morrison *et al*., 2003), caprine (Ichimaru *et al*., 2001) and non-

human primates (Plant and Barker-Gibb, 2004). In gonadectomized male rats both routes of infusion (intravenous and intraventricular) cause decline in concentration of plasma LH (Kerkarian *et al.,* 1985). Profound inhibition of gonadotropic axis has also been observed after chronic central infusion of NPY in intact male and female rats which caused delay in sexual maturation (Catzeflis *et al*., 1993; Pierroz *et al*., 1995; Pierroz *et al*., 1996). Ovariectomy with steroid replacement also caused suppression of LH release in ewes (McShane *et al.,* 1992; Malven *et al.,* 1992). Presence or absence of estradiol does not affect dominant role of NPY in inhibiting LH secretions in ruminants (Gazal *et al*., 1998; Morrison *et al*., 2003; Estrada *et al*., 2003). It was suggested that this inhibition of LH may be mediated due to direct inhibition of GnRH release via NPY1R (Gazal *et al*., 1998; Klenke *et al*., 2010). In case of non-human higher primates, central injection of NPY halts pulsatile release of GnRH in post-pubertal male (Shahab *et al*., 2003) and female (Kaynard *et al*., 1990) monkeys. Recently, Chemo genetic activation of NPY neurons also demonstrated suppression in LH secretion which consequently prolonged estrous cycle duration (Padilla *et al*., 2017). Above cited data indicate NPY as the vital factor in controlling the HPG axis activity by repressing GnRH release (Gruaz *et al.,* 1993).

Immunohistochemistry also revealed the expression of NPY in prenatal and mature human gonads (Jorgenson *et al.,* 1996) and Y1 receptor on testicular blood vessels of adult male rodent (Allen *et al.,* 2011). Likewise, higher expression of NPY and NPY1R has been observed in non-human primate (Frungieri *et al*., 2000) and human (Korner *et al*., 2011) testes, respectively. Direct injection of NPY in testes of male rat cause inhibition of testosterone secretion (Allen *et al.,* 2011). These observations highlighted the local role of NPY in regulating testicular functions.

NPY mediated inhibition of the HPG axis enchanted different groups of reproductive neuroendocrinologists to evaluate the possible role of NPY as neurobiological brake which keeps GnRH pulse generator activity in quiescent mode during juvenile period after brief stimulation during infant stage. Most of the researchers imagined that NPY may act as a major neuro inhibitory factor to keep protracted release of pulsatile secretion of GnRH during pubertal development (Kaynard *et al.,* 1990; Pau *et al.,* 1995; El Majdoubi *et al.,* 2000a). In this regard, significant role of NPY as neurobiological brake to keep GnRH

pulse generator in quiescent mode during juvenile stage of development was critically evaluated by El Majdoubi *et al*. 2000a. In this exquisite research, an inverse relationship between GnRH pulse generator activity and NPY mRNA/protein was noticed in male agonadal monkeys during juvenile-pubertal and neonatal-juvenile transition. In addition, NPY also suppressed LH release in adult male monkeys (Shahab *et al.,* 2003). This NPY based inhibition in GnRH release was thought to be mediated by the NPY1R in adult male monkeys (Plant and Shahab, 2002). Different antagonists were tested to clarify the critical role of NPY to inhibit GnRH release via NPY1R signaling. The data from rodents and primates indicated that NPY-NPY1R signaling plays a major role in prepubertal hiatus of the HPG axis. For instance, in case of agonadal male juvenile monkeys, it has been demonstrated that NPY based inhibition of LH release could be reversed by *icv* administration of NPY1R antagonist (El Majdoubi *et al*., 2000a; Shahab *et al*., 2003). Early pubertal development has been observed in female rats after chronic *icv* administration of NPY1R antagonist. This has been manifested by earlier vaginal cornification with elevated LH content indicating potential involvement of NPY1R in mediating inhibitory effect of NPY on gonadotropic axis (Pralong *et al*., 2000).

Nevertheless, it has also been documented that two more highly specific NPY1R antagonist failed to elicit LH discharge in juvenile castrated male monkeys (Shahab *et al*., 2003). It has also been noted that expression of NPY mRNA gradually increases with age in either the gender of rats with highest expression from pre-pubertal to early pubertal period (Nowak and Gore, 1999) implicating potential role of NPY in keeping GnRH neuronal activity at minimum before pubertal onset. Furthermore, fluorophore tagged dual labelled GnRH and NPY immunostaining revealed a close juxtaposition between NPY axon and GnRH cell bodies and this intimate association between the two was much greater in juvenile male monkeys as compared to pubertal animals (Plant and Shahab, 2002). Corresponding to the observations in male monkeys, significant elevation in immunoreactive contacts between NPY/AgRP neuronal fibers onto GnRH neurons has also been observed in pre-pubertal as compared to adult female monkeys (True *et al*., 2017). Similar observations are also documented in heifers (Alves *et al*., 2015). Hence, the above-

cited data suggests that NPY may act as plausible neurobiological brake restraining pubertal onset.

In case of humans, one isolated case report showed higher levels of circulating NPY associated with delayed puberty (Błogowska *et al*., 2004). In the study, NPY levels were higher in girls with constitutional delay of puberty as compared to girls undergone normal pubertal development. However, two genetic studies on patients suffering from pubertal disorders indicated that mutation in NPY1R is not involved in regulating pubertal disorders (Barker-Gibb *et al*., 2004; Freitas *et al*., 2007).

Both kisspeptin and NPY are shown to be potent stimulator and inhibitor of the HPG axis respectively (Terasawa *et al.,* 2013). Since both the neuropeptides showed antagonizing effect on the HPG axis activity in general and particularly during pubertal development, therefore, it may be speculated that NPY may regulate the HPG axis activity indirectly via kisspeptinergic pathway. In this regard close reciprocal association between NPY and kisspeptin neurons has been observed in ewes (Backholer *et al*., 2010; Polkowska *et al*., 2014). These appositions between NPY and kisspeptin neurons are thought to form synaptic connection (Amstalden *et al*., 2011). Interaction of NPY fibers with kisspeptin soma suggests direct regulation of kisspeptin neurons by NPY. In this context, contradictory reports about NPY regulation of kisspeptin (both stimulatory and inhibitory) have been presented. NPY based elevation in kisspeptin gene expression under the ex-vivo setting (N6 cell line) was reversed in NPY knockout mice (Luque *et al.,* 2007), discloses facilitatory role of NPY on kisspeptin neuronal activity. NPY injection also potently enhanced mean LH levels in male wistar rats (Azizi *et al*., 2020). Moreover, positive effect of NPY on LH and testosterone secretion and on male sexual behavior by enhancing *Gnrh1*, *Kiss1* and *Tac3* and by decreasing the *Pdyn* gene expression is recently reported in rats. These effects of NPY reverted by the application of NPY1R antagonist BIBP 3226 (Azizi *et al*., 2020; Azizi *et al*., 2021). As far as inhibitory role of NPY on kisspeptin neuronal population is concerned, two elegant studies using chemo genetic and electrophysiological approaches unveiled the notion that the removal of endogenous constrain of NPY is necessary to activate or upregulate kisspeptin's excitatory input to GnRH neurons to initiate a cascade of the reproductive process (Padilla *et al*., 2017).

It has been demonstrated that selective chemo genetic activation of NPY/AgRP neurons suppressed pulsatile secretion of LH which resulted in prolonging estrus cycle duration (Padilla *et al*., 2017). Furthermore, electrophysiological data by a recent in vitro study also demonstrated that NPY has direct effect in inhibition of arcuate kisspeptin neuronal activity (Hessler *et al*., 2020). Based on RNA sequencing, it was also revealed that arcuate kisspeptin neurons in rodents harbor NPY1R transcripts (unpublished data as cited in Hessler *et al*., 2020). The study also corroborated that direct inhibition of kisspeptin neuronal activity imposed by NPY can be reversed partially by applying NPY1R antagonist BIBO 3304 (Hessler *et al*., 2020), potentiating the role of NPY1R in inhibiting kisspeptin neuronal activity. In rodents, comparison of *Npy* and *Kiss1* gene expression profile revealed lower expression of *Npy* and greater expression of *Kiss1* in adult male mice (Molnar *et al*., 2016).

Since the discovery of kisspeptin different physiological, pharmacological, clinical, and genetic approaches have been implemented to decipher the prime importance of kisspeptin as component of the GnRH pulse generator to trigger pubertal onset in primate and non-primate mammals. However, kisspeptin is considered as a component of the pulse generator but not the trigger for pubertal onset. Particularly the enigma about nadir activity of the GnRH pulse generator during juvenile stage is still included in the hundred unsolved questions in science. Aforementioned data indicated that both kisspeptin and NPY are potential stimulator and inhibitor, respectively, to regulate the HPG axis activity and the antagonists of both behave antagonistically on gonadotropins secretion. Therefore, the neuroendocrine mechanism underlying postnatal intermittent release of GnRH which is high at infantile and adult stage characterizing mini and true puberty separated by low levels at juvenile stage still needs to be explicitly defined. In this context, NPY regulation of kisspeptin neurons and their morphological interaction during pubertal development has not been studied yet in non-human higher primates. In this regard, recently, NPY based inhibition of kisspeptin neurons harkens back to the concept that NPY may serve as upstream central neurobiological brake for suppression of kisspeptin neurons keeping GnRH pulse generator in check during pre-pubertal period, while alleviation of NPY brake

may likely to occasion kisspeptin dependent resurgence in the HPG axis activity at the onset of puberty.

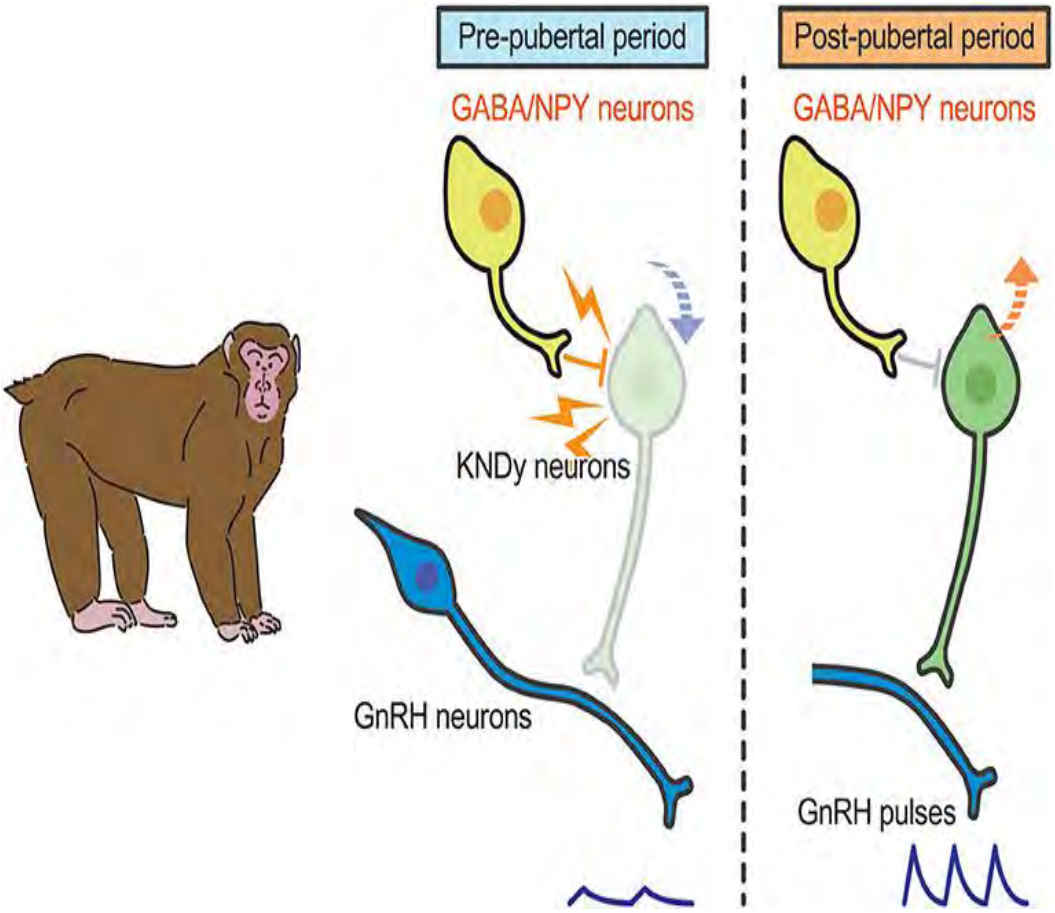

Figure x. Schematic illustration showing a current proposed interpretation for brain mechanism controlling pubertal onset in primates. (Plant, 2019; Terasawa, 2019; Uenoyama *et al*., 2019).

The present experimental work was formulated to study the developmental variation between NPY and kisspeptin across pubertal stages in non-human higher primates and to study the role of NPY in occasioning pre-pubertal suppression of GnRH pulse generator in male rhesus monkey. Following three experiments were conducted to achieve the objectives.

1. By quantifying fluctuations in the relative mRNA expression of both key neuropeptides (NPY and KISS1 genes) along with receptor genes of both neuropeptides across four developmental stages of male monkeys by using real time-qPCR.
2. By the assessment of developmental variation in NPY and NPY1R expression onto kisspeptin neurons in male rhesus macaque by using dual-label immunofluorescence.
3. By evaluating the response of the HPG axis after intravenous administration of NPY1R antagonist in intact juvenile male rhesus monkeys.

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

GENERAL MATERIALS

AND

METHODS

GENERAL MATERIALS AND METHODS

A total of nineteen intact male rhesus monkeys (*Macaca mulatta*) were used to conduct three experiments in the present research work. Fifteen animals assigned into four different developmental groups [infant (4-7 months)], [juvenile (10-14 months)], [pre-pubertal (21-24 months)], [adult (7-10 years)] were employed to study differential expression of *KISS1* and *NPY* along with *KISS1R* and *NPY1R* through real-time quantitative PCR (RT-qPCR) while morphological interactions and variations in NPY and NPY1R expression on kisspeptin neurons were observed through fluorescent immunocytochemistry. Four juvenile intact male rhesus monkeys (*Macaca mulatta*) 10-14 months old were used to decipher the effect of intravenous (*iv*) administration of NPY1R specific antagonist BIBO 3304 on the HPG axis activity indirectly via testosterone secretion as a surrogate of LH. Individual cage was designated for each animal under semi-controlled environmental conditions (25±3°C) temperature, 12-hour light/dark photoperiod with lights on between 06:00 and 18:00 hr in the Primate Facility of Zoology Department, Quaid-i-Azam University, Islamabad in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. The daily meal comprised of boiled potato (09:00-09:30 hr), boiled eggs (11:00 hr), bread (13:00-13:30 hr) and fresh fruits, nuts (15:00-16:00 hr). Additionally, vitamins supplements were also given to account for vitamin deficiency. *Ad libitum* access to water was ensured. Protocols and procedure of all experimentation were approved by the Animal Care and Use Committee of the Department of Zoology.

Tissue Collection

Fifteen animals assigned into four different developmental groups [infant (4-7 months)], [juvenile (10-14 months)], [pre-pubertal (21-24 months)], [adult (7-10 years)] based upon physical (body weight and testicular volume) and hormonal (testosterone) parameters were sacrificed to obtain hypothalamic blocks. Three animals (n=3) in the infant group, while four animals (n=4) each in the rest of the groups were employed for dissecting hypothalamic tissue. Before dissection, animals were sedated

with intramuscular (*im*) injection of ketamine hydrochloride (Ketamax, Rotex Medica, Trittau, Germany; 10-20 mg kg^{-1} BW). After sedation body weight was taken. A sharp sterilized razor was used to shave off hair from head. Povidone with 70% ethanol was used to clean area of skin. After that, brains were exposed by cutting skull bone along the circumference of the head with the help of sharp bone cutter. Animals were euthanized with sodium thiopental (Abbott Laboratories, Karachi, Pakistan; approximately 30 mg kg^{-1} BW) injected intravenously (*iv*) after opening the skull, after which the brains were removed from the cranium instantly. Dissected brains were immediately placed on a cold glass plate upside down i.e., ventral side up. Hypothalamic blocks from ventral side of the brain were dissected out by executing details of the manoeuvre as described earlier (Ramaswamy *et al.,* 2008). In summary, taking optic chiasm as anterior point and mammillary bodies as posterior landmarks, cuts were made coronally. On both sides of the midline approximately 5 mm cut in parasagittal plane was observed. Finally horizontal cut at dorsal to the anterior commissure gave square hypothalamic block (Figure vi). Taking medial line as reference a cut was made to obtain hemi-hypothalamic blocks encompassing preoptic area (POA) and mediobasal hypothalamus (MBH) area. Each hemi-hypothalamic block from all animals was quickly immersed in liquid nitrogen and then placed at -80 ºC before proceeding for RNA isolation. The other hemi-hypothalamic block of animals was processed for fixation in 4% paraformaldehyde (PFA) and dehydration in sucrose (20% and 30% solution) before executing cryosectioning and immunocytochemistry.

Testicular dimensions were noted after bilateral orchidectomy with the help of vernier caliper. In addition, the middle portion (4mm) from one testis of each animal from all developmental stages except infant was obtained to observe testicular morphology. In the case of the infant group, half testis after longitudinal bisection was used. Morphometric data of testicular histology (epithelial height and seminiferous tubule diameter) and spermatogenic status obtained from hematoxylin and eosin staining was also employed to gauge a particular developmental stage as depicted in previous studies (Plant *et al.,* 2005; Simorangkir *et al.,* 2012).

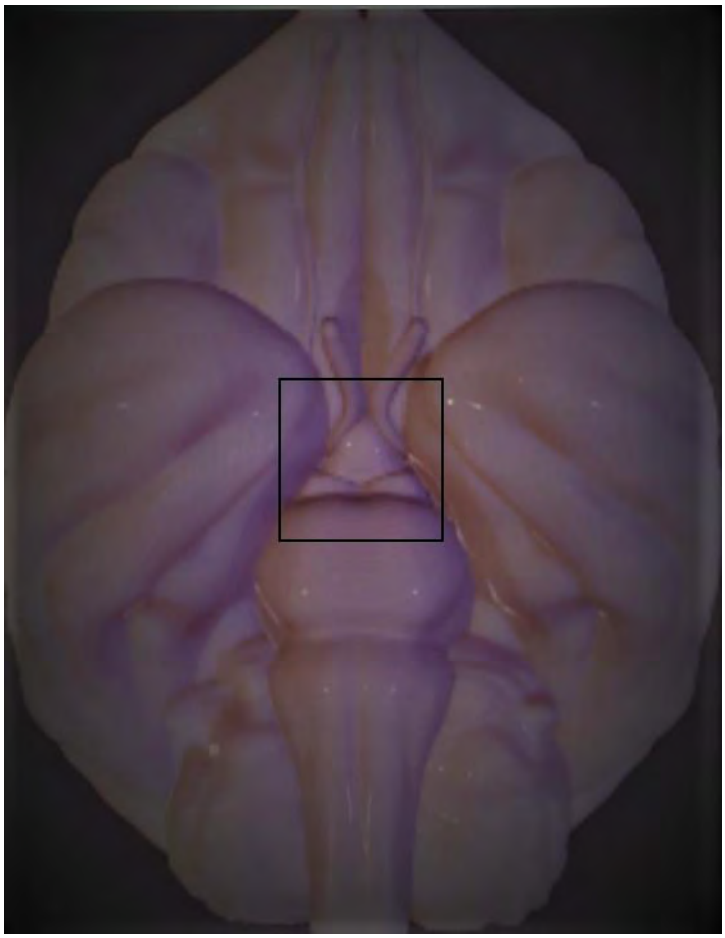

Figure xi. Ventral view of a 3D model of rhesus monkey brain (https://sketchfab.com/kelly.bullock.art). Inset shows demarcation lines for the dissection of the hypothalamic block.

Blood sample from the saphenous vein was also taken into heparinized syringe from each animal to measure testosterone concentration before euthanasia. Animals were sacrificed in the morning (10:00 - 12:00 hr) to avoid any confounding effect of time to observe morphological interactions and variations in NPY and NPY1R expression on kisspeptin neurons through fluorescent immunocytochemistry and to quantify differential expression of *KISS1* and *NPY* along with *KISS1R* and *NPY1R* through real-time quantitative PCR.

SYBR Green RT-qPCR

Real-time quantitative PCR (RT-qPCR) was performed for the relative quantification of mRNA of *KISS1* and *NPY* and their receptors (*KISS1R* and *NPY1R*) from hemi-hypothalamic tissues of fifteen intact male rhesus monkeys grouped into four developmental stages. Dissected hemi-hypothalamic blocks placed at -80 ºC, after immersion in liquid nitrogen as described above, were proceeded to isolate total RNA.

I. Isolation of Total RNA

Total RNA was extracted with Wizol™ Reagent (Wizbiosolutions, Seongnam, South Korea) according to manual instructions provided by the manufacturer from hemi-hypothalamic tissues of all animals. Briefly, for hypothalamic block weighing 100 mg 1 ml of Wizol ™ reagent was added. With the help of mortar and pestle proper grinding of the tissue was done. After that, homogenized content was mixed by adding 200 µl chloroform. The content was vortexed for 15 sec. After 3 min short incubation at room temperature, centrifugation of the samples was done for 15 min at 4 ºC at the speed of 12000 rpm. New tube was taken to separate upper aqueous phase. However, lower phase and interphase were discarded. Next step was to add 500 µl chilled isopropanol. After gentle inversion samples were incubated at room temperature for 10 min and then centrifugation was done at 12000 rpm at 4 ºC for 10 min. After centrifugation, supernatant was discarded. After all this processing, a pellet at the bottom of the tube was easily seen. Washing of the pellet was done with 1 ml of 75% ethanol and vortexed briefly. Last centrifugation was done at 7500 rpm at 4 ºC for 5 min. Pellets were dried to remove ethanol.

Lastly pellets were dissolved in Diethylpyrocarbonate (DEPC) treated water and resuspend RNA by pipetting up and down few times.

II. RNA Quantification

RNA quantity was estimated by using UV/ Vis Nano-Drop TM Spectrophotometer (Nanodrop, Wilmington, DE, USA) with units ng μl^{-1}.

III. Complimentary DNA Synthesis

One µg of total RNA per 20 µl of reaction was used to synthesize complimentary DNA (cDNA) by using WizScript™ First Strand cDNA Synthesis kit (Wizbiosolutions, Seongnam, South Korea) with the random hexamer primers according to the protocol given by the kit manufacturer in a thermocycler (T100 Bio-Rad Thermocycler, Hercules, CA, USA). Reaction conditions are reported in detail in chapter 1.

IV. Primers and Dilutions

Diethylpyrocarbonate (DEPC) treated water was used to make 100 µM stock solution of each primer. Brief centrifugation was done at 13000 rpm for 30 sec. Prepared primers stock was incubated at room temperature for 15 min. To prepare working stock of primers, 10 µl of the stock primers were mixed with 90 µl of DEPC water.

V. Relative Quantitative Real-time PCR

RT-qPCR was performed to evaluate developmental fluctuations in the relative expression of *NPY, NPY1R, KISS1*, and *KISS1R*. The sequence of all primers is given in chapter 1. The reaction was carried out by using Wizpure™ qPCR Master (SYBR) kit (Wizbiosolutions, Seongnam, South Korea). For each gene, the reaction was conducted in duplicate manner with a final volume of 10 µl included 5 µl SYBR Green, 4 µl of cDNA (with 5x dilution), and 0.5 µl of primer set (each forward and reverse primer quantity was equal to 0.25 µl). Services of Thermo Fisher Scientific (Invitrogen) (Waltham,Massachusetts, USA) were taken for the synthesis of primers. Details of reaction

conditions and calculation of relative fold changes in genes expression is described in chapter 1.

Fluorescence Immunocytochemistry

Fifteen hemi-hypothalamic blocks which were dissected out as described above were used to conduct fluorescent immunocytochemistry. Following two sets of different dual labeled immunostainings were performed:

i. to observe developmental variation in kisspeptin and NPY expression and to examine the changes in close apposition between NPY and kisspeptin neurons.
ii. to observe developmental changes in NPY1R expression onto kisspeptin neurons.

I. Tissue Fixation

Hemi-hypothalamic blocks of all animals were obtained according to the scheme described above. These hemi-hypothalamic blocks were quickly placed in freshly prepared paraformaldehyde (4% PFA, Scharlau Chemie SA, Barcelona, Spain) solution in 0.1M phosphate buffer saline (PBS), (pH 7.4) to do fixation. Tissues were kept overnight at 4 ºC. After fixation, dehydration of the hemi-hypothalamic blocks was carried out in 20% sucrose solution (Fischer Chemical, Leicestershire, UK) in 0.1M PBS at 4 ºC until they sank to the bottom of the vial. Tissues were then immersed in a freshly prepared 30% sucrose solution until they sank again.

II. Serial Sectioning

Each of the hemi-hypothalamic block was sectioned using a cryostat (Bright OTF5000, A-M Systems, Sequim,Washington, USA; temperature -25 ºC). 20 µm thick sections were cut. The sections were stored in cryopreservative solution (30% ethylene glycol, 1% polyvinylpyrrolidone and 30% sucrose in PBS) at -20 ºC until processed for immunocytochemical detection of a particular peptide.

III. Antibodies

Details of primary and secondary antibodies used to conduct two different immunostainings on 20 µm free-floating hemi-hypothalamic sections are shown in Table 2.1 of chapter 2.

IV. Procedure

Free floating 20 µm thick sections were treated with specific primary and fluorescently tagged secondary antibodies for two sets of fluorescent immunocytochemistry. Three sections per animal were used for each staining while one section per animal was used as primary antibody omitted control for each staining. Protocol to conduct both immunostainings was same. Sterile cell culture plates (Nest Biotechnology Co. Ltd, China) were used for all washings and incubations. The general procedure which was opted to carry out two different sets of staining was as follows. First, sections were washed with PBS solution. After that blocking was carried out by adding normal serum from animals in which secondary antibody was raised. Primary incubation of 48 hours was done in cocktail of specific primary antibodies. Following primary incubation, second incubation of about two hours in cocktail of specific fluorescent secondary antibodies was done. Lastly, sections were mounted on super frosted slides. More details about solution composition, incubation timings, antibodies, and procedure are mentioned in chapter 2.

V. Microscopy

Imaging of fluorescent labeling for kisspeptin, NPY and NPY1R was examined using fluorescent microscope (Olympus BX51, Tokyo, Japan) by appropriate filters. A camera connected to microscope helped to take images. Quantification criteria and procedure is described in chapter 2.

Pharmacological Study

Four juvenile intact male rhesus monkeys (*Macaca mulatta*) 10-14 months old were used to study the effect of *iv* administration of NPY1R antagonist (BIBO 3304; SML2094, St. Louis, Missouri, USA) on the HPG axis activity indirectly via testosterone secretion to determine the role of NPY1R in mediating putative inhibitory action of NPY on the HPG axis during juvenile period. Before starting of the experiment, GnRH priming was done to enhance pituitary responsiveness. For this purpose, intramuscular (*im*) injection of GnRH agonist (Lutrate Depot; GP-Pharma, S.A. Sant Quinti de Mediona, Spain) 1.8 µg per 6 hr was given for a week. Blood samples on two time points (7:00 am and 13:30 pm) were taken on alternative days during priming to observe pituitary responsiveness in terms of testosterone secretion. Actual experiment comprised of two days of sequential blood sampling after one week of priming. In the first day of sequential blood sampling, vehicle was administered while after the gap of one day, second serial sampling with NPY1R antagonist administration was done. Blood samples (~ 0.5-0.7ml/sample) were taken at 30 min interval. Blood sampling details with respect to time interval, duration, and dose administration are described in chapter 3.

I. Venous Catheterization for Serial Blood Sampling

For serial blood sampling and administration of drugs (Vehicle/BIBO 3304/KP-10) via *iv* route, ketamine hydrochloride (Ketamax, Rotex Medica, Trittau, Germany; 5 mg kg^{-1} BW, *im*) was injected into animals to anesthetized them. A teflon cannula (Vasocan Branule, 0.7 mm / 24 G O.D, B. Braun Melsungen AG, Belgium) was placed into the saphenous vein. At the distal end of the cannula, a butterfly tube (Length 300 mm, volume 0.20 ml, 22 GX3 / 4 ″, JMS, Singapore) was linked. A syringe (1cc 26G X 1/2") was connected at proximal end of butterfly tube. This infusion set settings greatly helped to withdraw sequential blood samples. In addition, *iv* administration of drugs were also feasible due to such instrumental settings. Sampling was initiated after impact of sedation was completely removed.

II. Pharmacological Reagents

The following drugs were used.

GnRH agonist: Lutrate Depot (Leuprorelin acetate 3.75 mg; GP-Pharma, S.A. Sant Quinti de Mediona, Spain)

Heparin (Rotex media, Trittau, Germany)

Ketamine hydrochloride (Ketamax, Ketler, Astarapin, Germany)

Dimethyl Sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri, USA)

Normal saline (0.9 % NaCl; A-Z Pharmaceuticals, Kasur, Pakistan)

Human KP-10 (amino acids 112 – 121; Calbiochem, La Jolla, CA, USA).

NPY1R antagonist: BIBO 3304 (N-[(1R)-1-[[[[4-[[(aminocarbonyl)amino]methyl] phenyl]methyl]amino]carbonyl]-4-[(aminoiminomethyl)amino]butyl]-a′-phenyl benzeneacetamide ditrifluoroacetate; SML2094, Sigma-Aldrich, St. Louis, Missouri, USA).

III. Blood Sampling

All blood samples (0.5-0.7ml) were obtained in heparinized syringes at 30 min interval. An equal amount of heparinized (5 IU ml^{-1}) normal saline (0.9 % NaCl) was administered to maintain blood volume to avoid hypovolemic shock to the animals following withdrawal of each sample. Moreover, during blood sampling fruits/juices were provided to the animals. Samples were transferred to eppendorf tubes kept on ice. Blood sampling was conducted between 10:30 – 18:30 hr. Centrifugation of the blood samples was done at 3000 rpm at 4 ºC for 15 min. Plasma was extracted and stored at – 20 ºC until hormonal analysis.

Plasma Testosterone Measurement

Plasma testosterone measurement was done in two different assays. Enzyme linked immunosorbent assay (ELISA) was used to measure plasma testosterone levels in samples obtained in pharmacological study (before and after *iv* administration of BIBO 3304 and vehicle and of priming regime) by using a human testosterone ELISA kit. Plasma testosterone concentration in samples from fifteen male rhesus monkeys (*Macaca mulatta*) categorized into four developmental groups (infant, juvenile, pre-pubertal and adult) was measured by using a human testosterone enzyme immune assay (EIA) kit. The detailed procedure and principle of each assay is as follows.

I. Plasma Testosterone Measurement by ELISA

Testosterone concentration in plasma of samples (before and after *iv* administration of BIBO 3304 and vehicle and of priming regime) was estimated by using a human testosterone ELISA kit (JTC Diagnosemittel UG, Schulweg, Voehl, Germany). The assay was performed according to the standard protocol provided in the kit. All samples, controls and standards were brought to room temperature before the start of the assay. The reported assay sensitivity was 1.16 ng ml^{-1}. Performance of the assay was documented through assay coefficients of variation. For intra assay coefficients of variation was <5%. While <9% coefficient of variation was recorded for inter assay.

i. Assay Principle

The kit is designed on the principle of solid phase competitive ELISA. Streptavidin coated wells are incubated with working testosterone-horse reddish peroxidase (HRP) conjugate, samples, and anti-testosterone-biotin solution. Competition occurs between unknown concentration of testosterone in the sample and testosterone enzyme (HRP) conjugate. After washing, addition of substrate leads to development of color. Color intensity is inversely proportional to the concentration of testosterone in the samples. Color intensity shows an inverse relation to the testosterone concentration which is plotted in the form of standard curve.

ii. Assay Procedure

The assay was done according to the instructions of the manufacturer. All samples, controls, standards, and all reagents were settled down to room temperature before the assay was started. First of all, standards, controls and samples 50 µl each were pipetted into the assigned wells. After that testosterone-enzyme conjugate reagent 100 µl was dispensed out into every well. Afterwards, 50 µl biotin reagent was added into each well. To mix the reagents, microplate was gently swirl for 20-30 seconds. One hour incubation of the plate was carried out at room temperature after wrapping with aluminum foil. Content of the microplate was discarded and then washing was done by 300 µl /well with 1X wash buffer for three times. 100 µl of 3, 3', 5, 5'- tetramethylbenzidine (TMB) substrate was added into each well. Again, plate was resealed and incubated for 30 min at room temperature. After 30 min incubation, 50 µl of stop solution was added into each well to stop the reaction. Micro plate reader (680xR, BIO-RAD, Tokyo, Japan) at 450 nm was used to get absorbance (O.D) values within 15 min after completion of the assay.

iii. Result Calculation

By plotting standard concentrations on abscissas and respective absorbance on ordinates, a standard curve was constructed. Plasma testosterone concentrations were estimated by using 4PL model given in microtiter plate reader software (Bio Rad Laboratories, Hercules, CA, USA).

II. EIA to Measure Plasma Testosterone

A human testosterone EIA kit (Astra Biotech GmbH, Luckenwalde, Germany) was used to measure plasma testosterone concentration in samples from fifteen male rhesus monkeys (*Macaca mulatta*) categorized into four developmental groups (infant, juvenile, pre-pubertal and adult). The procedure and instructions provided within the kit were carefully employed to run the assay. Technical details of the kit were as follows:

Inter assay coefficient of variation <9%

Intra assay coefficient of variation <10%

Sensitivity of the assay was 0.05 ng ml^{-1}

i. Assay Principle

Testosterone EIA is a competitive assay. Testosterone present in the sample competes with testosterone-horseradish peroxidase (HRP) conjugates to bind with rabbit anti-testosterone, the amount of which is constant. The wells are coated with goat anti-rabbit IgG. All the contents (samples, standards, testosterone-HRP conjugate reagent, controls, and rabbit-anti testosterone reagent) are added into wells for 90 min incubation. After washing, the addition of TMB leads to the development of blue color. Sample testosterone concentration shows inverse relation with color intensity which is manifested by standard curve. This curve is used to read the concentration of testosterone in the sample.

ii. Assay Procedure

In the first step, 10 µl of standards, samples, and controls were dispensed into desired wells. Then 100 µl of testosterone-HRP conjugate reagent was added into each well. In the next step, rabbit anti- testosterone reagent (50 µl) was poured into each well. Incubation of the plate for 90 min at 37 ºC was done after the contents were thoroughly mixed. Next, incubation mixture was discarded by flicking plate contents into a waste container. Microplate wells were then washed and flicked with sterile distilled water. To avoid any water contamination, plate was sharply strike on paper to absorb residual water. TMB substrate solution (100 µl) was the next to add into all wells. After addition of TMB, content of the plate was mixed gently about 10 sec. Aluminum foil was used to cover the plate and then plate was incubated at 25 ºC for 20 min under dark. Reaction was stopped by adding stop solution (100 µl). The contents were mixed for 30 sec. Microtiter reader (680xR, BIO-RAD, Tokyo, Japan) was used to take absorbance values using 450 nm filter within 15 min after completion of the assay.

iii. Result Calculation

By plotting standard concentrations on abscissas and respective absorbance on ordinates, a standard curve was constructed. Plasma testosterone concentrations were estimated by using 4PL model given in microtiter plate reader software (Bio Rad Laboratories, Hercules, CA, USA).

Testicular Volume

Vernier caliper was used to measure the volume of both the testicles (left and right) with the help of a formula given by Steiner and Bremner, 1981. The formula was $V = (\pi w^2 l)/6$, where width (w) and length (l) were measured in mm units. However, volume (V) was calculated in ml. Total volume was obtained by combining left and right testis volume.

Morphological Examination of Testes

Developmental changes in testicular morphology (tubular diameter with epithelial height) were measured through hematoxylin and eosin staining.

I. Tissue Fixation and Processing

Excised testicular tissues were immediately placed in Bouins's fixative (75 ml of aqueous solution of picric acid, 25 ml of formalin and 5 ml of glacial acetic acid) overnight. After that tissues were dehydrated and cleared in ascending grades of alcohol and xylene, respectively. The tissues were then embedded in paraffin wax. 5 µm thick sections obtained on microtome were stained with standard eosin and hematoxylin staining technique.

II. Hematoxylin and Eosin Staining

Tissue sections were stained with eosin and hematoxylin for assessing the spermatogenic status and for observation of testicular histology during different developmental stages (infant, juvenile, pre-pubertal and adult). Deparaffinization was done by giving two washes with xylene for five min each. Then, in descending grades of alcohol all the sections were rehydrated for one min each (100%, 90%, 80%, 70%). The sections were then stained for 5 min in hematoxylin. Next processing of the sections was done with water wash followed by rinsing in 1% acid alcohol. In the last, sections were treated with eosin for 2 min. Washing, dehydration and xylol washes were next three steps which were performed. The sections were then cover slipped with gelvatol mounting media. Diameter of seminiferous tubules and epithelial height were measured through compound microscope (Nikon SE, Shinagawa, Tokyo, Japan).

Statistical Analyses

All the data were expressed as mean±SEM. Statistical comparison of relative expression of *KISS1*, *KISS1R*, *NPY* and *NPY1R* mRNA in different developmental groups was evaluated by one-way ANOVA followed by Tukey's multiple comparison test. Linear association between any two genes on natural log transformed fold change data was calculated through Pearson correlation. Changes in number of immunoreactive cell bodies of kisspeptin, dual label kisspeptin neurons with NPY, and expression of NPY1R on kisspeptin neurons during pubertal development were also compared by one-way ANOVA followed by Tukey's multiple comparison test. Comparison of mean testosterone release across different time points after vehicle and antagonist administration and mean pre and post testosterone release in vehicle and antagonist treated animals was evaluated by two-way ANOVA followed by Tukey's multiple comparison test. Comparison for the overall mean±SEM testosterone levels in vehicle and antagonist treated animals was also done by using student's t-test. Testosterone response to vehicle and antagonist treated monkeys was also calculated through area under the curve (AUC) method (Abbud and Smith, 1993). After that t-test was applied. In addition, effect of KP-10 administration in vehicle and antagonist treated monkeys on pre and post testosterone concentration (mean±SEM) was also measured by using paired t-test. All statistical analyses were performed by using GraphPad Prism Software version 8.3.0 (GraphPad software, La Jolla, CA, USA). Statistical significance level was set at $p < 0.05$.

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

CHAPTER # 01

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

Inverse correlative fluctuations in KISS1 and NPY gene expression: an off-on-off switch regulating timing of pubertal initiation in male rhesus monkey

ABSTRACT

Introduction: The neuroendocrine mechanism underpinning the postnatal sinusoidal wave pattern of gonadotropin releasing hormone (GnRH) pulse generator activity from infantile to adult age still requires punctilious explanation. Compelling evidence has identified kisspeptin as a major component of pulse generator that is involved at mini and true puberty. However, no variation in apposition of kisspeptin fibers onto GnRH neurons was observed in pre-pubertal and adult non-human primates. Moreover, the inability of the hypothalamic-pituitary-gonadal (HPG) axis to respond against kisspeptin injection during earlier Tanner stages in boys suggests dormancy of the axis might be due to some inhibitory factor. Apart from the inhibitory effects of NPY on the HPG axis reported earlier, recently, the intimate relationship between NPY and kisspeptin neurons and the direct inhibition of arcuate kisspeptin neurons by NPY highlight the significance of these two neuropeptides in regulating reproductive axis activity. Therefore, depending upon foregoing data it was hypothesized that before pubertal commencement, NPY acts as a neurobiological brake to keep kisspeptin neurons in hibernation state; as this brake vanishes out, kisspeptin-dependent HPG axis activation occurs at puberty. Thus, the goal of the current work was to characterize correlative changes in relative expression of both these neuropeptides (KISS1 and NPY), and their receptor genes from the mediobasal hypothalamus (MBH) of male rhesus monkeys (*Macaca mulatta*) during sexual maturation and to understand concurrent changes in these gene expression patterns.

Materials and Methods: A total of fifteen intact male rhesus monkey (*Macaca mulatta*) categorize into four developmental stages (infant, juvenile, pre-pubertal and adult) depending on physiological and hormonal indexes were used. Three animals were used in infant group while four animals were used in rest of the groups. Messenger ribonucleic acid (mRNA) was extracted from MBH from all animals. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to quantify the levels of *KISS1*, *KISS1R*, *NPY* and *NPY1R* mRNA, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Results: Significant uprise in mRNA of both *KISS1* and its receptor *(KISS1R)* ($p<0.05$-0.01) was noted in adult animals in comparison to pre-pubertal animals. Contrasting to kisspeptin and its receptor, *NPY* and its receptor *NPY1R* showed significant decrease ($p<0.05$) in mRNA expression in adult group with respect to preceding age group. Likewise, in comparison to juvenile animals, adult monkeys showed significantly higher ($p<0.01$) and lower ($p<0.05$) expression of KISS1 and NPY mRNA, respectively. Comparison between juvenile and infant group showed significant elevation ($p<0.05$) in *NPY* mRNA and non-significant ($p>0.05$) uprise in *NPY1R* mRNA in juvenile animals as compared to infant animals. Furthermore, inverse correlative age-associated fluctuations across pubertal development stages were also evident in pre-pubertal group with respect to infant animals. As pre-pubertal group manifested significantly higher ($p<0.05$) relative expression of *NPY* mRNA and insignificant ($p>0.05$) decrease in *KISS1* relative mRNA. The same pattern of expression was also observed for *NPY1R* and *KISS1R* mRNA in pre-pubertal animals with respect to infant animals.

Conclusion: NPY may serve as upstream central neurobiological brake for kisspeptin neurons keeping GnRH pulse generator in check during pre-pubertal period causing dormant state of the generator while alleviation of NPY brake may play a permissive role for kisspeptinergic excitatory inputs for resurrection of GnRH secretion to drive HPG axis for the onset of puberty. These findings may be advantageous in designing pharmacological, therapeutic, and clinical approaches to regulate fertility problems in humans.

INTRODUCTION

Puberty is an intricate process that is essential for the initiation and maintenance of reproductive phenomena in mammals. Maturity of the reproductive neuroendocrine axis is the prerequisite for this fascinating event to set up. The commanding position of this hierarchical axis is bestowed to gonadotropin releasing hormone (GnRH) neurons releasing decapeptide (GnRH) from the hypothalamus, which directs pituitary gonadotrophs to release gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH). These gonadotropins reach the distant target sites (testes and ovaries) to trigger steroidogenesis and gametogenesis, ensuring the reproductive capability to be achieved (Terasawa and Garcia, 2020). The prologue of the pubertal process is GnRH which is released because of synchronous activity of hypothalamic GnRH neurons controlled by GnRH pulse generator, which remains in a dormant state during the juvenile period after brief stimulation in the infant stage, thus, opting for on-off-on mode characterizing infant (mini puberty), juvenile (hypogonadotropic state), and pubertal state (adolescent puberty), respectively (Plant, 2019). Several lines of evidence try to manifest multiple players, both stimulatory [kisspeptin, glutamate, etc.] and inhibitory [neuropeptide Y (NPY), gamma aminobutyric acid (GABA), etc.] to uncover disguise neuroendocrine mechanism controlling pubertal onset (Terasawa and Fernandez, 2001). The prime agent involved in the pre-pubertal hiatus of the GnRH pulse generator is still vague (Plant, 2015); hence the puberty question is included in unsolved enigma (so much more to know. Science, 2005)

Among stimulatory regulators, kisspeptin is identified as a potent secretagogue of GnRH secretion in mammals, including rodents, ruminants, and primates, by applying a plethora of experimental strategies, both pharmacological and physiological (Terasawa *et al*., 2013; Uenoyama *et al*., 2019). The dynamic role of kisspeptin in regulating the reproductive axis has been well defined (Plant, 2019; Uenoyama *et al*., 2019; Terasawa and Garcia, 2020). The critical role of kisspeptin and its receptor in pubertal onset has been implicated by observing inactivating mutation in *KISS1* or *KISS1R* lead to delayed or absence of puberty (de Roux *et al*., 2003; Seminara *et al.,* 2003; Topaloglu *et al*., 2012). While activating mutation in *KISS1R* caused precocious puberty (Teles *et al*., 2008). In rodents, *Kiss1*

expression has been shown to increase during pubertal progression (Navarro *et al*., 2004a; Takumi *et al*., 2011). A similar increase in *KISS1* expression during pubertal progression was also observed in primates (Shahab *et al*., 2005). More precisely, an upsurge in *KISS1* expression along with kisspeptin neuronal population and peptide content with secretion at median eminence (ME) is evident in male and female monkeys during puberty (Shahab *et al*., 2005; Keen *et al*., 2008; Guerriero *et al*., 2012). Although the close intimate association between kisspeptin fibers with GnRH cell bodies as well as with GnRH fibers has been documented (Ramaswamy *et al*., 2008). However, no variation in apposition of kisspeptin fibers onto GnRH neurons was observed in pre-pubertal and adult non-human primates (True *et al*., 2017). In both male and female monkeys, kisspeptin agonist administration into the ME during the pre-pubertal and pubertal stages showed dose-dependent GnRH release. In this set of experiments, pubertal animals showed a greater response against kisspeptin agonist than pre-pubertal animals. Interestingly, a higher dose of agonist is required in male monkeys than in female monkeys (Guerriero *et al.,* 2012; Garcia *et al.,* 2017; Garcia *et al.,* 2018). Less sensitivity of the pre-pubertal male monkeys against kisspeptin agonist indicates the presence of some neurobiological inhibitory factor that may impair kisspeptin-dependent activity of the reproductive axis before pubertal onset. In another study, the inability of the HPG axis to respond against kisspeptin administration during earlier Tanner stages in boys was noted (Nabi *et al*., 2018). The authors speculated that non-responsiveness of the pituitary gonadal component upon kisspeptin administration during pre-pubertal age was due to some inhibitory factor.

In addition to kisspeptin, neurokinin B (NKB) and dynorphin has been shown to coexist within the cohort of arcuate neurons called KNDy neurons in different species including, non-human higher primates (Goodman *et al*., 2007; Navarro *et al*., 2009; Ramaswamy *et al*., 2010). Among these three peptides, kisspeptin and neurokinin B play a stimulatory role while dynorphin is considered inhibitory (Plant, 2019). Interestingly, mutations of the gene encoding NKB (*TAC3*), and its receptor (*TAC3R*) caused hypogonadotropic hypogonadism (Topaloglu *et al.,* 2009) like those in kisspeptin and its receptor. In non-human primates, the role of neurokinin B signaling has been systematically studied (Garcia *et al*., 2017; Garcia *et al*., 2018). Notably, in male monkeys, no difference in GnRH/kisspeptin response

in pre-pubertal and pubertal animals against neurokinin B agonist (senktide) was observed. However, the GnRH response to kisspeptin in male and female monkeys is dose-dependent during pubertal development (Garcia *et al.*, 2019). These results suggest the importance of kisspeptin signaling in controlling the HPG axis activity during pubertal development.

The second neuronal population that plays a pivotal role in regulating the HPG axis before and after puberty is neuropeptide Y (NPY). NPY suppresses GnRH/LH secretion in different species including non-human higher primates (McDonald *et al*., 1989; Estrada *et al*., 2003; Shahab *at al*., 2003). NPY neurons in the arcuate have been shown to project GnRH neurons in mice (Turi *et al.,* 2003) and monkeys (Plant and Shahab, 2002). GnRH neurons express neuropeptide Y1 receptor (NPY1R) (Li *et al.*, 1999; Klenke *et al*., 2010). NPY1R antagonist provokes LH release in pre-pubertal and adult male monkeys (Shahab *et al*., 2003., El Majdoubi *et al.*, 2000a), rats (Pralong *et al.*, 2000), and in prenatal GnRH nasal explant of mouse (Klenke *et al*., 2010). These observations implicate regulation of gonadotropins secretion through direct modulation of GnRH neurons. Moreover, NPY gene and protein expression are inversely related to GnRH pulse generator activity (El Majdoubi *et al*., 2000a), suggesting it as a plausible neurobiological brake restraining pubertal onset in higher primates.

Both kisspeptin and NPY have been studied extensively for their stimulatory and inhibitory properties, respectively, on the HPG axis. Owing to their antagonizing effect on GnRH secretion, it may be speculated that NPY may influence GnRH activity indirectly through kisspeptin neurons. Recent studies have highlighted this possible notion where a complex reciprocal connection between kisspeptin and NPY neurons was observed in arcuate nuclei of sheep (Backholer *et al.,* 2010; Polkowska *et al.,* 2014), indicating NPY may regulate kisspeptin neuronal activity. In this regard, two studies showed that NPY induced GnRH neurosecretion inhibition by exerting a direct inhibitory effect on arcuate kisspeptin neurons (Padilla *et al*., 2017; Hessler e*t al.*, 2020). A direct inhibitory synaptic input from NPY to kisspeptin neurons was observed. Moreover, chemo genetic activation of NPY neurons demonstrated decreased pulsatile LH secretion with increasing estrous cycle duration in female mice (Padilla *et al.,*2017) suggests possible direct projection of NPY to kisspeptin neurons. In addition, the direct inhibitory effect on arcuate kisspeptin neurons

was slightly blocked by the NPY1R antagonist (Hessler *et al*., 2020) potentiating the role of NPY in suppressing kisspeptin neuronal activity is mediated by the NPY1R. Pubertal development data showed an increase in *Kiss1* and a decrease in *Npy* expression in adult (postnatal day 60) as compared to infantile (postnatal day 14) male mice (Molnar *et al*., 2016). However, such concurrent developmental profile of NPY and kisspeptin has not been studied yet in a non-human higher primate.

From the above-cited data, it is reasonable to hypothesize that before pubertal commencement, NPY acts as a neurobiological brake to keep kisspeptin neurons in hibernation state; as this brake vanishes out, kisspeptin-dependent HPG axis activation occurs at puberty. Thus, the present study was undertaken to investigate the above hypothesis by examining the differential expression of *KISS1* and *NPY* along with *KISS1R* and *NPY1R* through real-time quantitative PCR to decipher the correlative changes of these neuropeptides during sexual development in a representative higher primate.

MATERIALS AND METHODS

Animals

A total of fifteen intact male rhesus monkeys (*Macaca mulatta*) assigned into four different developmental groups [infant (4-7 months)], [juvenile (10-14 months)], [pre-pubertal (21-24 months)], [adult (7-10 years)] were used in the present study. Three animals were in the infant group, while four animals were employed each in the rest of the groups. Initially, the study employed characterization of animals into four different developmental stages, i.e., infant, juvenile, pre-pubertal, and adult, based upon physical (body weight and testicular volume) and hormonal (testosterone) data. The same classification criteria were also adopted earlier (Garcia *et al.,* 2018). In addition, morphometric data of testicular histology (epithelial height and seminiferous tubule diameter) and spermatogenic status obtained from hematoxylin and eosin staining was also employed to gauge a particular developmental stage as depicted in previous studies (Plant *et al*., 2005; Simorangkir *et al*., 2012). Since suppression of gonadotropin release during the pre-pubertal period is under control of central neurobiological mechanism and not influenced by gonadal steroids in male monkeys (Plant and Barker-Gibb, 2004; Ramaswamy *et al.,* 2013; Garcia *et al*., 2018), intact animals were utilized in the present study to observe developmental variations in hypothalamic neuropeptides under normal physiological settings. Each animal was kept in a separate cage under semi-controlled environmental conditions (25±3°C) temperature, 12-hour light/dark photoperiod with lights on between 06:00 and 18:00 hr in the Primate Facility of the Department of Zoology, Quaid-i-Azam University, Islamabad in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. The daily meal comprised of fresh fruits, nuts (09:00-09:30 hr), boiled eggs (11:00 hr), and bread (13:00-13:30 hr). *Ad libitum* access to water was ensured. Protocols and procedure of all experimentation was approved by the Animal Care and Use Committee of the Department of Zoology.

Tissue Collection and Blood Sampling

Before dissection, animals were sedated with intramuscular injection of ketamine hydrochloride (Ketamax, Rotex Medica, Trittau, Germany; 10-20 mg kg^{-1} BW, *im*). After sedation body weight was taken. Testicular dimensions were also noted after bilateral orchidectomy with the help of vernier caliper. Blood sample from the saphenous vein was taken into heparinized syringes from each animal to measure testosterone concentration. Testicular tissues were also excised to make blocks to observe testicular morphological parameters. Animals were sacrificed in the morning (10:00–12:00 hr) to avoid any confounding effect of time. The detailed scheme to dissect out whole hypothalamic block in squre and to further obtain hemi-hypothalamic block surrounding preoptic area (POA) and mediobasal hypothalamus (MBH) area is described in general materials and methods section. Finally, each hemi-hypothalamus was quickly immersed in liquid nitrogen and then placed at -80 ºC before proceeding for RNA isolation.

Testicular Volume

Vernier caliper was used to measure the volume of both the testicles (left and right) with the help of a formula given by Steiner and Bremner, 1981. The formula and calculation method are already described in general materials and methods section.

Morphological Examination of Testes

Developmental changes in testicular morphology (tubular diameter with epithelial height) were measured through hematoxylin and eosin staining.

I. Tissue Fixation and Processing

Testicular tissues were excised at dissection. Tissues were immediately placed in Bouins's fixative for overnight fixation. The fixative was prepared by mixing aqueous solution of picric acid, glacial acetic acid, and formalin in a ratio 15:1:5 respectively. All these chemicals were purchased from Merck, Darmstadt, Germany. Dehydration was carried out in increasing grades of alcohol for 1 hr each (70%, 80%, 90%, 100%). After all the water

had been removed, the tissues were treated with xylene two times for 1 hr each. Tissues were embedded in paraffin wax at 62 ºC. Testicular blocks (4mm) were cut at 5 µm thickness on a microtome. The sections were stained with standard eosin and hematoxylin staining technique. Staining procedure is already described in general materials and methods section. The diameter and epithelial height of 25 round seminiferous tubules were measured with a calibrated ocular micrometer.

EIA to Measure Plasma Testosterone

A human testosterone EIA kit (Astra Biotech GmbH, Luckenwalde, Germany) was used to measure plasma testosterone concentration in samples from fifteen male rhesus monkeys (*Macaca mulatta*) categorized into four developmental groups (infant, juvenile, pre-pubertal and adult). The procedure and instructions provided within the kit were carefully employed to run the assay. Technical details of the kit were as follows:

Inter assay coefficient of variation <9%

Intra assay coefficient of variation <10%

Sensitivity = 0.05 ng ml^{-1}

SYBR Green RT-qPCR

Real-time quantitative PCR (RT-qPCR) was performed for the relative quantification of mRNA of *KISS1* and *NPY* and their receptors (*KISS1R* and *NPY1R*) from hemi-hypothalamic tissues of fifteen intact male rhesus monkeys grouped into four developmental stages.

I. Isolation of Total RNA

Total RNA was extracted with Wizol^{TM} Reagent (Wizbiosolutions, Seongnam, South Korea) according to manual instructions provided by the manufacturer. RNA quantity was estimated by using UV/ Vis Nano-Drop TM Spectrophotometer (Nanodrop, V3.7, Thermo-Fisher Scientific, Wilmington, DE, USA) in ng μl^{-1} units.

II. Complimentary DNA Synthesis

one µg of total RNA per 20 µl of reaction was used to synthesize complimentary DNA (cDNA) by using WizScript^{TM} First Strand cDNA Synthesis kit (Wizbiosolutions, Seongnam, South Korea) with the random hexamer primers according to the protocol given by the kit manufacturer in a thermocycler (T100 Bio-Rad Thermocycler, Hercules, CA, USA). Briefly, primer/RNA mix was initially incubated for 5 min at 65 ºC, then by adding buffer/enzyme components to primer/RNA mix further incubation was carried out at 37 ºC for 60 min followed by termination for 10 min at 70 ºC. The cDNA samples were stored at -20˚C until analysis.

III. Relative Quantitative Real-time PCR

RT-qPCR was performed to evaluate developmental changes in the relative expression of *KISS1*, *NPY, KISS1R,* and *NPY1R*. The reaction was carried out by using Wizpure^{TM} qPCR Master (SYBR) kit (Wizbiosolutions, Seongnam, South Korea). For each gene, the reaction was conducted in duplicate manner with a final volume of 10 µl included 5 µl SYBR Green, 0.5 µl of primer set, and 4 µl of cDNA (with 5x dilution). Services of Thermo Fisher Scientific (Invitrogen) (Waltham, Massachusetts, USA) were taken for the synthesis of primers. The sequence of all primers is given in Table 1.1. Thermocycler conditions were first hold at 95 ºC for 10 minutes then 45 cycles each at 95 ºC for 10 sec followed by 60 ºC for 45 sec and 72 ºC for 17 sec. MyGo Pro Software 3.4 was used to calculate cycle threshold (Ct) values (MyGo Pro real-time PCR system, Dublin, Republic of Ireland). Each gene in each sample was normalized to endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). $2^{-\Delta\Delta CT}$ method was used for calculation of relative fold change quantification considering infant group as calibrator (Livak and Schmittgen, 2001).

Table 1.1. List of primers used for real-time qPCR with primer positions and GenBank accession numbers.

Gene	Accession#		Primer sequences (5′–3′)	Primer position	Product Size
KISS1	XM028852143.1	Forward	CTGGAATCCCTGGACCTCTC	356-375	226
		Reverse	TTGTAGTTCGGCAGGTC CTT	582-563	
KISS1R	XM015125638.2	Forward	CACATTCAGTCCCAAACCGC	168-187	105
		Reverse	ACCCTGAGTGGGGATAGCTT	273-254	
NPY	NM001032814.1	Forward	GTACCCTTCCAAACCGGACAA	94-114	73
		Reverse	GCAGCGCCGAGTAGTATCTG	167-148	
NPY1R	NM001032866.1	Forward	TCTGCTGGCTACCTCTTACCA	821-841	99
		Reverse	AGGTGGCAGAGCAGGAATAAC	920-900	
GAPDH	NM001195426.1	Forward	TGTTGCCATCAATGACCCCT	150-169	119
		Reverse	TTGATGACGAGCTTCCCGTT	269-250	

Statistical analyses

All the data were expressed as mean±SEM. Statistical comparison was evaluated by one-way ANOVA followed by Tukey's multiple comparison test. Linear association between any two genes on natural log transformed fold change data was calculated through Pearson correlation. All statistical analyses were performed by using GraphPad Prism Software version 8.3.0 (GraphPad software, La Jolla, CA, USA). Statistical significance level was set at $p < 0.05$.

RESULTS

Pubertal Stages in Monkey

Physical parameters (body weight and testicular volume) and hormonal index (plasma testosterone concentration) showed a gradual increase from infant to adult group as the age of animals progresses. Statistically, adults showed significantly higher body weight and testicular volume ($p<0.0001$) with respect to all other stages animals (Figure 1.1A, Figure 1.1B). Hormonal index estimating testosterone levels showed significant ($P<0.01$) elevation in pubertal/adult group as compared to rest of the preceding developmental groups (Figure 1.1C). Morphometric data obtained from hematoxylin and eosin-stained testicular sections showed marked changes in testicular morphology in comparison of adults to younger groups (Figure 1.2). In the infant to the pre-pubertal group, imperceptible gradual change in luminal space, epithelial height, and tubular diameter was discernable. However, active spermatogenesis with differentiated spermatogonia, spermatocytes, and spermatids was only seen in adults. More precisely, a statistically significant ($p<0.0001$) increase in morphometric parameters like seminiferous tubule diameter and epithelial height was observed in adults with respect to all other developmental groups (Figure 1.3A, and 1.3B).

Developmental Expression of *NPY* and *NPY1R*

A collateral increase in *NPY* and *NPY1R* relative mRNA expression has been observed from infant to pre-pubertal group, while a sharp decrease in expression was observed in the adult group (Figure 1.4A, and 1.4B). Statistically, both juvenile and pre-pubertal groups showed higher expression ($p<0.05$) of *NPY* mRNA as compared to the infant group, while with the same significance ($p<0.05$) adult group showed a decline in *NPY* mRNA expression as compared to juvenile and pre-pubertal group (Figure 1.4A). In concordance with *NPY*, although a gradual increase in *NPY1R* mRNA expression has been noticed across the first three developmental stages (infant, juvenile, and pre-pubertal), however, only pre-pubertal monkeys showed statistically significant augmentation ($p<0.05$) in relative NPY1R mRNA expression in comparison to infant group.

In general, suppression in the relative expression of *NPY1R* mRNA was evident in the adult group with respect to the juvenile and pre-pubertal group, but a statistically significant decrease ($p<0.05$) was only manifested in adults relative to pre-pubertal animals (Figure 1.4B).

Developmental Expression of *KISS1* and *KISS1R*

Variations in relative mRNA expression of *KISS1* and *KISS1R* were in contrast with relative mRNA expression of *NPY* and *NPY1R*. Relative *KISS1* mRNA levels indicated reduced expression from infant to pre-pubertal stage with no statistical difference. In contrast, elevation in *KISS1* mRNA expression was observed in the adult group as compared to infant ($p<0.05$), juvenile, and pre-pubertal ($p<0.01$) stage (Figure 1.5A). Relative expression of *KISS1R* mRNA indicated a non-significant ($p>0.05$) decline in the pre-pubertal group as compared to the infant and juvenile group, whereas the adult group showed a statistically significant ($p<0.05$) rise in *KISS1R* relative mRNA expression in comparison to the pre-pubertal group (Figure 1.5B).

Correlation between *KISS1* and *NPY*, *KISS1* and *NPY1R*, and *KISS1R* and *NPY1R* relative mRNA

Correlation on natural log transformed fold change values between any two genes was performed. Analysis showed that *KISS1* expression was highly correlated ($p<0.01$) with *NPY* and *NPY1R* expression (Figure 1.6A, and 1.6B). In addition, a significant association ($p<0.05$) was only observed between *KISS1R* and *NPY1R* mRNA expression (Figure 1.6C), while a non-significant correlation was depicted between *KISS1R* and *NPY* mRNA (data not shown).

Figure 1.1. Comparison of mean±SEM body weight (A), testicular volume (B), and plasma testosterone concentration (C) across four developmental stages of male monkey (*Macaca mulatta*). One-way ANOVA followed by Tukey's multiple comparison showed that the adult group has significantly higher body weight, testicular volume ($p<0.0001$), and testosterone ($p<0.01$) as compared to other groups.

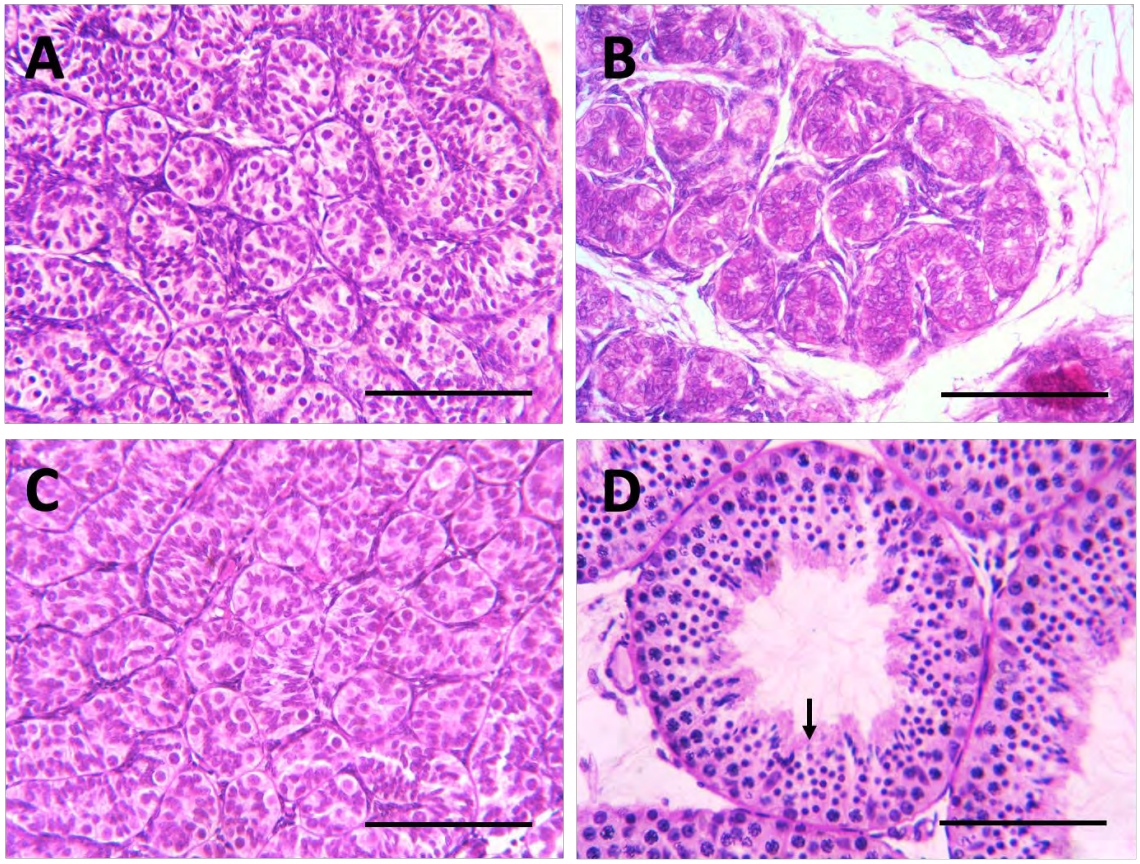

Figure 1.2. Representative photomicrographs demonstrating testicular histology of animals across four developmental stages. In the panel above A represents infant, B represents juvenile, C represents pre-pubertal, and D represents adult monkey testicular section. Wide lumen with active spermatogenesis (arrow indicated) is seen in mature monkeys. Scale bar=120 µm.

Figure 1.3. Comparison of mean±SEM of epithelial height (A) and seminiferous tubule diameter per tubule (B) across four developmental stages of male monkey (*Macaca mulatta*). One-way ANOVA followed by Tukey's multiple comparison showed that the adult group has significantly higher ($p<0.0001$) epithelial height and tubular diameter as compared to other groups.

Figure 1.4. Hypothalamic fold change (Mean±SEM) mRNA expression of *NPY* (A) along with *NPY1R* (B) across developmental stages in male monkeys. A. One way ANOVA followed by Tukey's test showed hypothalamic fold change in *NPY* mRNA expression significantly increased ($p<0.05$) in juvenile and pre-pubertal group (n=4 in each) as compared to infant (n=3) and significant decline ($p<0.05$) in expression was observed in adult as compared to preceding two stages (n=4 in every group). B. One way ANOVA followed by Tukey's test showed non-significant fold change increase ($p>0.05$) in *NPY1R* mRNA expression in juvenile (n=4) as compared to infant (n=3). However, a significant increase ($p<0.05$) was observed in the pre-pubertal group (n=4) as compared to infant (n=3). Comparison between adult and pre-pubertal stage shown significantly reduced ($p<0.05$) expression in adult monkeys (n=4 in both stages).

Figure 1.5. Hypothalamic fold change (Mean±SEM) mRNA expression of *KISS1* (A) and *KISS1R* (B) across pubertal stages in male rhesus monkeys. A. One way ANOVA followed by Tukey's test showed hypothalamic fold change in *KISS1* mRNA expression significantly increased ($p<0.05$) in adult (n=4) as compared to infant (n=3) group. Adult group (n=4) also showed significantly increased ($p<0.01$) expression in comparison to juvenile and pre-pubertal animals (n=4 in each group), however, an insignificant decline ($p>0.05$) was observed in succeeding stages with respect to infant. B. One way ANOVA followed by Tukey's test showed non-significant ($p>0.05$) fold change in *KISS1R* mRNA expression among first three stages. However, non-statistical decline ($p>0.05$) was evident in the pre-pubertal group (n=4) as compared to other groups. Significantly increased ($p<0.05$) expression was only shown by adult monkeys in comparison to pre-pubertal animals (n=4).

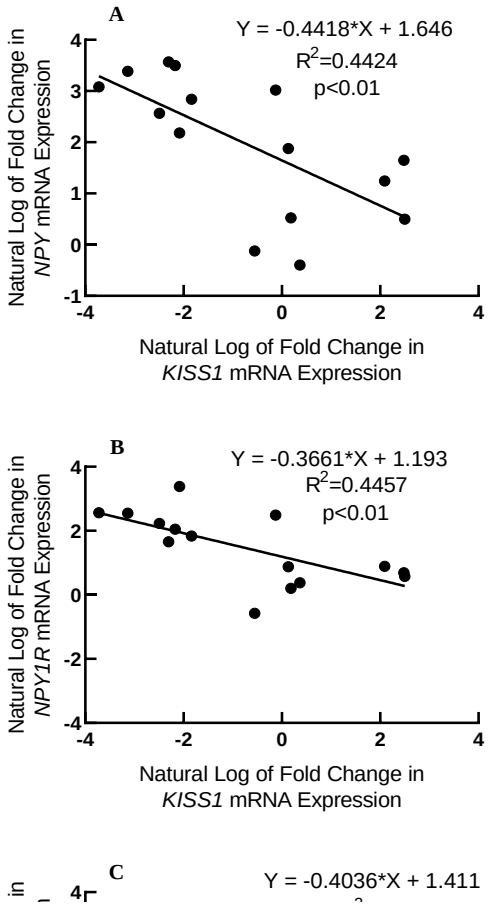

Figure 1.6. Correlation between hypothalamic fold change in *KISS1* and *NPY* (A), between *KISS1* and *NPY1R* (B), and between *KISS1R* and *NPY1R* mRNA expression (natural log transformed) during pubertal stages in male rhesus monkeys. A, B. Significant correlation ($p<0.01$) was observed between *KISS1* and *NPY*, and between *KISS1* and *NPY1R* fold change mRNA expression. C. *KISS1R* fold change mRNA was significantly ($p<0.05$) correlated with *NPY1R* fold change mRNA expression.

DISCUSSION

The present study demonstrates a gradual decline in *KISS1* mRNA expression from infant to the pre-pubertal stage, while again, a sharp rise is observed in the adult stage of development. These results corroborated the previous observations with respect to kisspeptin expression profile (Navarro *et al.,* 2004a; Takumi *et al.*, 2011) in rodents. The similarity between results also exists when compared with non-human higher representative primates i.e., monkeys. One of the critical studies on rhesus monkey conducted by Shahab and colleagues in 2005 showed pattern increase in kisspeptin expression in arcuate area of monkey hypothalamus. Another study on female monkeys conducted by Keen and colleagues in 2008 under the supervision of Ei Terasawa demonstrated higher levels of kisspeptin secretion in pubertal animals than pre-pubertal animals by using push pull perfusion technique. The observations of the current study are in the same direction as the previous ones in all mammalian models hence add a little more contribution in understanding the role of kisspeptin during pubertal phenomenon. Apart from animal studies humans were also evaluated to observe the role of kisspeptin during this critical phase of development of life. In comparison to pre-pubertal participants, greater amounts of kisspeptin were found in girls who experienced precocious puberty (De Vries *et al.*, 2009). Across developmental stages basal profile of kisspeptin in boys showed that only Tanner stage IV and V has higher kisspeptin levels as compared to Tanner stages I, II, and III (Bano *et al.*, 2009). Furthermore, kisspeptin induced GnRH response in both male and female pubertal monkeys is higher over pre-pubertal male and female monkeys (Guerriero *et al.*, 2012; Garcia *et al*., 2017; Garcia *et al*., 2018), suggesting that developmental maturation of kisspeptin progressively approaches at maximum during the latter part of the pubertal transition. This has also been shown in the present study where higher expression of *KISS1* was observed in adult monkeys as compared to juvenile and pre-pubertal animals. Moreover, relative expression of *KISS1* mRNA in the infant and juvenile stages of the current study corresponds to kisspeptin content observed in these two developmental stages (Ramaswamy *et al*., 2013). In contrast to developmental variations in *KISS1* expression in male monkeys observed in the present study particularly elevated expression in the adult group as compared to pre-pubertal group, no statistical distinction

in kisspeptin protein expression in pre-pubertal and ovariectomized (OVX) adult female monkeys was observed (True *et al*., 2017). Whether this discrepancy between the two results may lie either in gender difference or gonadal status is still unclear. Interestingly, kisspeptin induced GnRH response was relatively smaller in pubertal male monkeys than pubertal female monkeys (Guerriero *et al*., 2012., Garcia *et al*., 2017) which may imply that males are prone to more strong central inhibition. Nevertheless, equal intense co-staining of kisspeptin and GnRH between infant and juvenile male (Ramaswamy *et al*., 2008) and between pre-pubertal and gonadectomized adult female (True *et al*., 2017) monkeys suggests that the restraint which keeps GnRH pulse generator in check during juvenile/pre-pubertal stage lies upstream to kisspeptin neurons.

To ascertain the complete paradigm involving the kisspeptin system during pubertal progression quantification of *KISS1R* expression was also done in the present study. Statistically significant expression of *KISS1R* was observed in the adult group compared to pre-pubertal animals, which contrast with a previous report (Shahab *et al*., 2005) where no statistical difference in *KISS1R* expression has been reported between juvenile and pubertal male monkeys. This disparity may be explained by considering the difference in age and gonadal status of animals. In the former study, early pubertal animals (32-34 months of age) were used, but in the current study, late post-pubertal animals (7-10 years) were considered as adults. A slight increase in *KISS1R* expression during the juvenile stage compared to the infant stage is quite a contentious lending credence that continuous maturation of KISS1R may occur during the pubertal development. However, a precipitous drop in expression during the pre-pubertal stage was observed in the present study, which is compatible with an earlier study where the diminished activity of KISS1R has also been described in earlier Tanner stages of boys (Nabi *et al*., 2018). Thus, a progressive decrease in *KISS1R* expression up to the pre-pubertal stage may indicate a progressive increase in inhibitory tone, likely NPY1R signaling by its cognate ligand, which plausibly offset kisspeptin activity during the pre-pubertal period.

As far as inhibitory factor involving pre-pubertal hiatus of the HPG axis is concerned, we have tried to observe differential expression of *NPY* and *NPY1R* in addition to *KISS1* and *KISS1R*. Our results demonstrated higher levels of *NPY* during juvenile and pre-pubertal

stages, which are in concordance with previous reports in which an increase NPY gene/peptide and mRNA was observed in juvenile male (El Majdoubi *et al*., 2000a) and pre-pubertal female (True *et al.,* 2017) monkeys, respectively. Our results also showed a decline in *NPY* mRNA expression in adult monkeys compared to pre-pubertal animals. A similar decrease was also revealed in adult (early pubertal) male monkeys as compared to juvenile monkeys (El Majdoubi *et al.,* 2000a). Moreover, earlier findings also highlighted that NPY suppresses GnRH/LH secretion in different species, including non-human primates (Kaynard *et al*., 1990; Pierroz *et al.,* 1996; Shahab *et al*., 2003) suggesting that NPY may be considered a prime candidate to act as neurobiological brake.

Inverse correlative changes between *NPY* and *KISS1* expression observed in the present study thrive the notion that NPY may play an important role in regulating kisspeptin neurons during sexual development. From the current results, it may be suggested that reduction of NPY based inhibitory tone may be involved in establishing kisspeptin-dependent stimulatory drive onto GnRH neurons for the onset of puberty. Thus, it is plausible to conceive a direct interaction between NPY and kisspeptin neurons. In this regard, close intimacy between the two has been detected in mammals (Backholer *et al.*, 2010; Polkowska *et al*., 2014). However, morphological interaction data in rhesus monkeys in this regard would also be helpful to unwind the functional role of these peptides during the pubertal process. Moreover, the direct inhibitory effect of NPY on kisspeptin neuronal activity has also been demonstrated in vitro (Hessler *et al*., 2020) and in vivo, prolonging estrous cycle duration in female mice (Padilla *et al*., 2017) suggests some synchronicity may be involved between the two neuronal populations to cater reproductive profile.

Considering inhibitory action of NPY mediated by NPY1R and NPY1R antagonist provoke LH release in pre-pubertal and adult male monkeys (Shahab *et al*., 2003) and rats (Pralong *et al*., 2000), NPY1R gene expression profile during sexual maturation was also recorded. Results showed a gradual increase in *NPY1R* during pubertal progression up to the pre-pubertal stage, while a precipitous decline in expression was noticed in adults. These observations indicate the importance of NPY signaling via NPY1R to regulate the HPG axis. Based on above-cited literature about NPY regulation of kisspeptin neurons, it is imperative to observe the presence of NPY1R on kisspeptin neurons.

In this context, convincing evidence to support our hypothesis is provided by an elegant recent work where the direct inhibitory effect of NPY on kisspeptin neurons was partially reversed by NPY1R antagonist application in arcuate brain slices of adult male mice (Hessler *et al*., 2020). In addition, RNA-based sequencing revealed the presence of NPY1R transcripts in rodent arcuate kisspeptin neurons (Unpublished data as cited in Hessler *et al*., 2020), which highlights the role of NPY based suppression of kisspeptin neuronal activity is mediated by NPY1R. Nevertheless, further work is needed to determine the pre and postnatal developmental regulation of NPY1R on kisspeptin neurons and whether the NPY1R ablation of kisspeptin neurons may play a permissive role in activating the HPG axis during the juvenile period remains to be elucidated.

A significant inverse correlative trend between *KISS1* and *NPY* and between *KISS1* and *NPY1R* relative mRNA was observed in the current study. Likewise, an inverse correlative trend between *KISS1R* and *NPY1R* relative mRNA was also detected. Correlative data suggests that an upsurge in *NPY* and *NPY1R* mRNA expression highlights possibly increased concerted action of NPY signaling, which may likely cause subservience in kisspeptin signaling.

The possibility of direct inhibition of GnRH neurons via NPY during pubertal development can also not be excluded owing to the intimate association of NPY fibers to GnRH neurons in rodents (Li *et al*., 1999; Turi *et al.,* 2003) and non-human primates (Plant and Shahab, 2002; True *et al.*, 2017) with NPY1R presence on GnRH neurons (Li *et al*., 1999). Prenatal GnRH nasal explant model also revealed a dose-dependent inhibitory effect of exogenous NPY via NPY1R, which was antagonized by NPY1R antagonist (Klenke *et al*., 2010). It is important to mention that association between two neuronal populations is noticed to be increased in juvenile and pre-pubertal monkeys (Plant and Shahab, 2002; True *et al*., 2017). Hence, recapitulation of the prenatal kisspeptin neuronal model would be helpful to resolve the functional relationships between NPY and kisspeptin. Parenthetically, it is worthy to mention that one isolated case report showed involvement of circulating NPY contributing to delayed puberty (Blogowska *et al.,* 2004). However, on the other hand, two different studies indicated no causal involvement of NPY receptor mutation in inducing precocious HPG axis activity (Barker-Gibb *et al.,* 2004; Freitas *et al*., 2007).

As a potential role of hypothalamic NPY signaling in the initiation of puberty is being suggested, it remains to be studied whether the putative NPY off-on-off switch is constitutive or, in turn, regulated by other signals. While the former idea cannot be excluded, available information is highly suggestive in favor of the latter idea. The presence of leptin (Baskin *et al.,* 1999), insulin (Maejima *et al.,* 2011), and ghrelin (Willesen *et al.*, 1999), receptors on NPY neurons indicates that NPY neuronal population is sensitive to several metabolic signals, particularly during pubertal initiation. In this regard, an elegant study demonstrated that ablation of leptin receptors from NPY neurons caused the delay in pubertal onset (Egan *et al*., 2017). The foregoing study suggests NPY as a plausible candidate to sense and relay energy status information to GnRH neurons during pubertal development.

In summary, our results demonstrate that inverse correlative changes between hypothalamic *NPY* and *KISS1* and the expression of their receptors occur during pubertal development in male rhesus monkeys. Particularly, higher expression of *NPY* and *NPY1R* along with lower expression of *KISS1* and *KISS1R* during juvenile and pre-pubertal stages and vice versa during the infant and adult stages appears to suggest that when NPY levels decline, kisspeptin-dependent HPG axis activation at puberty is expected to occur in higher primates. NPY may operate as a neurobiological suppressor for the dormancy of kisspeptin neurons prior to pubertal onset. However, the involvement of multiple neuropeptides regulating the neuroendocrine reproductive axis during pubertal development cannot be ruled out. Nonetheless, based on previous literature, current findings may implicate NPY as a neurobiological brake for pubertal onset. More comprehensive studies in the future are required to confirm this hypothesis to delineate functional significance and causal relationship between these two neuronal populations. Further sophisticated genetic and pharmacological approaches in this regard would help to resolve enigma related to pubertal development. These findings may be advantageous in designing pharmacological, therapeutic, and clinical approaches to regulate fertility problems in humans.

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

CHAPTER # 02

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

Immunocytochemical evidence for developmental variation in kisspeptin, NPY and NPY1R expression and interaction of NPY and NPY1R with kisspeptin neurons in the hypothalamus of male rhesus monkey (*Macaca mulatta*): A plausible role of NPY-NPY1R signalling in attenuating kisspeptin neuronal activity during pre-pubertal period

ABSTRACT

Introduction: Both Neuropeptide Y (NPY) and kisspeptin have been implicated to play a pivotal role in regulating the hypothalamic-pituitary-gonadal (HPG) axis activity in various species including non-human higher primates. Before the advent of kisspeptinocentric era, the search for the neurobiological inhibitory factor that restrain GnRH pulse generator activity during juvenile period has been the most fascinating question for the reproductive neuroendocrinologist. In this regard, NPY has been considered as one of the potent neuroinhibitory factors in restricting GnRH/LH release. Particularly, an inverse relationship between GnRH pulse generator activity and NPY mRNA/peptide was noticed in male rhesus monkey during pubertal transition. Moreover, immunohistochemical data in non-human higher primates also indicated that intimate association between NPY and GnRH neurons is much higher in juvenile/pre-pubertal monkeys as compared to pubertal/adult monkeys. These observations suggest that NPY may act as a putative neurobiological brake which keeps GnRH pulse generator activity at nadir during pre-pubertal/juvenile stage. However, after the discovery about novel role of kisspeptin in regulating pubertal initiation, extensive research has been done to decode importance of kisspeptin signaling in regulating the HPG axis activity during pubertal development in many species including higher primates. Despite of the fact that a wealth of research has been shown the prime importance of the kisspeptin as integral component of the GnRH pulse generator but failed to draw consensus about its role as the initiator for pubertal onset. Therefore, the quest to search for the neural substrate acting as neurobiological brake to halt GnRH pulse generator activity during juvenile/pre-pubertal stage remains incognito. Recent data in ewes showed that a close intimate association exists between kisspeptin and NPY. In addition, NPY based inhibition of kisspeptin neuronal activity has also been demonstrated by in vivo and ex vivo experiments. Recent studies about NPY suppressions of kisspeptin neurons thrives the notion that NPY may be the plausible neurobiological restrain in diminishing kisspeptin neuronal activity thus keeping GnRH pulse generator in check during juvenile/pre-pubertal stages. Therefore, it was hypothesized that decreased kisspeptin signaling during juvenile/pre-pubertal stage occurs due to increase in NPY inhibitory inputs to kisspeptin neurons with elevated levels of

NPY1R. The present study was designed to examine the developmental variations of NPY, NPY1R, and kisspeptin expression in the mediobasal hypothalamus (MBH) of male rhesus monkeys across different pubertal stages and to observe changes in dual labeled kisspeptin neurons with either NPY or NPY1R during pubertal progression.

Materials and Methods: Fifteen hemi-hypothalamic blocks from four developmental groups (infant group comprised of three animals while four animals were used in other developmental groups) were collected. After fixation and dehydration, blocks were cut at 20 µm thickness on cryostat. Randomly selected sections (n=3) from MBH of all animals were processed to conduct immunocytochemistry. Dual labeled immunofluorescence using cocktail of specific primary antibodies was conducted to examine developmental variations in kisspeptin, NPY and NPY1R expression and to observe variation in interaction of kisspeptin neurons with NPY/NPY1R across four developmental stages.

Results: A significant ($p<0.01$ and $p<0.001$) decrease in kisspeptin immunoreactive cell bodies expression was observed in juvenile and pre-pubertal animals in comparison to infant animals. While an increase ($p<0.05$ and $p<0.001$) in expression was observed in adult group as compared to juvenile and pre-pubertal group. With respect to dual labeling, significantly increased ($p<0.001$ and $p<0.05$) percentage interaction of kisspeptin neurons with NPY was observed in juvenile and pre-pubertal monkeys as compared to infant monkeys. In contrast, significantly decrease ($p<0.01$ and $p<0.05$) percentage of kisspeptin and NPY was also observed in adult and pre-pubertal group respectively as compared to juvenile group. Dual labelled immunofluorescence of kisspeptin and NPY1R expression showed significant percentage increase ($p<0.01$ and $p<0.001$) in juvenile and pre-pubertal animals in comparison to infant. In contrast, the percentage of dual label kisspeptin neurons with NPY1R showed significant decrease ($p<0.001$ and $p<0.01$) in adult group as compared to pre-pubertal and juvenile group, respectively.

Conclusion: Findings of the present study suggest that decreased kisspeptin signaling during juvenile/pre-pubertal stage may occur due to increase in NPY inhibitory input to kisspeptin neurons with elevated levels of NPY1R onto kisspeptin neurons. Increased

percentage of kisspeptin neurons expressing NPY and NPY1R during pre-pubertal stage also highlights the possibly increased concerted action of NPY signaling which may likely cause subservience in kisspeptin signaling before pubertal onset. Current immunohistochemical data suggests that NPY may be the plausible neurobiological restrain that attenuates kisspeptin neuronal activity thus keeping GnRH pulse generator in check during juvenile/pre-pubertal stages. Nevertheless, a potential direct involvement of NPY in inhibiting kisspeptin neuronal activity can be ascertained through detailed confocal analyses. In addition, genetic and pharmacological strategies are required to further delineate the role of NPY-NPY1R in mediating putative inhibitory action on GnRH pulse generator via kisspeptinergic pathway during pubertal development.

INTRODUCTION

Prior to the advent of kisspeptinocentric period, neuropeptide Y (NPY) has been contemplated as one of the essential factors regulating the HPG axis activity during the pubertal development. NPY, a 36 amino acids long peptide has been isolated from porcine brain in 1982 (Tatemoto, 1982). Copious expression of NPY neurons in different hypothalamic nuclei has been observed in different animal models including non-human and human higher primates (Campbell *et al.,* 2001). The most prominent expression was noted in arcuate nuclei, dorsomedial hypothalamic nuclei, medial preoptic area, paraventricular nucleus, suprachiasmatic nucleus, and in median eminence (Chronwall *et al*., 1985). As far as NPY neuronal innervations are concerned, extensive expression of NPY fibers and neuro terminals were also documented throughout the hypothalamus (Dudas *et al*., 2000) innervating the paraventricular nucleus, medial preoptic, supraoptic nucleus, preoptic nucleus, arcuate nuclei, and in the internal zone of the median eminence (Chronwall *et al*., 1985; de Quidt and Emson 1986; Sahu *et al*., 1988a; Ciofi *et al*., 1991; Contijoch *et al.,* 1993; Chaillou *et al.,* 2002).

Among plethora of receptors which are involved in mediating NPY effects, NPY1R has been implicated to regulate reproductive neuroendocrine function (Kalra *et al*., 1992; Blomqvist and Herzog, 1997; Gehlert, 1998; Raposinho *et al*., 2000). An analogous topographic expression of NPY1R like NPY expression, has also been observed in different hypothalamic nuclei such as arcuate nucleus, paraventricular nucleus, dorsomedial nucleus, the medial preoptic area, and ventromedial nuclei (Caberlotto *et al.,* 1997; Kopp *et al*., 2002).

The major physiological role of NPY has been shown to inhibit reproductive axis activity by suppressing GnRH/LH release in various animal models including non-human higher primates (Pierroz *et al*., 1995; Plant and Barker-Gibb, 2004). Particularly, prepubertal hiatus in GnRH pulse generator activity has been shown to inversely related with mRNA/protein expression of NPY in male monkeys (EI Majdoubi *et al*., 2000a). This inverse relationship between NPY and GnRH led to speculate a neuro morphological

connection between two neuronal entities. Immunofluorescence based confocal microscopic observations revealed that the close association between NPY neuronal varicosities onto GnRH neurons are much higher in juvenile male monkeys as compared to pubertal male monkeys (Plant and Shahab, 2002). Corresponding to the observations in male monkeys, a similar trend has also been observed in female monkeys. Higher expression of NPY and significantly higher contacts between NPY fibers and GnRH neurons in pre-pubertal female monkeys (True *et al*., 2017) conferring the notion that NPY may likely involve in inhibition of GnRH release before pubertal onset and diminishing of this brake plausibly the cause to trigger pubertal initiation. Likewise, higher expression of NPY and enhanced NPY innervations with GnRH neurons were also observed in heifers during juvenile period (Alves *et al*., 2015). This direct morpho structural association between NPY fibers and GnRH neurons was also seen in other species like rodent (Campbell *et al*., 2001; Ward *et al*., 2009), and ovine (Norgren and Lehman, 1989; Tillet *et al*., 1989). Moreover, NPY neurons also projected to presynaptic terminals of GnRH neurons in the median eminence (Li *et al*., 1999).

Furthermore, direct NPY inhibition of GnRH secretion is thought to be mediated by NPY1R (Klenke *et al*., 2010; Roa and Herbison, 2012). This facet of NPY1R presence on GnRH neurons has been manifested in adult mice and in GnRH neuronal population extracted from nasal explant of mice (Klenke *et al*., 2010; Roa and Herbison, 2012) respectively. Immunocytochemical data also revealed co-localization of NPY1R onto GnRH neurons (Li *et al*., 1999). NPY induced inhibition of the HPG axis thought to be mediated by NPY1R (Gazal *et al*., 1998; Gonzales *et al.,* 2004). NPY1R has also been considered a potential receptor to mediate NPY inhibition of GnRH release in monkeys (Plant and Shahab, 2002; Shahab *et al.,* 2003).

Numerous experimental strategies have demonstrated that kisspeptin signaling is not only involved in onset of puberty but also in the regulation of the HPG axis activity during adulthood. It has been observed that kisspeptin administration (peripheral and central) caused robust discharge of LH in different species like rodents and non-human primates. Central administration of kisspeptin caused early pubertal onset as evident by vaginal

opening in female and preputial separation in male rats (Navarro *et al*., 2004b; Navarro *et al*., 2005a). Similarly central and peripheral administration of KP increased LH levels in juvenile male rhesus monkey (Shahab *et al*., 2005). In addition, push pull perfusion samples from the stalk-median eminence of pubertal female monkeys indicated an association and correlation of pulsatile release between kisspeptin and GnRH (Keen *et al*., 2008). Both kisspeptin and its receptor have shown higher pubertal hypothalamic expression in rodent (Navarro *et al*., 2004a), and non-human primates (Shahab *et al.,* 2005). A similar developmental profile of kisspeptin levels has also been shown in humans (Bano *et al*., 2009) across Tanner stages. Pharmacological studies also demonstrated that kisspeptin antagonist diminished kisspeptin induced gonadotropins secretion in rodents and macaques (Roseweir *et al*., 2009) and delayed vaginal opening in female rodents (Pineda *et al*., 2010). All these studies show an integral role of kisspeptin in pubertal onset.

Although the push-pull perfusion and immunocytochemical data have revealed lower kisspeptin levels along with lower kisspeptin content in female and male rhesus monkeys during juvenile and pre-pubertal stages respectively, however, it is interesting to note that kisspeptin content in MBH of infant and adult male monkeys was comparable (Kurian *et al.,* 2012; Ramaswamy *et al.,* 2013). Moreover, a similar pattern of GnRH fibers expression at the median eminence in male monkeys (Ramaswamy *et al.,* 2013) and similar association between kisspeptin fibers onto GnRH neurons in female monkeys (True *et al*., 2017) along developmental trajectory i.e., between infantile and juvenile stage and between pre-pubertal and adult stage respectively, suggest that a neurobiological brake restricting GnRH release during juvenile period by restraining kisspeptin drive.

Studies in different animal models have generated the idea that kisspeptin enhances HPG axis activity while the role of NPY is to suppress the axis (Terasawa *et al*., 2013; Manfredi-Lozano *et al*., 2018; Plant, 2019). However, NPY regulation of kisspeptin neurons is shown by two recent studies. Using optogenetic technique, it has been demonstrated that arcuate kisspeptin neurons are the direct target of NPY inhibitory inputs (Padilla *et al*., 2017). Electrophysiological data of arcuate kisspeptin neurons tagged with green fluorescent protein from intact adult male and female mice showed suppression of kisspeptin neuronal

firing by NPY (Hessler *et al*., 2020). This inhibitory effect of NPY on kisspeptin neuronal activity as shown by voltage-clamped data was reversed by the application of NPY1R antagonist (Hessler *et al*., 2020). In addition, chemo genetic activation of NPY neurons caused attenuation of fertility as indicated by decrease in LH secretion and stretch in time of estrous cyclicity (Padilla *et al*., 2017). These observations emphasized the idea that kisspeptin neurons are the direct target of NPY and kisspeptin dependent HPG axis activity is primarily modulated by NPY signaling. This has also been manifested by gene expression data in non-primate and primate model. In adult male mice an opposite expression profile of kisspeptin and Npy gene was observed (Molnar *et al.,* 2016). Likewise, previous study also demonstrated inverse correlative changes between hypothalamic kisspeptin and NPY gene expression in male monkeys during pubertal progression (Bano *et al.,* 2022). Hence, the above-cited data suggests that NPY may act as plausible neurobiological brake restraining kisspeptin drive onto GnRH neurons during pubertal onset.

The physiological regulation of kisspeptin neurons by NPY and opposite gene expression profile of the two suggest neuroanatomical association between these two neuronal populations. The hierarchical role of NPY and kisspeptin neurons in regulating the HPG axis activity has been demonstrated in ewes (Backholer *et al*., 2010; Polkowska *et al*., 2014). It has been demonstrated that kisspeptin neurons are closely apposed by NPY neuronal fibers in female lambs. Almost thirty percent kisspeptin neurons have been shown to be innervated by NPY fibers (Polkowska *et al*., 2014). In another study, a mutual association between kisspeptin neuronal cells with NPY fibers and vice versa has been indicated in intact and ovariectomized ewes. Percentage apposition of kisspeptin fibers with NPY cells has shown to be less as compared to percentage apposition of NPY fibers with kisspeptin cells. In addition, NPY fibers association with kisspeptin neurons were more in ovariectomized condition with respect to intact animals (Backholer *et al*., 2010). This structural association between NPY fibers with kisspeptin neurons is in view of synaptic connection (Amstalden *et al*., 2011). This shows commanding position of NPY in regulating GnRH release via kisspeptinergic neurons. However, this neuroanatomical association has not been yet studied in higher primates.

Albeit the continuous efforts have been done to search plausible neurobiological substrate which acts as a brake to keep GnRH pulse generator activity in quiescent mode during juvenile period, the irrefutable neurobiological factor keeping dormant state of GnRH pulse generator in juvenile period after brief stimulation in infant stage remains incognito. Recent studies about NPY suppression of kisspeptin neurons and inverse correlative gene expression of the two thrives the notion that NPY may be the plausible neurobiological restrain in diminishing kisspeptin neuronal activity thus keeping GnRH pulse generator in check during juvenile/pre-pubertal stage. Hence, to check whether gene expression data correspond to protein data, it was hypothesized that decreased kisspeptin signaling during juvenile/pre-pubertal stage occurs due to increase in NPY inhibitory inputs to kisspeptin neurons with elevated levels of NPY1R. Therefore, the present study was designed to examine the developmental variations of NPY, NPY1R, and kisspeptin expression in the MBH of male rhesus monkeys across different pubertal stages and to observe changes in dual labeled kisspeptin neurons with either NPY or NPY1R.

MATERIALS AND METHODS

Animals

A total of fifteen intact male rhesus monkeys (*Macaca mulatta*) were used to conduct the present research experiment. Fifteen animals assigned into four different developmental groups [infant (4-7 months)], [juvenile (10-14 months)], [pre-pubertal (21-24 months)], [adult (7-10 years)] were employed to study morphological interaction and variations in NPY and NPY1R expression on kisspeptin neurons through fluorescent immunocytochemistry. Each animal was kept in a separate cage under semi-controlled environmental conditions (25±3°C) temperature, 12-hour light/dark photoperiod with lights on between 06:00 and 18:00 hr in the Primate Facility of the Department of Zoology, Quaid-i-Azam University, Islamabad in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. The daily meal comprised of boiled potato (09:00-09:30 hr), boiled eggs (11:00 hr), bread (13:00-13:30 hr) and fresh fruits, nuts (15:00-16:00 hr). *Ad libitum* access to water was ensured. Protocols and procedure of all experimentation was approved by the Animal Care and Use Committee of the Department of Zoology.

Tissue Collection

Fifteen animals assigned into four different developmental groups [infant (4-7 months)], [juvenile (10-14 months)], [pre-pubertal (21-24 months)], [adult (7-10 years)] based upon physical (body weight and testicular volume) and hormonal (testosterone) parameters were sacrificed to obtain hypothalamic blocks. Three animals (n=3) in the infant group, while four animals (n=4) each in the rest of the groups were employed for dissecting hypothalamic tissue. Before dissection, animals were sedated with intramuscular (*im*) injection of ketamine hydrochloride (Ketamax, Rotex Medica, Trittau, Germany; 10-20 mg kg^{-1} BW). After sedation body weight was taken. Detailed description regarding hypothalamic dissection with respect to landmarks as identified in monkey brain atlas is mentioned in general materials and methods section.

Fluorescence Immunocytochemistry

Fifteen hemi-hypothalamic blocks which were dissected out as described in detail in general materials and methods section were used to conduct fluorescent immunocytochemistry. Following two sets of different dual labeled immunostainings were performed:

i. to observe developmental variation in kisspeptin and NPY expression and to examine the changes in close apposition between NPY and kisspeptin neurons.
ii. to observe developmental changes in NPY1R expression onto kisspeptin neurons.

I. Tissue Fixation

Fixation of hemi-hypothalamic block was carried out in freshly prepared paraformaldehyde (4% PFA, Scharlau Chemie SA, Barcelona, Spain) solution in 0.1M phosphate buffer saline (PBS), (pH 7.4). After fixation, dehydration was carried out two times, first in 20% sucrose solution (Fischer Chemical, Leicestershire, UK) in 0.1M PBS at 4 ºC and second 30% sucrose solution in 0.1M PBS at 4 ºC. Sectioning was not proceeded until tissues were completely sank at the bottom of the vial.

II. Tissue Sectioning

Each of the hemi-hypothalamic block was sectioned using a cryostat (Bright OTF 5000, A-M Systems, Sequim, Washington, USA; temperature -25 ºC). 20 µm thick sections were cut. The sections were stored in cryopreservative solution (30% ethylene glycol, 1% polyvinylpyrrolidone and 30% sucrose in PBS) at -20 ºC until processed for immunocytochemical detection of a particular peptide.

III. Antibodies

Details of primary and secondary antibodies used to conduct two different immunostainings on free-floating hemi-hypothalamic sections are shown in Table 2.1.

Table 2.1. List of primary and secondary antibodies used in immunocytochemistry.

Antibody	Dilution	Catalogue #	Source
Sheep anti-KISS1 (GQ2)	1:120,000		gifted by Dr. Bloom (Imperial College London, Hammersmith Hospital, London, UK)
Rabbit anti-NPY	1:500	ab30914	Abcam, Boston, MA, USA
Mouse anti-NPY1R	1:200	sc393192	Santa Cruz Biotechnology, Dallas, Texas USA
Donkey anti sheep AF-488	1:500	ab150177	Abcam, Boston, MA, USA
Goat anti rabbit Cy-3	1:500	AS007	ABclonal, Wuhan, Hubei, China
Goat anti mouse TR	1:400	sc2781	Santa Cruz Biotechnology, Dallas, Texas USA

IV. Procedure

Free floating 20 µm thick sections were treated with specific primary and fluorescently tagged secondary antibodies for two sets of fluorescent immunocytochemistry. Three sections per animal were used for each staining while one section per animal was used as primary antibody omitted control for each staining. Protocol to conduct both immunostainings was same. Sterile cell culture plates (Nest Biotechnology Co. Ltd, China) were used for all washings and incubations. Initially, phosphate buffer saline solution, prepared with the help of Omnipur PBS tablets **(**Calbiochem, EMD chemicals Inc, New Jersey, USA**)** with approximate pH 7.2 to 7.4, was used to wash sections eight times for 15 min at room temperature on a shaker (Serono Diagnostic, London, UK). To block non-specific binding, incubation of sections was carried out on shaker for 120 min at room temperature in incubation solution containing 10% normal goat and donkey serum (NGS and NDS, respectively), Triton-X 100 (0.05%) and bovine serum albumin (BSA) (0.1%) in PBS. After that the sections were washed three times for 15 min each with PBS solution. For first set of immunostaining, sections were then incubated in a cocktail of primary antibodies for kisspeptin [GQ2; 1:120,000; generously gifted by Dr. Bloom (Imperial College London, Hammersmith Hospital, London, UK)] and NPY [ab30914; 1:500; (Abcam, Boston, MA, USA)] diluted in an incubation solution. The constitution of the incubation solution was same as described above. After addition of cocktail solution of primary antibodies, two days incubation (48 hr) at 4 ºC on a shaker was done. Similarly, for second set of staining, incubation of sections at 4 ºC for 48 hours was carried out in a cocktail of specific primary antibodies for NPY1R [sc393192; 1:200; (Santa Cruz Biotechnology, Dallas, Texas USA)] and kisspeptin [GQ2; 1:120,000; generously gifted by Dr. Bloom (Imperial College London, Hammersmith Hospital, London, UK)]. After 48 hours, washing was done in PBS three times for 15 min with same scheme mentioned above. Then, cocktail of secondary antibodies was added after washing. Secondary antibody incubation was carried out at room temperature on shaker for 2 hours in complete darkness. For kisspeptin and NPY detection, mixture of secondary antibodies (Alexa fluor 488 Donkey anti sheep at 1:500 and goat anti rabbit Cy-3 at 1:500) in incubation solution was used. Similarly, for second set of staining to detect kisspeptin and NPY1R, mixture of

secondary antibodies (Alexa fluor 488 Donkey anti sheep at 1:500 and goat anti mouse Texas red at 1:400) was also diluted in incubation solution. Secondary antibodies were also added in wells containing primary antibody omitted control sections. Following incubation, washing was again done for 15 minutes each. After that, sections were mounted on super frosted slides (12-550-15, Fisher Scientific, Waltham, MA, USA). The slides were dried by keeping them overnight in the darkness. After overnight drying, cover slips were placed using gelvatol as mounting medium. Then slides were wrapped in aluminum foil, kept in wooden box in refrigerator at 4 ºC, and stored till fluorescent microscopy was performed.

V. Microscopy

Imaging of fluorescent labeling for kisspeptin, NPY and NPY1R was examined using fluorescent microscope (Olympus BX51, Tokyo, Japan) by appropriate filters. A camera connected to microscope helped to take images. All sections containing MBH area from each animal were thoroughly scanned. Quantification of total and mean numbers of kisspeptin cells was done in each section from all animals. In both immunostainings, number of kisspeptin cells expressing NPY and NPY1R were also counted as indicated by yellow color immunoreactivity in merged photos.

Statistical Analyses

All the data were expressed as mean±SEM. Changes in number of immunoreactive expression of kisspeptin and dual label kisspeptin with NPY and NPY1R during pubertal development were compared by one-way ANOVA followed by Tukey's multiple comparison test. All statistical analyses were performed by using GraphPad Prism Software version 8.3.0 (GraphPad software, La Jolla, CA, USA). Statistical significance level was set at $p < 0.05$.

RESULTS

In the current study developmental variations in NPY-like immunoreactive (IR), kisspeptin-like IR neurons were observed. In addition, potential colocalization between the two neuropeptides was also observed in MBH of male rhesus monkeys across different developmental stages. Representative immunofluorescent photomicrographs showing immunoreactive expression of kisspeptin and NPY and their colocalization across four developmental stages are depicted in Figures 2.1-2.4. Individual and mean number of kisspeptin neurons along with mean dual labelled kisspeptin and NPY neurons with percentage are shown in Table 2.2-2.5.

Kisspeptin like-IR neurons have been observed to decrease from infant to pre-pubertal stage while an increase in kisspeptin-like IR neurons from pre-pubertal to adult stage was evident. Comparable immunoreactive expression of kisspeptin has been observed in infant and adult stage indicating involvement of kisspeptin to drive GnRH pulse generator activity in regulating reproductive neuroendocrine axis. Particularly, in juvenile and pre-pubertal animals significant decrease ($p<0.01$ and $p<0.001$, respectively) in kisspeptin immunoreactive expression was manifested as compared to infant animals. However, higher immunoreactive kisspeptin expression in adult group was noticed in comparison to juvenile ($p<0.05$) and pre-pubertal ($p<0.001$) groups as shown in Figure 2.5A.

Colocalization between NPY and kisspeptin showed higher percentage in juvenile monkeys as compared to infant ($p<0.001$) while adult animals showed significant ($p<0.01$) decrease in percentage of kisspeptin neurons colocalised with NPY as compared to juvenile monkeys. Significant increased ($p<0.05$) in percentage colocalization of NPY and kisspeptin neurons was also observed in pre-pubertal group as compared to infant. Albeit the higher colocalization of NPY and kisspeptin neurons in pre-pubertal group with respect to infant group was observed, interestingly, the significant ($p<0.05$) percentage decrease of colocalization was observed in pre-pubertal animals in comparison to juvenile group as shown in Figure 2.5B. Primary antibody was not added in control sections to ensure the non-specific cross reactivity of the primary antibodies. Complete absence of IR expression

in primary antibody omitted control sections verified the specificity of the primary antibodies (Figures 2.1-2.4).

Similarly, developmental variations in NPY1R-like (IR) and kisspeptin-like IR and colocalization between the kisspeptin and NPY1R has also been observed. Representative immunofluorescent photomicrographs showing immunoreactive expression of kisspeptin and NPY1R and their colocalization across four developmental stages are represented in Figures 2.6-2.9. Individual and mean number of kisspeptin neurons along with dual labelled kisspeptin with NPY1R and their percentages are shown in Table 2.6-2.9.

A gradual decline in kisspeptin like-IR neurons from infant to pre-pubertal stage has been observed while an increase in kisspeptin-like IR neurons from pre-pubertal to adult stage was noticed. In juvenile and pre-pubertal animals, significant decline in kisspeptin immunoreactivity ($p<0.01$ and $p<0.001$, respectively) was noted as compared to infant animals. However, higher immunoreactive kisspeptin expression in adult group has been observed in comparison to juvenile ($p<0.05$) and pre-pubertal ($p<0.01$) group (Figure 2.10A).

Dual labelled immunofluorescence of NPY1R and kisspeptin showed higher percentage ($p<0.001$) of kisspeptin neurons with NPY1R in pre-pubertal monkeys as compared to infant. Likewise, higher percentage of kisspeptin neurons with NPY1R was observed in juveniles in comparison to infants ($p<0.01$). Non-significant percentage increase in colocalization of NPY1R and kisspeptin neurons was also observed in pre-pubertal group as compared to juvenile group. Adult animals showed significant ($p<0.001$) decrease in percentage of kisspeptin neurons with NPY1R as compared to pre-pubertal monkeys and the significant ($p<0.01$) decrease of this colocalization was also observed in adult animals in comparison to juvenile group (Figure 2.10B). Control sections devoid of primary antibody were also processed to ensure the validity of the primary antibodies. Complete absence of IR expression in primary antibody omitted control sections verified the specificity of the kisspeptin and NPY1R antibodies (Figures 2.6-2.9).

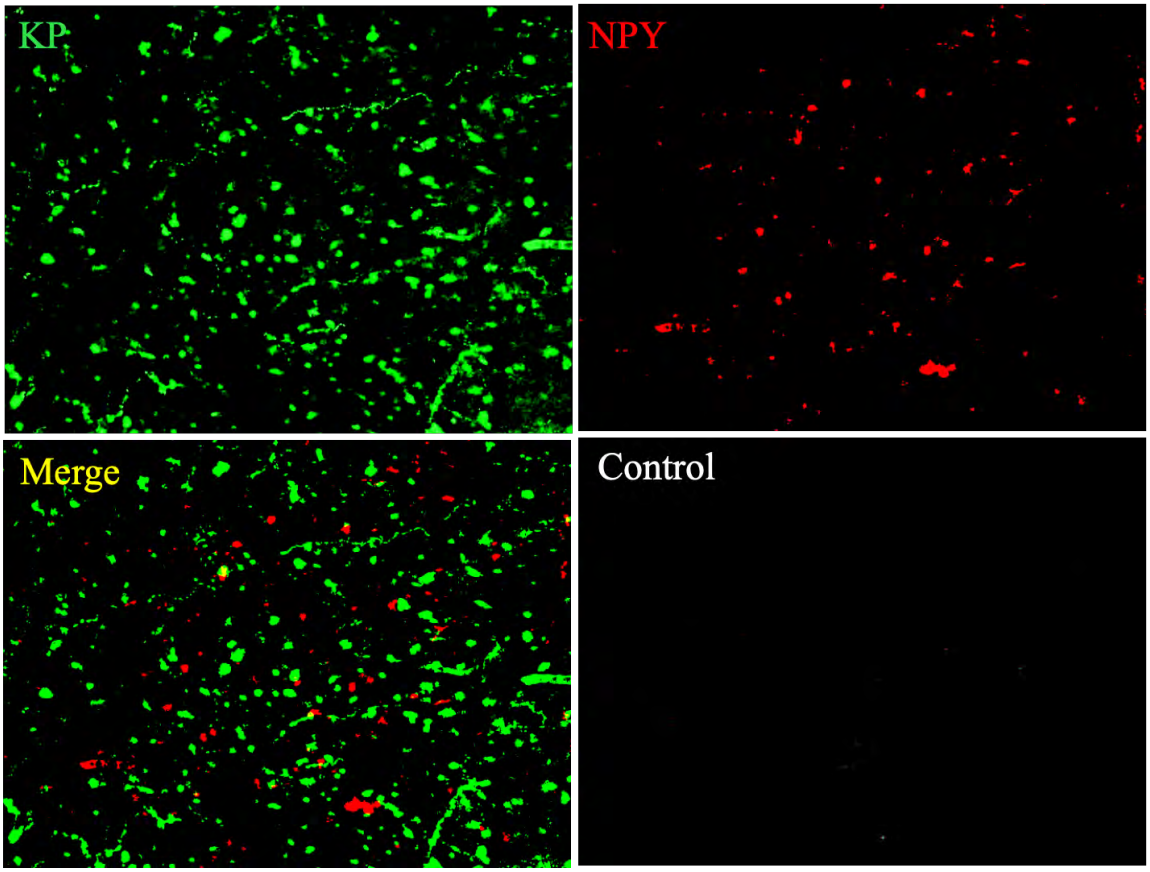

Figure 2.1. Representative fluorescent photomicrograph showing kisspeptin immunoreactivity (KP-IR, green) and NPY immunoreactivity (NPY-IR, red) in the mediobasal hypothalamic sections of infant male monkey. Colocalization between two neuropeptides is presented as yellow color in merged image. Primary antibody omitted control sections showed no immunofluorescence. Photographs were taken at 20x objective by using Olympus BX51.

Table 2.2. Total and mean number of single kisspeptin like-IR and double labelled (dbl) kisspeptin neurons expressing NPY in three random sections of MBH in infant male rhesus monkeys.

Animal No.	Section A		Section B		Section C		Mean KP	Mean dbl	% dbl
	KP	dbl	KP	dbl	KP	dbl			
2M-18	198	16	150	15	159	14	169.00	15.00	8.88
3M-17	213	15	186	17	204	18	201.00	16.67	8.29
7M-16	240	23	252	24	216	23	236.00	23.33	9.89
Mean ±SEM							202.00 ±19.34		09.01 ±0.46

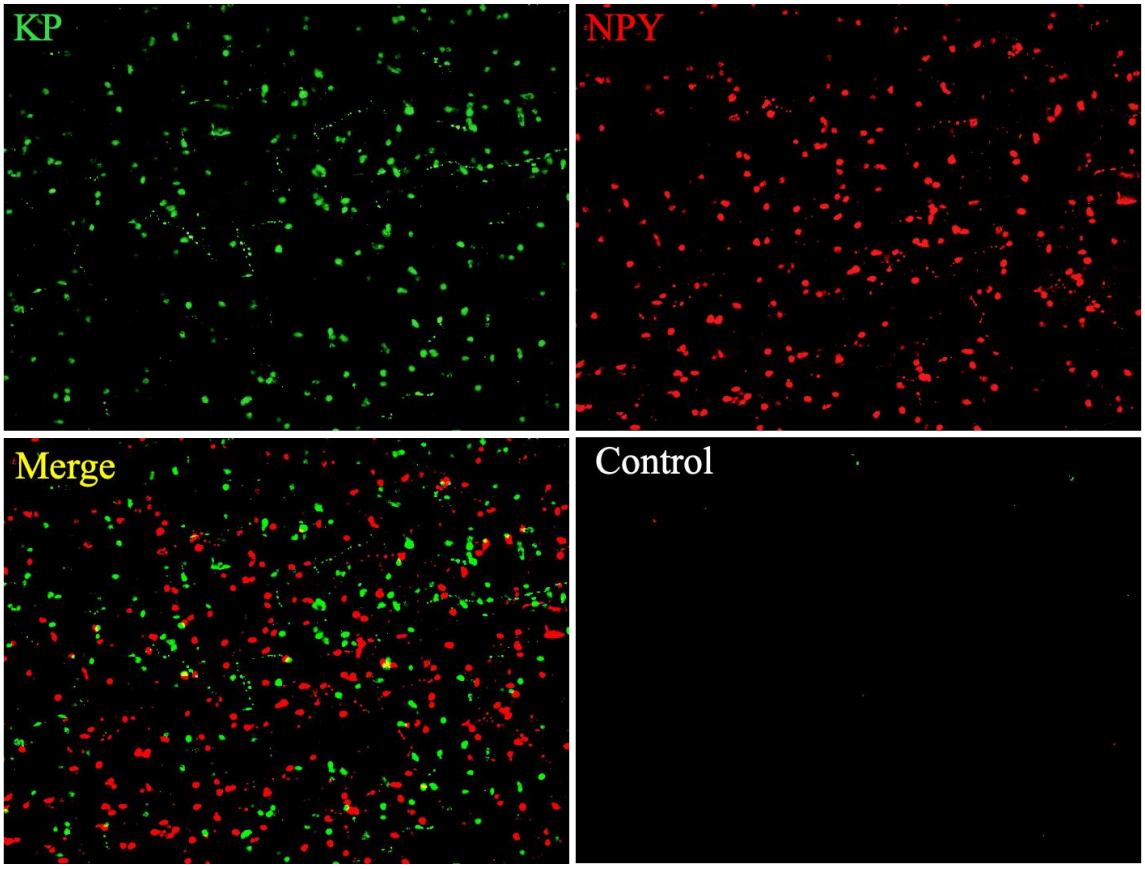

Figure 2.2. Representative fluorescent photomicrograph showing kisspeptin immunoreactivity (KP-IR, green) and NPY immunoreactivity (NPY-IR, red) in the mediobasal hypothalamic sections of juvenile male monkey. Colocalization between two neuropeptides is presented as yellow color in merged image. Primary antibody omitted control sections showed no immunofluorescence. Photographs were taken at 20x objective by using Olympus BX51.

Table 2.3. Total and mean number of single kisspeptin like-IR and double labelled (dbl) kisspeptin neurons expressing NPY in three random sections of MBH in juvenile male rhesus monkeys.

Animal No.	Section A		Section B		Section C		Mean KP	Mean dbl	% dbl
	KP	dbl	KP	dbl	KP	dbl			
3M-16	135	17	153	18	147	20	145	18.33	12.64
4M-16	162	23	141	24	147	23	150	23.33	15.56
5M-16	138	17	165	20	123	18	142	18.33	12.91
6M-16	144	25	162	24	180	26	162	25.00	15.43
Mean ±SEM							149.75 ±04.40		14.14 ±0.78

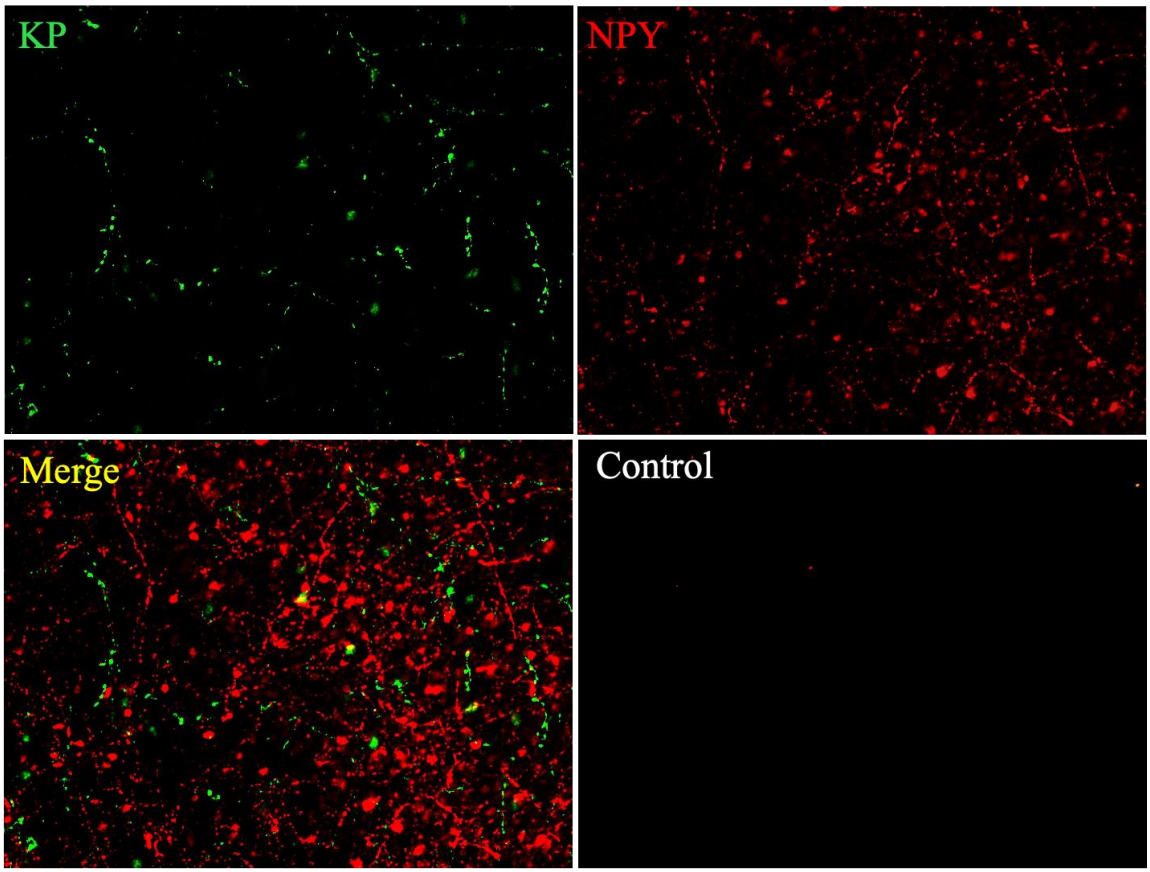

Figure 2.3. Representative fluorescent photomicrograph showing kisspeptin immunoreactivity (KP-IR, green) and NPY immunoreactivity (NPY-IR, red) in the mediobasal hypothalamic sections of pre-pubertal male monkey. Colocalization between two neuropeptides is presented as yellow color in merged image. Primary antibody omitted control sections showed no immunofluorescence. Photographs were taken at 20x objective by using Olympus BX51.

Table 2.4. Total and mean number of single kisspeptin like-IR and double labelled (dbl) kisspeptin neurons expressing NPY in three random sections of MBH in pre-pubertal male rhesus monkeys.

Animal No.	Section A		Section B		Section C		Mean KP	Mean dbl	% dbl
	KP	dbl	KP	dbl	KP	dbl			
4M-18	99	14	150	15	126	16	125	15.00	12.00
5M-18	117	17	141	16	147	17	135	16.67	12.35
6M-18	120	14	114	13	144	13	126	13.33	10.58
7M-18	123	15	111	14	141	16	125	15.00	12.00
Mean ±SEM							127.75 ±02.42		11.73 ±0.39

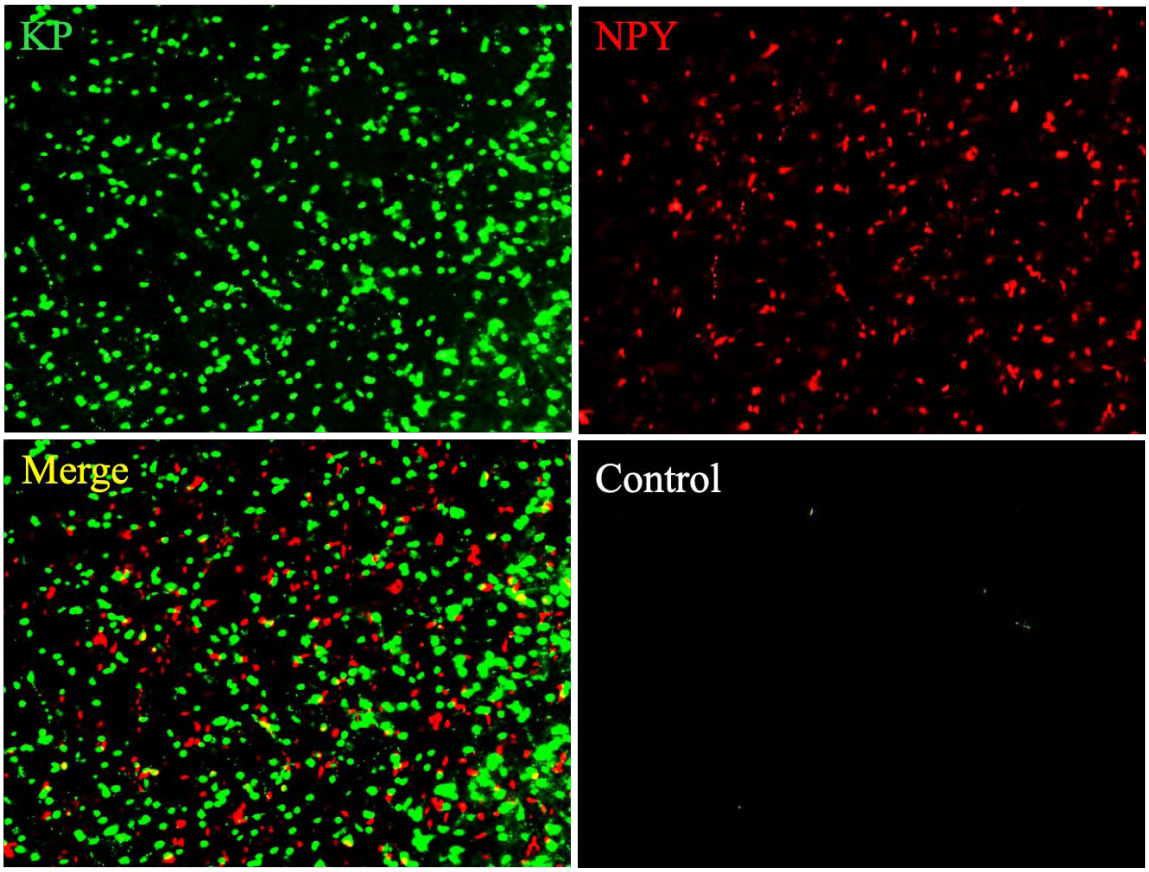

Figure 2.4. Representative fluorescent photomicrograph showing kisspeptin immunoreactivity (KP-IR, green) and NPY immunoreactivity (NPY-IR, red) in the mediobasal hypothalamic sections of adult male monkey. Colocalization between two neuropeptides is presented as yellow color in merged image. Primary antibody omitted control sections showed no immunofluorescence. Photographs were taken at 20x objective by using Olympus BX51.

Table 2.5. Total and mean number of single kisspeptin like-IR and double labelled (dbl) kisspeptin neurons expressing NPY in three random sections of MBH in adult male rhesus monkeys.

Animal No.	Section A		Section B		Section C		Mean KP	Mean dbl	% dbl
	KP	dbl	KP	dbl	KP	dbl			
1M-14	195	19	200	21	185	20	193.33	20.00	10.34
2M-14	176	19	191	21	188	20	185.00	20.00	10.81
1M-16	190	20	174	22	179	23	181.00	21.67	11.97
2M-16	184	18	193	21	207	21	194.67	20.00	10.27
Mean ±SEM							188.50 ±03.28		10.85 ±0.39

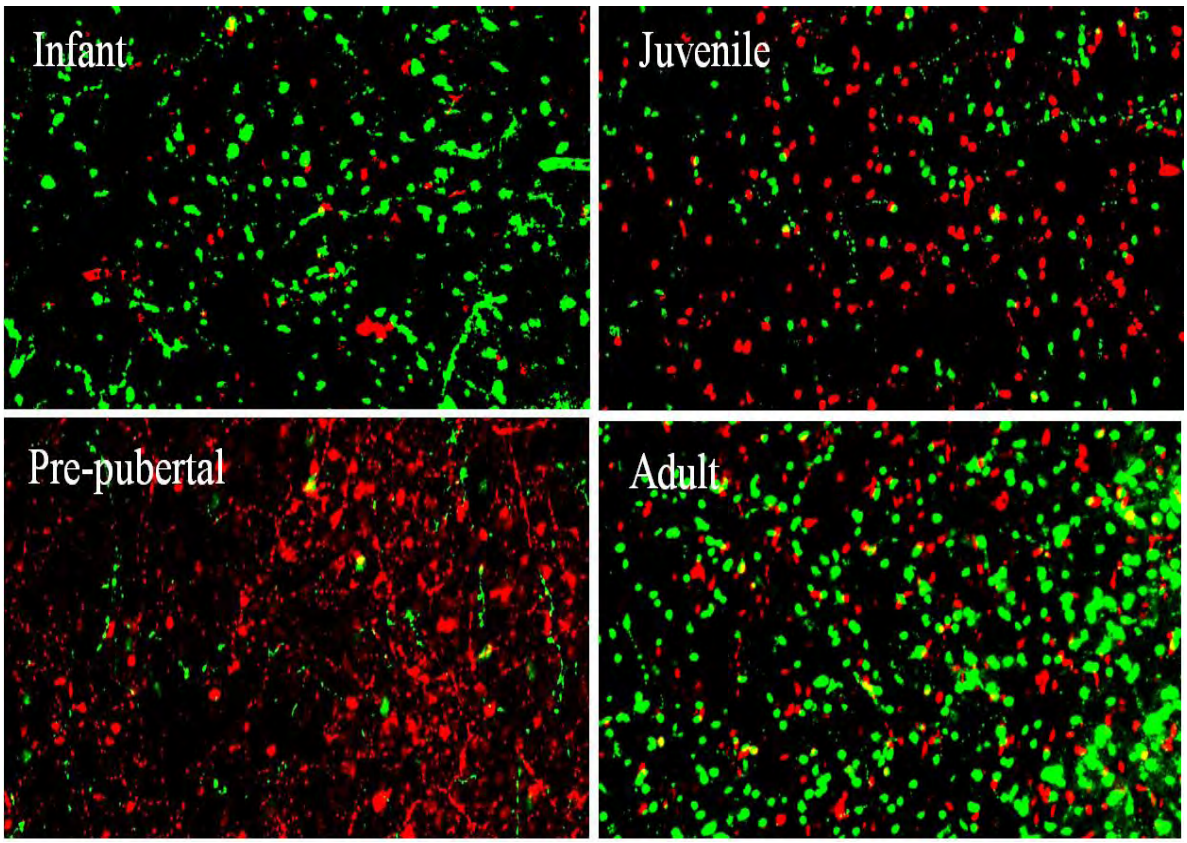

Figure 2.5. Representative fluorescent photomicrographs showing colocalization between kisspeptin immunoreactivity (KP-IR, green) and NPY immunoreactivity (NPY-IR, red) as yellow color in merged images in the mediobasal hypothalamic sections of male rhesus monkey across four developmental stages (infant, juvenile, pre-pubertal, and adult).

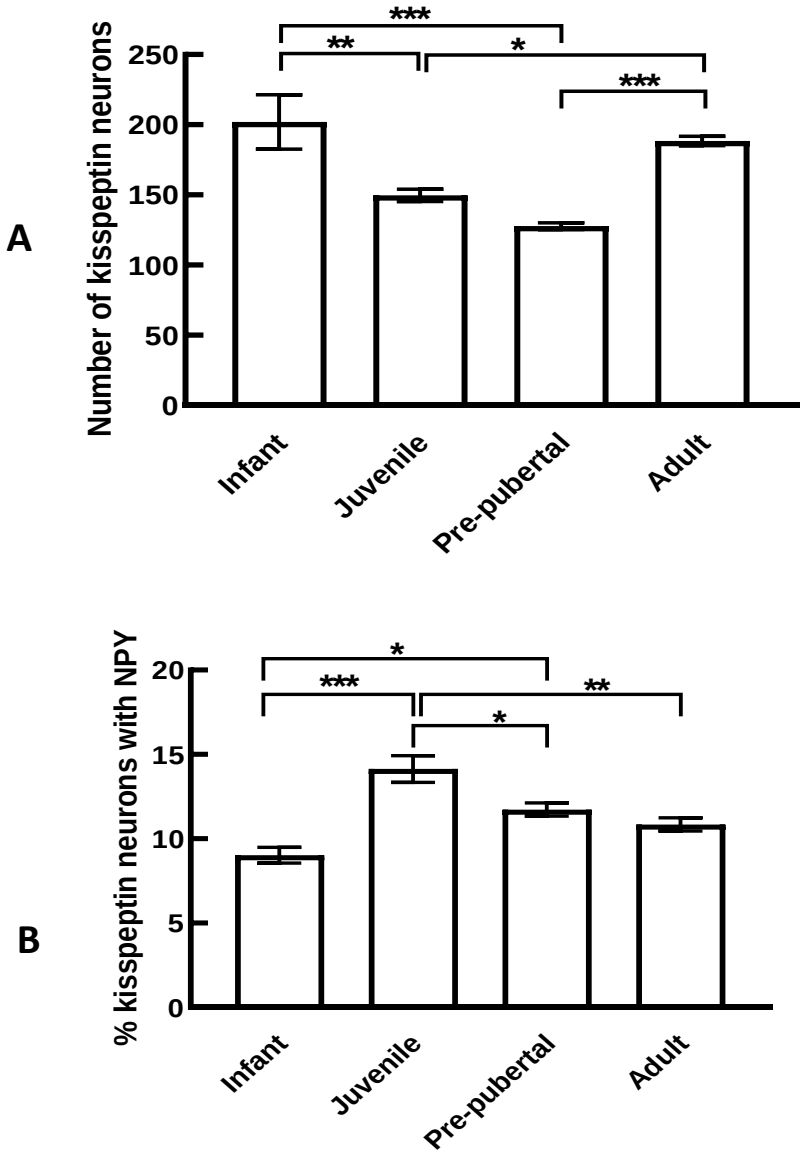

Figure 2.6. Comparison of mean±SEM of kisspeptin-like IR neurons in MBH of male monkeys during different stages of pubertal development. Analysis of variance (One way) followed by post-hoc multiple comparison (Tukey's test) showed significant decrease ($p<0.01$ and $p<0.001$) in number of kisspeptin-like IR neurons in juvenile and pre-pubertal animals respectively as compared to infant. However, an increase in kisspeptin-like IR neurons ($p< 0.001$) from pre-pubertal to adult stage has been observed (A). Comparison of mean±SEM percentage of kisspeptin neurons expressing NPY showed higher percentage ($p<0.001$ and $p<0.01$) in juvenile monkeys as compared to infant and adult monkeys respectively (B).

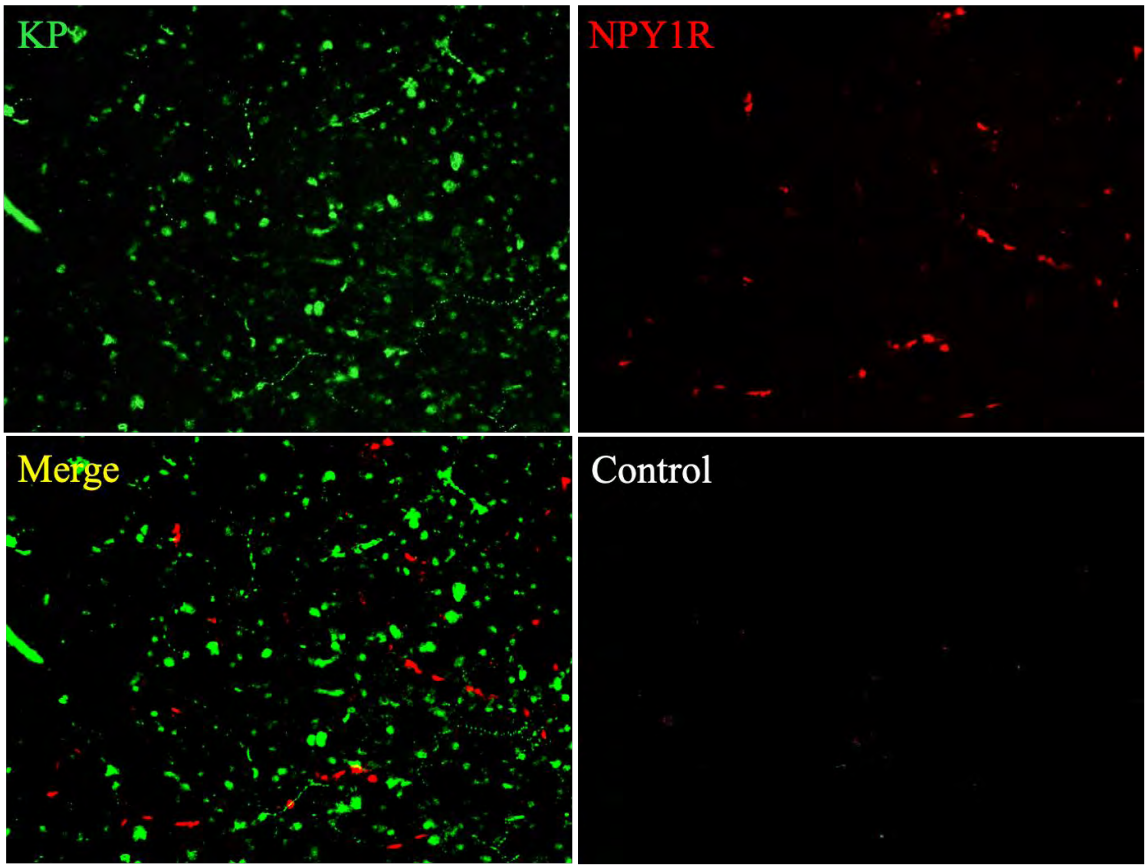

Figure 2.7. Representative fluorescent photomicrograph showing kisspeptin immunoreactivity (KP-IR, green) and NPY1R immunoreactivity (NPY1R-IR, red) in the mediobasal hypothalamic sections of infant male monkey. Colocalization between two is presented as yellow color in merged image. Primary antibody omitted control sections showed no immunofluorescence. Photographs were taken at 20x objective by using Olympus BX51.

Table 2.6. Total and mean number of single kisspeptin like-IR and double labelled (dbl) kisspeptin neurons expressing NPY1R in three random sections of MBH in infant male rhesus monkeys.

Animal No.	Section A		Section B		Section C		Mean KP	Mean dbl	% dbl
	KP	dbl	KP	dbl	KP	dbl			
2M-18	201	26	192	24	198	25	197.00	25.00	12.69
3M-17	167	15	170	14	158	16	165.00	15.00	9.09
7M-16	234	23	239	25	223	23	232.00	23.67	10.20
Mean ±SEM							198.00 ±19.34		10.66 ±01.06

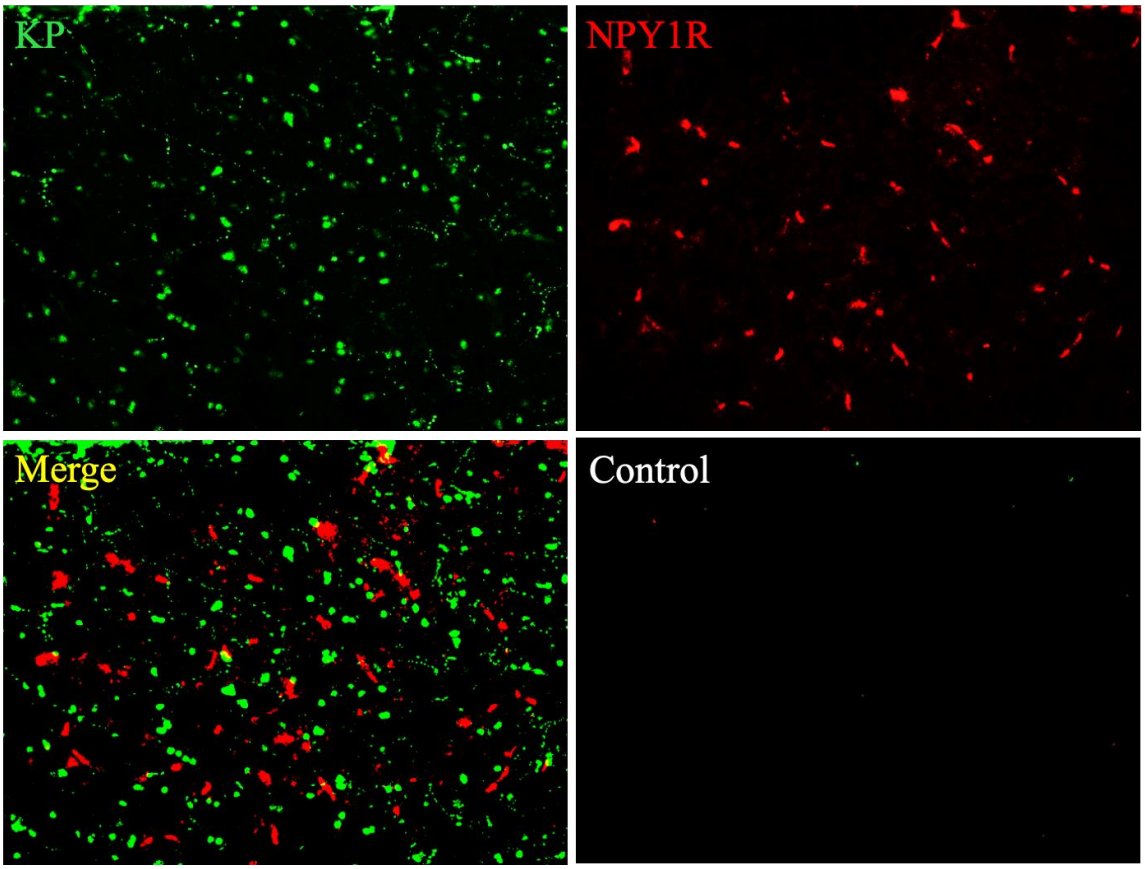

Figure 2.8. Representative fluorescent photomicrograph showing kisspeptin immunoreactivity (KP-IR, green) and NPY1R immunoreactivity (NPY1R-IR, red) in the mediobasal hypothalamic sections of juvenile male monkey. Colocalization between two is presented as yellow color in merged image. Primary antibody omitted control sections showed no immunofluorescence. Photographs were taken at 20x objective by using Olympus BX51.

Table 2.7. Total and mean number of single kisspeptin like-IR and double labelled (dbl) kisspeptin neurons expressing NPY1R in three random sections of MBH in juvenile male rhesus monkeys.

Animal No.	Section A		Section B		Section C		Mean KP	Mean dbl	% dbl
	KP	dbl	KP	dbl	KP	dbl			
3M-16	150	25	148	25	140	23	146.00	24.33	16.67
4M-16	132	26	138	28	144	26	138.00	26.67	19.32
5M-16	157	36	154	37	163	36	158.00	36.33	23.00
6M-16	135	23	147	25	141	23	141.00	23.67	16.78
Mean ±SEM							145.75 ±04.40		18.94 ±1.48

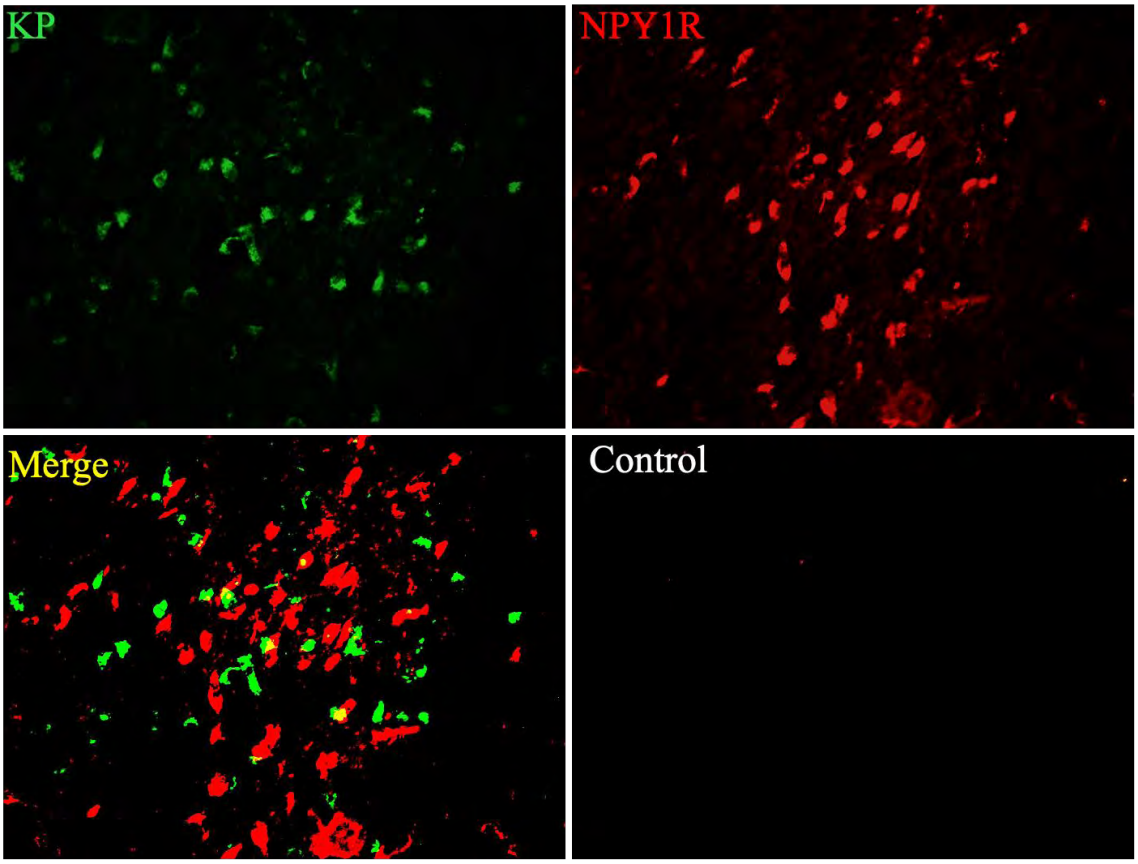

Figure 2.9. Representative fluorescent photomicrograph showing kisspeptin immunoreactivity (KP-IR, green) and NPY1R immunoreactivity (NPY1R-IR, red) in the mediobasal hypothalamic sections of pre-pubertal male monkey. Colocalization between two is presented as yellow color in merged image. Primary antibody omitted control sections showed no immunofluorescence. Photographs were taken at 20x objective by using Olympus BX51.

Table 2.8. Total and mean number of single kisspeptin like-IR and double labelled (dbl) kisspeptin neurons expressing NPY1R in three random sections of MBH in pre-pubertal male rhesus monkeys.

Animal No.	Section A		Section B		Section C		Mean KP	Mean dbl	% dbl
	KP	dbl	KP	dbl	KP	dbl			
4M-18	126	23	117	25	120	25	121.00	24.33	20.11
5M-18	118	28	121	29	127	30	122.00	29.00	23.77
6M-18	125	30	132	28	136	27	131.00	28.33	21.63
7M-18	110	27	134	25	119	26	121.00	26.00	21.49
Mean ±SEM							123.75 ±02.42		21.75 ±0.75

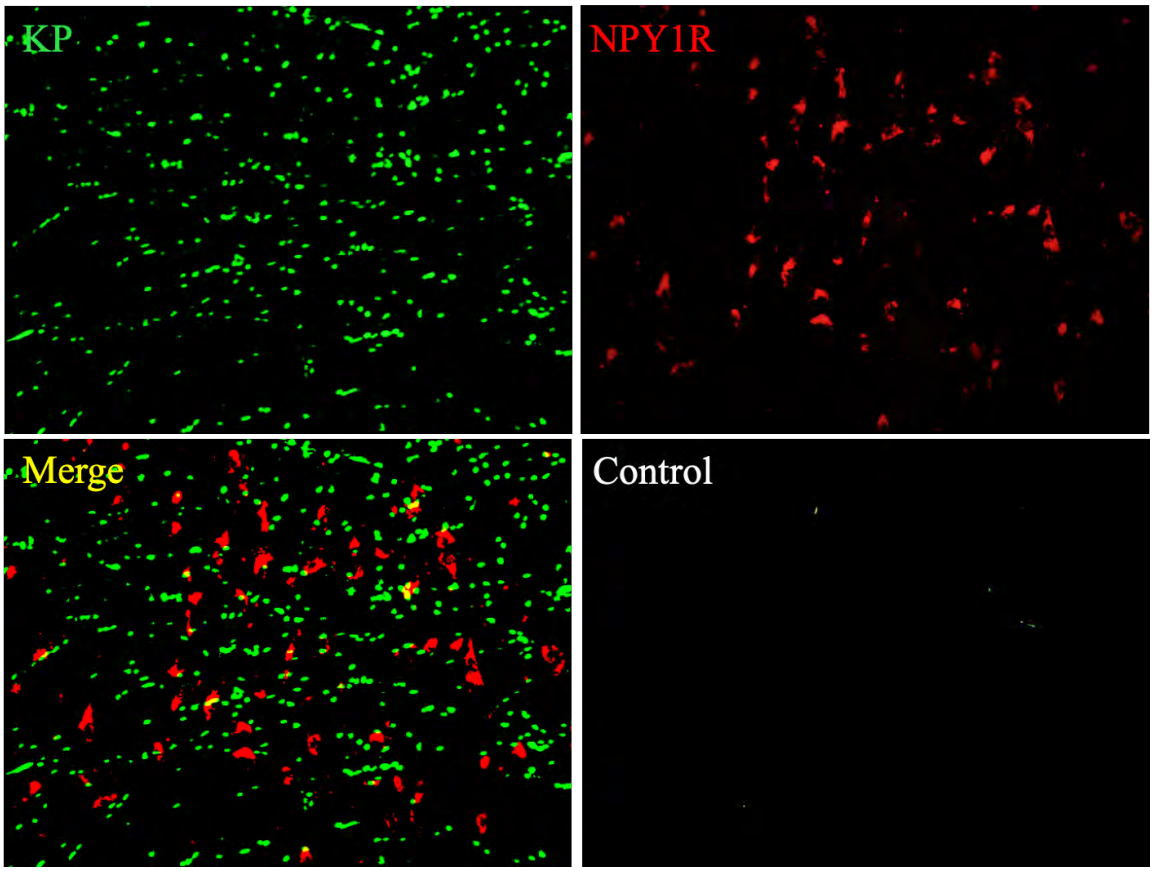

Figure 2.10. Representative fluorescent photomicrograph showing kisspeptin immunoreactivity (KP-IR, green) and NPY1R immunoreactivity (NPY1R-IR, red) in the mediobasal hypothalamic sections of adult male monkey. Colocalization between two is presented as yellow color in merged image. Primary antibody omitted control sections showed no immunofluorescence. Photographs were taken at 20x objective by using Olympus BX51.

Table 2.9. Total and mean number of single kisspeptin like-IR and double labelled (dbl) kisspeptin neurons expressing NPY1R in three random sections of MBH in adult male rhesus monkeys.

Animal No.	Section A		Section B		Section C		Mean KP	Mean dbl	% dbl
	KP	dbl	KP	dbl	KP	dbl			
1M-14	181	22	187	22	175	23	181.00	22.33	12.34
2M-14	170	25	173	27	188	27	177.00	26.33	14.88
1M-16	192	18	200	16	181	16	191.00	16.67	8.73
2M-16	175	17	178	19	190	19	181.00	18.33	10.13
Mean ±SEM							182.50 ±02.98		11.52 ±1.34

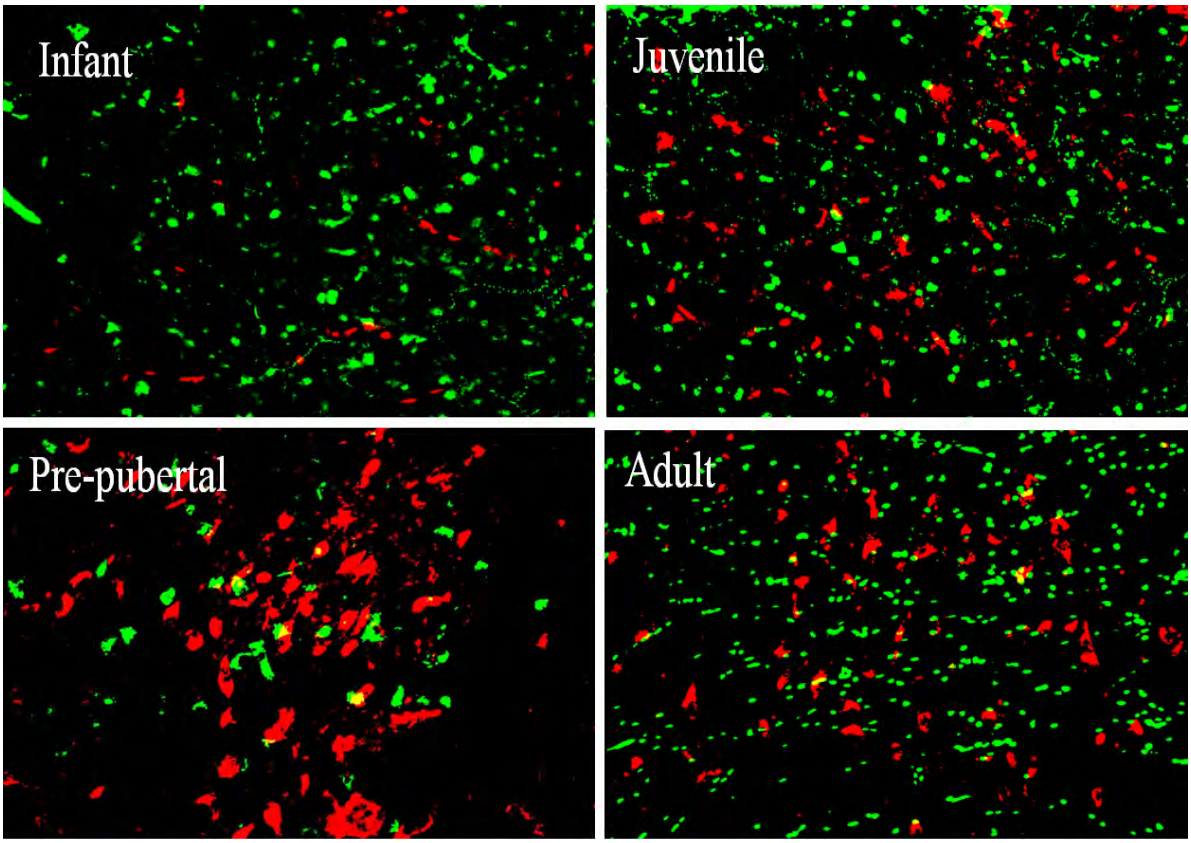

Figure 2.11. Representative fluorescent photomicrographs showing colocalization between kisspeptin immunoreactivity (KP-IR, green) and NPY1R immunoreactivity (NPY1R-IR, red) as yellow color in merged images in the mediobasal hypothalamic sections of male rhesus monkey across four developmental stages (infant, juvenile, pre-pubertal, and adult).

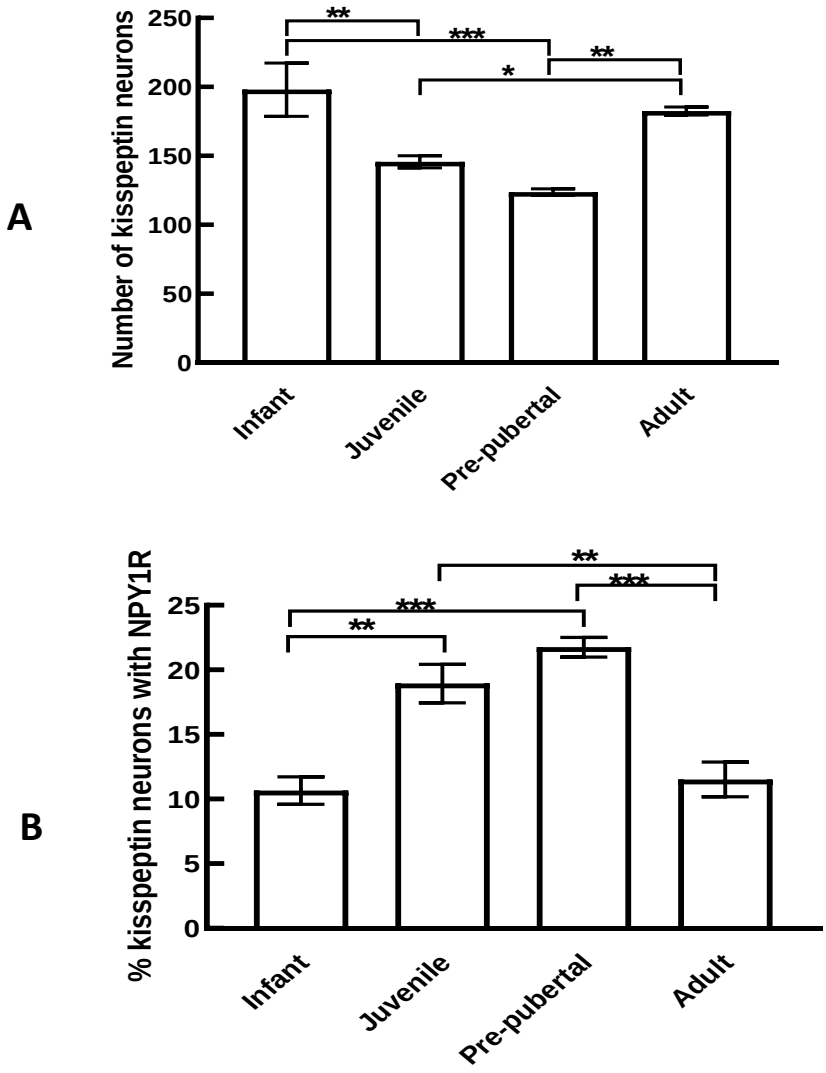

Figure 2.12. Comparison of mean±SEM of kisspeptin-like IR neurons in MBH of male monkeys during different stages of pubertal development. Analysis of variance (One way) followed by post-hoc multiple comparison (Tukey's test) showed significant decrease ($p<0.01$ and $p<0.001$) in number of kisspeptin-like IR neurons from infant to pre-pubertal stage. However, an increase in kisspeptin-like IR neurons ($p < 0.01$) from pre-pubertal to adult stage has been observed (A). Comparison of mean±SEM percentage of kisspeptin neurons expressing NPY1R showed higher percentage ($p<0.001$) in pre-pubertal monkeys as compared to infant and adult monkeys respectively (B).

DISCUSSION

The findings of the present study demonstrate that kisspeptin-like immunoreactive neuronal expression gradually decline from infant to pre-pubertal stage while an uplift in expression was evident in the adult stage as compared to juvenile and pre-pubertal stages in MBH of male rhesus monkeys. These findings are consistent with the previous study where developmental variation of kisspeptin expression between infant and juvenile monkeys was compared (Ramaswamy *et al*., 2013). It has been observed that kisspeptin cells in arcuate of infant are greater in number than in juvenile rhesus macaque suggesting that kisspeptin dependent GnRH pulse generator activity becomes quiescent due to low levels of kisspeptin peptide during juvenile stage (Ramaswamy *et al*., 2013). Studies in rodents (Navarro *et al*., 2004a; Takumi *et al*., 2011) and non-human primates (Shahab *et al*., 2005) also corroborated the kisspeptin variations during pubertal development indicating higher levels of kisspeptin expression in pubertal animals as compared to juvenile/pre-pubertal animals.

Significant decrease in kisspeptin expression in juvenile and pre-pubertal animals as compared to infant animals on one hand and increase expression in adult animals with respect to juvenile and pre-pubertal animals on the other raised the possibility that some neurobiological factor may play a crucial role as a switch that restrains kisspeptin-GnRH pulse generator activity during juvenile/pre-pubertal period. Data from the previous literature suggest that NPY may act as a plausible neurobiological brake that guarantee the quiescent mode of GnRH pulse generator during juvenile/pre-pubertal stages. In this regard, earlier findings highlighted that NPY suppresses GnRH/LH secretion in different species, including non-human primates (Kaynard *et al*., 1990; Pau *et al.,* 1995; Pierroz *et al*., 1996; El Majdoubi *et al.,* 2000a; Shahab *et al*., 2003). Colocalization between NPY and GnRH neurons has also been observed in arcuate nucleus of different animal models including human and non-human primates (Tillet *et al*., 1989; Li *et al*., 1999; Dudás *et al*., 2000; Plant and Shahab, 2002; Turi *et al*., 2003). Studies suggest that increased NPY signaling may plausibly offset kisspeptin neuronal activity during juvenile/pre-pubertal stage.

The key findings that bring about the concept for NPY signaling as neurobiological brake in higher primates to halt GnRH pulse generation showed an inverse relationship between GnRH pulse generator activity and NPY mRNA/protein in male agonadal monkeys during juvenile-pubertal and neonatal-juvenile transition (EI Majdoubi *et al*., 2000a). Moreover, intimate association between NPY neuronal varicosities onto GnRH neurons seemed to be greater in juvenile as compared to pubertal monkeys (Plant and Shahab, 2002). Corresponding to the observations in male monkeys, significant elevation in immunoreactive contacts between NPY/AgRP neuronal fibers onto GnRH neurons has also been observed in pre-pubertal as compared to adult female monkeys (True *et al*., 2017). These observations lend credence to consider NPY a prime candidate to act as neurobiological brake.

To find out possible morpho-functional involvement of NPY during pre-pubertal hiatus of kisspeptin dependent GnRH pulse generator activity, double label immunofluorescent cytochemistry between NPY and kisspeptin was conducted. Possible variation in colocalization between the two neuropeptides across four developmental stages was evaluated to justify the hypothesis that NPY may regulate GnRH release indirectly via kisspeptinergic pathway during pubertal development.

Double labelled kisspeptin neurons with NPY showed significantly increased percentage expression in juvenile and pre-pubertal animals as compared to infant animals. These results indicate that NPY may be the potential factor in restraining kisspeptin neuronal activity at minimum during these developmental stages. Additionally, significant decrease in percentage of kisspeptin neurons with NPY was also observed in adult group as compared to juvenile group. Current cited findings are in concordance with the earlier findings where close reciprocal association between NPY and kisspeptin neurons has been observed in ewes (Backholer *et al*., 2010; Polkowska *et al*., 2014). These NPY apposition are proposed to form synaptic inputs with kisspeptin neurons (Amstalden *et al*., 2011). In addition, optogenetic and electrophysiological data revealed that activation of NPY/AgRP neurons caused direct inhibition of arcuate kisspeptin neuronal activity (Padilla *et al*., 2017; Hessler *et al*., 2020). These results suggest that reduction of NPY based inhibitory tone

may be involved in establishing kisspeptin-dependent stimulatory drive onto GnRH neurons for the onset of puberty.

Interestingly, lower percentage colocalization of kisspeptin with NPY was observed in pre-pubertal animals as compared to juvenile animals. Whilst the observation is conundrum regarding neuro inhibitory role of NPY in diminishing kisspeptin/GnRH pulse generator activity during this critical period of development i.e., pre-pubertal. However, following tenable explanations may put forth for this anomalous percentage decrease of colocalization of kisspeptin neurons with NPY during pre-pubertal period. First, relinquishing of NPY may promulgate another neuro inhibitory factor. The procurement of this repressive factor may help to perpetuate the pre-pubertal hiatus of GnRH pulse generator. Consequently, a concomitant increase in other neuro inhibitory substrate which may subserve as compensatory mechanism in replacement of NPY continue to halt reawakening of the HPG axis during this stage. Secondly, although potential colocalization between these two neuropeptides becomes less but it is possible that NPY mediated signaling would enhance during this time by enhancing NPY1R expression onto kisspeptin neurons which may offset kisspeptin signaling. Therefore, it was imperative to observe latter idea during pubertal development.

Regarding direct inhibition of GnRH secretion by NPY, it has been demonstrated that NPY based inhibition is mediated by NPY1R (Klenke *et al*., 2010; Roa and Herbison, 2012). In adult mice, it was shown that NPY inhibition of GnRH neuronal activity is mediated by NPY1R (Roa and Herbison, 2012). Moreover, prenatal GnRH nasal explant also revealed that NPY inhibitory effects are mediated via NPY1R, and these effects can be reversed by the application of NPY1R antagonist (Klenke *et al*., 2010). Immunocytochemical data also support the potential involvement of NPY1R in mediating the putative inhibitory action of NPY on the HPG axis. Colocalization of NPY1R onto GnRH neurons was observed in rats (Li *et al*., 1999). Pharmacological data with respect to involvement of NPY1R in mediating NPY inhibitory effects in regulating the HPG axis activity has also been unmasked by utilizing multiple NPY1R antagonists. Both rodents (Pralong *et al*., 2000) and non-human primate (EI Majdoubi *et al*., 2000a; Shahab *et al*., 2003) models manifested increase in LH secretion after *icv* administration of NPY1R antagonist.

Based on importance of NPY signaling via NPY1R, it was pertinent to observe developmental variations in NPY1R and kisspeptin expression in the MBH of male rhesus monkeys across different pubertal stages and to observe changes in dual labeled kisspeptin neurons with NPY1R during different stages of pubertal progression.

Double label semi-quantitative immunofluorescence observations indicated significantly higher percentage expression of kisspeptin neurons with NPY1R during pre-pubertal and juvenile stages in comparison to infant stage. However, precipitous decline in dual labeled kisspeptin neurons with NPY1R was noticed in adult animals as compared to preceding two stages i.e., juvenile, and pre-pubertal. The presence of NPY1R onto kisspeptin neurons and enhanced colocalization between the two during juvenile and pre-pubertal stages corroborated the assumption that NPY based inhibition of GnRH release may be indirectly regulated via kisspeptinergic output. Our results are in line with the recent invitro study where electrophysiological and RNA sequencing data also revealed that NPY inhibition of kisspeptin neuronal firing activity can be partially reversed by applying NPY1R antagonist (Hessler *et al*., 2020). These observations indicated that NPY inhibition of kisspeptin neurons are mediated by NPY1R. Interestingly, non-significant increase in NPY1R expression onto kisspeptin neurons in pre-pubertal animals as compared to juvenile animals may imply that increased concerted action of NPY-NPY1R signaling may likely to tone down kisspeptin signaling. Hence, the above-cited data suggests that NPY may act as plausible neurobiological brake restraining kisspeptin drive onto GnRH neurons during pubertal onset.

In general, postnatal developmental changes in *KISS1* and *NPY* gene expression as demonstrated in previous study (Bano *et al.,* 2022) analogize with current immunohistochemical data connoting that NPY may be the plausible brake in attenuating kisspeptin neuronal activity before pubertal onset.

Foregoing results appear to indicate that higher percentage of colocalization between kisspeptin and NPY, and kisspeptin and NPY1R during juvenile and pre-pubertal stages and vice versa during adult stage perhaps be interpreted to suggest that decreased kisspeptin signaling during juvenile/pre-pubertal stage may occur due to increase in NPY inhibitory

inputs to kisspeptin neurons with elevated levels of NPY1R. Increased percentage of kisspeptin neurons expressing NPY and NPY1R during pre-pubertal stage also highlights the possibly increased concerted action of NPY signaling which may likely cause subservience in kisspeptin signaling before pubertal onset. Current immunohistochemical data suggests that NPY may be the plausible neurobiological restrain that attenuates kisspeptin neuronal activity thus keeping GnRH pulse generator in check during juvenile/pre-pubertal stages. However, more sophisticated advance techniques are required to delineate further the functional relationship between these two neuronal populations. Furthermore, confocal analysis will also be helpful to improve our understanding regarding morpho-functional relationship between these two neuronal populations.

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

CHAPTER # 03

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

Study of the role of NPY in occasioning pre-pubertal suppression of GnRH pulse generator in rhesus monkey

ABSTRACT

Introduction: Neuropeptide Y (NPY) has been considered as a neurobiological brake keeping gonadotropin-releasing hormone (GnRH) pulse generator activity at its nadir mimicking hypogonadotropic state during the juvenile period after its brief stimulation in the infant stage in higher primates. The underlying neurobiological mechanism by which NPY keeps protracted release of GnRH in juvenile is not fully known owing to the heterogeneity of NPY receptors. Pharmacological intervention with various antagonists partially supports NPY inhibition of GnRH/LH release via NPY 1 receptor (NPY1R); however, elevated levels of *NPY* and *NPY1R* mRNA in the mediobasal hypothalamus of pre-pubertal male monkeys resurrect the physiological importance of NPY signaling through NPY1R. Therefore, the present study was designed to delineate further the role of NPY1R in mediating the putative inhibitory action of NPY on the HPG axis by testosterone measurement as a surrogate of LH secretion after intravenous (*iv*) administration of highly selective NPY1R antagonist BIBO 3304 in the juvenile male rhesus monkey.

Materials and Methods: Four juvenile intact male rhesus monkeys (n=4) were used to observe the effect of NPY1R antagonist on testosterone secretion. First of all, pituitary responsiveness to GnRH was first heightened with a priming treatment of GnRH agonist. Intramuscular (*im*) injection of GnRH agonist 1.8 µg at 6 hr interval was given for a week to establish gonadotropins content within gonadotrophs. To observe the efficacy of priming and the response of the HPG axis, in terms of testosterone secretion was observed after a single *iv* KP-10 bolus (25 µg/800 µl). KP-10, a potent excitatory analog for GnRH, administration showed a significant increase in testosterone secretion, demonstrating intact GnRH-driven intensified activation of the gonadotrophs-Leydig cell axis. After one week of priming, four juvenile intact male rhesus monkeys, on separate occasions, were used for sequential blood sampling (~ 0.5-0.7ml) at 30 min interval following vehicle or NPY1R antagonist BIBO 3304 administration (three *iv* doses each of 1ml bolus of 2mg/animal). Three blood samples at 30 min interval for 60 min (at -60, -30, 0 min) before vehicle (0.9%

NaCl) or NPY1R antagonist administration were withdrawn. Three *iv* boli (1ml/animal) at 0, 60 and 120 min of vehicle or BIBO 3304 (each 1ml bolus of 2mg/animal) were administered and nine samples from 30 min up to 270 min were collected. Commercially available human testosterone ELISA kit was used to measure plasma testosterone concentration.

Results: Two-way ANOVA followed by Tukey's multiple comparison showed that neither treatment nor time has a significant effect on testosterone secretion. Moreover, no significant difference in mean testosterone levels and area under the curve (AUC) for testosterone between vehicle or antagonist administered animals was noted. No significant difference in mean pre- and post- BIBO 3304 or vehicle testosterone secretion was observed. Affirmation about the patency of the HPG axis and to interrogate the effect of NPY1R antagonist in inducing premature activation of the HPG axis, KP-10 (25 µg/800 µl, *iv*) injection after an interval of 1:30 hr, following 270 min sample, showed a significant increase in testosterone secretion. These results demonstrated that BIBO 3304 has no role in triggering the HPG axis activity in juvenile monkeys.

Conclusion: Current data contrast with the proposed hypothesis that pharmacological manipulation/blockage of NPY1R with highly specific antagonist in juvenile monkeys may instigate premature activation of the HPG axis. Present findings implicate that the putative inhibitory action of NPY on the HPG axis mediated by NPY1R, if any, posit concerns about the potency of the drug at the level of the hypothalamic monkey NPY1R or its accessibility to the monkey hypothalamus. However, an extensive exploration about the functionality of NPY1R in mediating NPY based inhibition of GnRH release during the juvenile period remains to be substantiated by different experimental paradigms to unwind twists and turns in the pubertal development story.

INTRODUCTION

The neuroendocrine strata of the hypothalamus, pituitary, and gonad constitute the hypothalamus-pituitary-gonadal (HPG) axis. Synchronized activity of scattered gonadotropin-releasing hormone (GnRH) neurons within the hypothalamus is the prerequisite for the pulsatile release of GnRH. The pulsatile GnRH secretion ensures the release of pituitary gonadotropins, luteinizing hormone (LH), and follicle stimulating hormone (FSH). These gonadotropins stimulate steroidogenesis and gametogenesis within gonads. Both these processes are necessary to achieve and sustain reproductive capacity, known as puberty (Terasawa and Garcia, 2020). Increase in pulse amplitude and frequency of GnRH at the time of puberty, which is the neuroendocrine hallmark, is dependent on GnRH pulse generator activity. The output of this pulse generator activity is mediated by one of its components i.e., kisspeptin. Kisspeptin has been shown to be the most potent GnRH secretagogue. Abundance of research studies in different animal models including highly evolved primates demonstrated the importance of kisspeptin in pubertal onset by inducing pulsatile GnRH secretion. In addition, pulsatile kisspeptin secretion is also necessary to maintain reproductive functions in adulthood (Plant, 2019). Postnatally GnRH pulse generator shows increase activity at infantile and adult stages designating as mini and true puberty, respectively. The period after infancy and before pubertal onset in which GnRH pulse generator activity becomes nadir known as juvenile. Despite the extensive involvement of kisspeptin in regulating the HPG axis functioning, the intriguing pattern of GnRH pulse generator activity from infancy to adulthood particularly the neurobiological rate limiting pivotal brake that encompasses plummet GnRH pulse generator activity during the juvenile phase needs to be defined explicitly (Terasawa *et al*., 2013; Terasawa, 2019; Plant and Steiner, 2022).

Neuropeptide Y (NPY) is one of the most copious neuropeptides of the hypothalamus, which plays a crucial role in regulating the gonadotropic neuroendocrine axis (Tatemoto, 1982; Allen *et al*., 1983; Terasawa and Fernandez, 2001). NPY neurons are abundantly expressed in major neuroendocrine hypothalamic areas like arcuate (ARC), preoptic area

(POA) and paraventricular hypothalamus (PVH) (Chronwall *et al.,* 1985; Chaillou *et al.,* 2002; Grove *et al*., 2003) which are involved in controlling reproductive functions (Kalra and Crowely, 1992). Majority of NPY fibers that are traced in the median eminence (Contijoch *et al.,* 1993) originate from ARH (Turi *et al*., 2003).

A plethora of G protein-coupled receptors, Y1, Y2, Y4, Y5 and y6, are available to mediate the actions of NPY (Blomqvist and Herzog, 1997; Balasubramaniam, 1997; Gehlert, 1998). Among these receptor subtypes, NPY Y1 (NPY1R) and NPY Y5 (NPY5R) are mandatory to supervise the HPG axis (Kalra *et al.,* 1992; Raposinho *et al.,* 1999; Raposinho *et al.,* 2000). NPY1R expression has been observed in ARC and PeVN of hypothalamus (Gerald *et al*., 1996; Kanatani *et al*., 2000). Highest expression of NPY1R among other NPY receptors in the hypothalamus suggests that it is a potential candidate to mediate the effects of NPY (Higuchi, 2012). NPY1R has been implicated inhibitory effects via membrane hyperpolarization (Sun *et al*., 1998).

NPY mediated inhibition of GnRH/LH release was shown in different mammals including rat (Kalra and Crowley, 1984), rabbit (Khorram *et al*., 1987), sheep (McShane *et al*., 1992; Barker-Gibb *et al*., 1995; Morrison *et al*., 2003), goat (Ichimaru *et al*., 2001) and monkey (Plant and Barker-Gibb, 2004). In the case of non-human higher primates, central administration of NPY halts GnRH pulsatile release in agonadal post pubertal monkeys of both sexes (Kaynard *et al*., 1990; Pau *et al*., 1995; Shahab *et al*., 2003). This NPY based suppression of LH was attributed by the neuronal association between NPY and GnRH neurons. The inter neuronal connection between NPY fibers and GnRH neurons was immunohistochemically seen at the level of arcuate and median eminence in various species including highly evolved primates both human and non-human (Dudás *et al*., 2000; Plant and Shahab, 2002). GnRH neurons from adult and nasal explant of mice and from rat have shown the presence of NPY1R on GnRH neurons (Li *et al*., 1999; Klenke *et al*., 2010; Roa and Herbison, 2012) indicating that the inhibitory functional impact of NPY on GnRH neurons is likely to occur by NPY1R.

Since KP is the most potent secretagogue of the GnRH, it is possible that NPY may inhibit GnRH pulsatility by inhibiting KP neurons. In this regard, intimate association between NPY and KP neurons was observed in sheep (Backholer *et al*., 2010; Polkowska *et al*., 2014). Moreover, decreased neuronal activity of KP neurons by NPY has been demonstrated by chemo genetic and electrophysiological technique (Padilla *et al*., 2017; Hessler *et al*., 2020). Based on RNA sequencing, it was revealed that arcuate kisspeptin neurons in rodents harbor NPY1R transcripts (unpublished data as cited by Hessler *et al*., 2020), potentiating the role of NPY1R in inhibiting kisspeptin neuronal activity.

Although the role of NPY in inhibiting HPG axis activity by decreasing LH/GnRH release was observed in many animals during post-pubertal period however the critical role of NPY as neurobiological restrain of the GnRH pulse generator has been evaluated during juvenile period in rodents and highly evolved non-human primates. It has been demonstrated that chronic infusion of NPY into lateral ventricles caused delayed vaginal opening with lower content of GnRH receptor of the pituitary (Pierroz *et al*., 1995). A similar reduction in pituitary testicular axis was also observed in male rats (Pierroz *et al*., 1996). Another study in male monkeys during postnatal developmental transition that is from neonatal to juvenile and from juvenile to pubertal showed that GnRH pulse generator activity was high as indicated by LH levels during neonatal and pubertal stages while NPY mRNA/protein expression was low in these two stages. However, the inverse relation between GnRH pulse generator activity and NPY mRNA/protein was seen during juvenile phase (EI Majdoubi *et al*., 2000a). Moreover, double labelled immunohistochemical analysis using fluorophore-tagged secondary antibodies against GnRH and NPY has shown interaction between NPY axons with GnRH cell bodies in monkey hypothalamus. Interestingly this axo-somatic interaction between these two neuronal entities was shown to be less in hypothalamus of pubertal male monkeys as compared to juvenile animals (Plant and Shahab, 2002). These observations indicate that NPY may act as a neurobiological brake to keep GnRH pulse generator in quiescent mode during the juvenile stage.

Interestingly, recent findings of our laboratory corroborated the idea, put forth by EI Majdoubi and colleagues (2000a), i-e. considering NPY as a hypothalamic brake during the juvenile period, one step further where we observed relative high expression of *NPY* and *NPY1R* and low expression of *KISS1/KISS1R* mRNA in juvenile/pre-pubertal male monkeys as compared to infant monkeys (Bano *et al*., 2022). Immunocytochemical data in previous study also provide sustenance to the idea.

NPY based inhibition of GnRH release is mediated by NPY1R in adult male monkeys (Plant and Shahab, 2002). Inverse correlation between relative gene expression of NPY and KP, NPY1R and KP has recently deciphered during pubertal development in male rhesus monkeys (Bano *et al*., 2022). The above cited data suggest that NPY regulation of GnRH secretion via kisspeptinergic neurons is mediated by NPY1R. However, the precise role of NPY1R in inhibiting the HPG axis activity generally and during pubertal development at the juvenile stage particularly either directly on GnRH neurons or indirectly via KP neurons, is still ambiguous. To delineate the precise physiological involvement of NPY1R in inhibiting the HPG axis, pharmacological intervention is the best tool. In continuation to the above-described notion, it is interesting to note that chronic *icv* infusion of highly specific NPY1R antagonist BIBP 3226 caused advancement in pubertal development in pre-pubertal female rats (Pralong *et al*., 2000). For non-human higher primates, inhibition in GnRH release induced by NPY was attenuated by *icv* administration of NPY1R antagonist (1229U91) in agonadal juvenile male monkeys (EI Majdoubi *et al*., 2000a; Shahab *et al*., 2003). Since this compound (1229U91) is effective on both NPY1R and NPY4R for antagonistic and agonistic properties, respectively, conclusive affirmation for NPY1R to mediate NPY based inhibitory effect on GnRH secretion/neurons remained contentious. In addition, a subsequent study showed that two more highly specific NPY1R antagonist (VD-11 and Compound A) failed to elicit LH discharge in juvenile castrated male monkeys (Shahab *et al*., 2003). Hence, the involvement of NPY1R in mediating NPY role as a major component of the brake that keeps pulsatile GnRH release in the protracted state during the juvenile stage is still dubious. However, the idea of the conceptual brake imposed by NPY is accentuated by a

recent in vitro study, which showed that direct inhibition of kisspeptin neuronal activity imposed by NPY can be reverse partially by applying NPY1R antagonist BIBO 3304 (Hessler *et al*., 2020). However, the effect of this highly specific NPY1R antagonist has not been studied in vivo in non-human male primates during pubertal development.

Tangible evidence from the above-cited literature indicates that NPY is the major neuroendocrine limiting factor acting as a neurobiological brake for keeping GnRH neurons in protracted state during the juvenile phase. However, convincing evidence about NPY based inhibition of the HPG axis via NPY1R in juvenile non-human higher primates by employing highly selective NPY1R antagonist still needs to be presented. Hence, it was hypothesized that pharmacological blockage of NPY1R with highly specific NPY1R antagonist in juvenile monkeys might instigate premature activation of the HPG axis. Thus, to test the hypothesis, the present study was carried out to delineate further the role of NPY1R in mediating the putative inhibitory action of NPY on the HPG axis in GnRH primed juvenile monkeys, three *iv* doses of a specific NPY1R antagonist were administered. The response of the HPG axis was assessed indirectly in terms of terminal neuroendocrine parameter testosterone secretion following peripheral administration of NPY1R specific antagonist BIBO 3304.

MATERIALS AND METHODS

Animals

Four juvenile intact male rhesus monkeys (*Macaca mulatta*) 10-14 months old, weighing 1.8-2.2 (2±0.11 mean±SEM) kg were used in the present study. Intact animals were utilized in the present study to observe the effect of intravenous (*iv*) NPY1R antagonist administration on testosterone secretion as a surrogate of LH release due to the absence of facility to measure rhesus monkey gonadotropins. Since the central neurobiological mechanism, not the gonads, controls the suppression of gonadotropin release from the infant-juvenile transition in male monkeys (El Majdoubi *et al*., 2000b; Plant and Barker-Gibb, 2004), therefore, the terminal product of the HPG axis (testosterone) was measured to gauge initiation of the activity of the hypothalamus and pituitary- two higher components of this hierarchical axis. Each animal was kept in a separate cage under semi-controlled environmental conditions (25±3°C) temperature, 12-hour light/dark photoperiod with lights on between 06:00 and 18:00 hr in the Primate Facility of the Department of Zoology, Quaid-i-Azam University, Islamabad in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. The daily meal comprised of boiled potato (09:00-09:30 hr), boiled eggs (11:00 hr), bread (13:00-13:30 hr), and fresh fruits, and nuts (15:00-16:00 hr). Additionally, vitamins supplements were also given to account for vitamin deficiency. *Ad libitum* access to water was ensured. To minimize the effect of stress on blood sampling, the animals were habituated to chair restraint two weeks before the start of experiment. The duration of restraint was gradually increased until 6 hr a week with the attainment of 3 hr for the first week and then up to 6 hr for the second week. The animals were sedated with ketamine hydrochloride (Ketler, Astarapin, Germany 5 mg/kg body weight, intramuscular) for placement in and removal from the monkey restraining chair. All the experimental procedures and protocols of the study were permitted by the Animal Care and Use Committee of the Department of Zoology.

Experimental Design

Four juvenile intact male rhesus monkeys were used to study the effect of *iv* administration of NPY1R antagonist (BIBO 3304) on testosterone secretion to determine the role of NPY1R in mediating putative inhibitory action of NPY on the HPG axis during the juvenile period. Before starting the experiment, GnRH priming was done to enhance the responsiveness of pituitary gonadotrophs to GnRH stimulation. This exogenous GnRH stimulation caused the robust activity of the HPG axis by increasing adult-like testosterone response as described previously (Abeyawardene *et al*., 1989). It has been documented that following cessation of GnRH priming, the response of the pituitary to GnRH remains intact for several days (Suter *et al*., 1998). For this purpose, intramuscular (*im*) injection of GnRH agonist (Lutrate Depot; GP-Pharma, S.A. Sant Quinti de Mediona, Spain) 1.8 µg at 6 hr interval was given for a week. The dose of the agonist was calculated based on previous literature (Shahab *et al*., 2005) to establish gonadotropins content within gonadotrophs. To observe the validity of priming/pituitary responsiveness, a single KP-10 bolus (25µg/800 µl per animal; *iv*) was given before and after priming. A single blood sample (0.5-0.7ml) before KP-10 prior to initiation of priming was taken, and immediately after the sample, KP-10 was injected. After the injection, 30 min later, again another blood sample was taken to observe testosterone response against KP-10 prior to priming protocol. From the next day, GnRH priming was initiated for one week. During the priming, two blood samples at 07:00 am and 01:30 pm on alternative days were collected to observe pituitary responsiveness against priming in terms of testosterone secretion. To check the efficacy of priming and pituitary responsiveness after one day break following seven days of priming, again KP-10 was injected, and the blood sample was collected with the same scheme as described above. The scheme of GnRH priming is depicted in Figure 3.1.

	Pre-Priming		Priming Session							Post-Priming	
	Before KP-10	After KP-10	GnRH agonist							Before KP-10	After KP-10
Dedicated days for blood sample collection	×	×	×		×		×		×	×	×
Days	0		1	2	3	4	5	6	7	9	

Figure 3.1. GnRH priming scheme to induce premature high content of gonadotropins within gonadotrophs of juvenile intact male rhesus monkeys (n=4). Heightened response of the pituitary against peripheral drug administration was assessed via testosterone secretion. Two blood samples at 07:00 am and 01:30 pm on alternative days were collected during priming regime. In addition, a single blood sample before and after KP-10 was collected before and at the completion of the priming protocol to ensure the validity of priming by comparing testosterone secretion.

The actual experiment comprised two days of sequential blood sampling after one week of priming. In the first day of sequential blood sampling, vehicle was administered, while after the gap of one day, second serial sampling with NPY1R antagonist administration was done. Blood samples (~ 0.5-0.7ml/sample) were taken at 30 min interval. Three blood samples at 30 min interval for 60 min (at -60, -30, 0 min) were withdrawn before vehicle (0.9% NaCl)/NPY1R antagonist (BIBO 3304) administration while nine samples (at 30, 60, 90, 120, 150, 180, 210, 240, 270 min) were taken after *iv* vehicle/BIBO 3304 administration. On the first day of sampling, three *boli* of vehicle (1ml/animal) were administered immediately after taking 0, 60 and 120 min samples at 1 hr interval, while on the second day of serial sampling, three *boli* of BIBO 3304 (each 1ml bolus contain 2mg antagonist/animal) were injected intravenously with the same scheme as described for vehicle. Following 270 min sample, a 1:30 hr rest was given; after that, two additional blood samples (at 360 and 390 min) were obtained before and after KP-10 administration, respectively, on both days of serial sampling. KP-10 was given immediately after 360 min sample. KP-10 (25 µg/800 µl) was used to check the responsiveness of the pituitary after priming and patency of the HPG axis. Experimental design for intravenous administration of BIBO 3304 and vehicle is shown in Figure 3.2

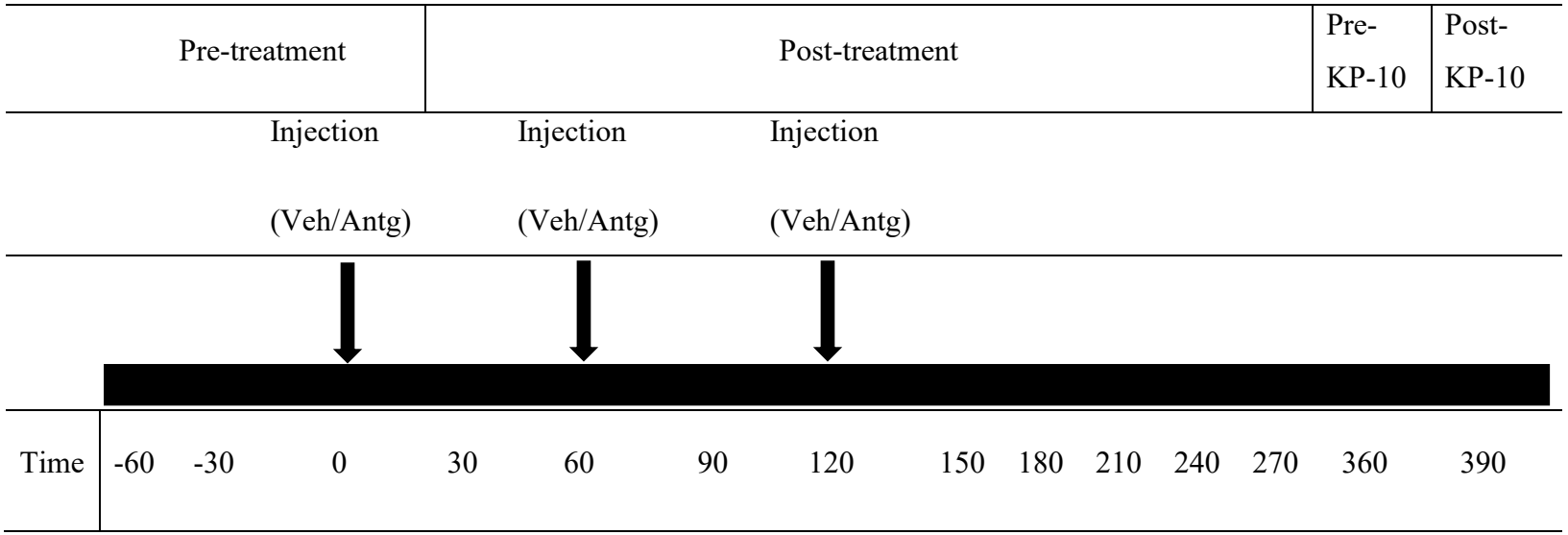

Figure 3.2. Experimental design for *iv* administration of vehicle (Veh) and NPY1R antagonist (Antg) (BIBO 3304). First *bolus* of vehicle (1ml/animal of 0.9% NaCl in 4.8% DMSO) or antagonist (1ml/animal at a concentration of 2mg/ml in 4.8% DMSO and 0.9 % NaCl) was administered one hour after the sequential bleeding. A total of three *boli* of vehicle or antagonist were administered intravenously at 0, 60 and 120 min as indicated by arrows. Three blood samples at 30 min interval for 60 min (at -60, -30, 0 min) were withdrawn before vehicle (0.9% NaCl)/NPY1R antagonist (BIBO 3304) administration while nine samples (at 30, 60, 90, 120, 150, 180, 210, 240, 270 min) were taken after *iv* vehicle/BIBO 3304 administration.

Blood sampling was done on two occasions in the following order.

I. Vehicle Administration (Day 1)

Normal saline with 4.8% DMSO was used as vehicle. Three intravenous injections each of 1ml at 1 hr interval at 0, 60, 120 min was administered. Sequential blood samples for 8 hr with 1:30 hr rest after 6 hr were obtained in heparinized syringes from four juvenile intact male monkeys. The sampling was done from 10:30 to 18:30 hr.

II. BIBO 3304 Administration (Day 2)

Three intravenous injections of NPY1R antagonist (BIBO 3304 in 4.8% DMSO and saline; each 1ml bolus, 2mg/animal) at 1 hr interval at 0, 60, 120 min was administered to all animals. Sequential blood samples for 8 hr (with 1:30 hr rest after 6 hr) were obtained in heparinized syringes from three juvenile intact male monkeys. From the fourth animal, a total of only seven blood samples; three samples at -60, -30, 0 min before antagonist, and four blood samples at 30, 60, 90, 120 min after antagonist, were collected. The sampling was done from 10:30 to 18:30 hr.

III. Venous Catheterization

For serial blood sampling and administration of drugs (Vehicle/BIBO 3304/KP-10) via *iv* route, ketamine hydrochloride (Ketamax, Rotex Medica, Trittau, Germany; 5 mg kg^{-1} BW, *im*) was injected into animals to anesthetized them. A teflon cannula (Vasocan Branule, 0.7 mm / 24 G O.D, B. Braun Melsungen AG, Belgium) was placed into the saphenous vein. At the distal end of the cannula, a butterfly tube (Length 300 mm, volume 0.20 ml, 22 GX3 / 4 ″, JMS, Singapore) was linked. A syringe (1cc 26G X 1/2") was connected at proximal end of butterfly tube. This infusion set settings greatly helped to withdraw sequential blood samples. In addition, *iv* administration of drugs were also feasible due to such instrumental settings. Sampling was initiated after impact of sedation was completely removed.

IV. Pharmacological Agents

GnRH agonist: Lutrate Depot (Leuprorelin acetate 3.75 mg; GP-Pharma, S.A. Sant Quinti de Mediona, Spain) was purchased from local pharmacy. Stock solution of Lutrate Depot was made by dissolving 3.75 mg powder into 2ml (0.8%) mannitol solvent provided within the pack. Working solution of GnRH agonist (1.8 µg/6hr at 200 µl/ injection) was prepared in normal saline (0.9 % NaCl).

Heparin (Rotex media, Trittau, Germany)

Ketamine hydrochloride (Ketamax, Ketler, Astarapin, Germany)

Dimethyl Sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri, USA)

Normal saline (0.9 % NaCl; A-Z Pharmaceuticals, Kasur, Pakistan)

Human KP-10 (amino acids 112 – 121) was supplied by the local agent of Calbiochem (La Jolla, CA, USA). Normal saline (0.9 % NaCl) and 1.4% DMSO was used to prepare stock solution (1mg/ml) of KP-10. From this stock solution, working solution (25µg/ani at 800 µl/ injection) was prepared in normal saline (0.9 % NaCl).

NPY1R antagonist: BIBO 3304 (N-[(1R)-1-[[[[4-[[(aminocarbonyl)amino]methyl]phenyl] methyl]amino]carbonyl]-4-[(aminoiminomethyl)amino]butyl]-a′-phenyl-benzeneacetamide ditrifluoroacetate; SML2094, Sigma-Aldrich, St. Louis, Missouri, USA). Working solution of BIBO 3304 was prepared in 4.8% DMSO and normal saline (0.9 % NaCl) to a concentration of 2mg/ml.

V. Blood Sampling

All blood samples (0.5-0.7ml) were obtained in heparinized syringes at 30 min interval. Same amount of heparinized (5 IU ml^{-1}) normal saline (0.9 % NaCl) was injected to maintain blood volume to avoid hypovolemic shock to the animals following withdrawal of each sample. Moreover, during blood sampling fruits/juices were provided to the animals. Samples were transferred to eppendorf tubes kept on ice. Blood sampling was

conducted between 10:30 – 18:30 hr. Centrifugation of the blood samples was done at 3000 rpm at 4 ºC for 15 min. Plasma was extracted and stored at – 20 ºC until hormonal analysis.

Plasma Testosterone Measurement

Plasma testosterone concentration was measured by using a human testosterone ELISA kit (JTC Diagnosemittel UG, Schulweg, VÖhl, Germany). The assay was performed according to the standard protocol provided in the kit. All samples, controls and standards were brought to room temperature before proceeding with the assay. The sensitivity of the assay was 1.16 ng ml^{-1} while inter and intra assay coefficients of variation were <9% and <5%, respectively.

Statistical Analyses

All the data of testosterone secretion after KP-10 injection before GnRH priming and after GnRH priming, after vehicle and antagonist administration were expressed as mean±SEM. Comparison of mean±SEM plasma testosterone secretion in pre-primed and post-primed animals after KP-10 administration and mean±SEM percentage plasma testosterone response after KP-10 administration before and after priming was evaluated by student's t-test. Comparison of mean testosterone release across different time points after vehicle and antagonist administration and mean pre- and post-testosterone release in vehicle and antagonist-treated animals was evaluated by two-way ANOVA followed by Tukey's multiple comparison test. The overall mean±SEM testosterone levels in vehicle and antagonist-treated animals was also compared by using student's t -test. Testosterone response to vehicle and antagonist-treated monkeys was also calculated by area under the curve (AUC) (Abbud and Smith, 1993) and statistically compared by t-test. In addition, a comparison of mean±SEM pre- and post-testosterone concentration after KP-10 administration in vehicle and antagonist-treated monkeys was also measured by using paired t-test. All statistical analyses were performed by using GraphPad Prism Software version 8.3.0 (GraphPad software, La Jolla, CA, USA). Statistical significance level was set at $p < 0.05$.

RESULTS

GnRH priming was carried out to induce premature transient activation of hypothalamic-pituitary-testicular axis. To check the accuracy of GnRH priming regime, a single *iv bolus* of KP-10 was given to all animals before initiation of the priming protocol and after one week of the priming regime. Overall changes in mean testosterone concentration before and after KP-10 administration in pre-primed and post-primed animals are shown in Figure 3.3. Significant increase ($p<0.05$) in testosterone (mean±SEM) response against KP-10 infusion was observed in both pre-primed and post-primed animals (Figure 3.4 and 3.5). However, GnRH-driven intensified activation of the gonadotrophs-Leydig cell axis of the juvenile monkeys was manifested by the testosterone secretion after priming. As expected, after 7 days of priming, the percentage response of testosterone after KP-10 administration was more pronounced and substantially significant ($p<0.05$) in post-primed animals as compared to testosterone response against KP-10 administration in pre-primed animals (Figure 3.6). During priming (day 1-7), the progressive activation of the pituitary-testicular axis by the GnRH treatment in individual animal was monitored in blood samples collected on alternative days by tracking plasma testosterone concentration and are depicted in (Figures 3.7-3.10).

After one week of priming, sequential blood sampling was undertaken to observe testosterone changes after vehicle and NPY1R antagonist (BIBO 3304) administration, respectively. Individual variations in plasma testosterone concentrations before and after vehicle and antagonist administration are shown in Table 3.1 and 3.2, respectively. Time course (-60 – 270 min) changes in testosterone concentration (mean±SEM) in vehicle and antagonist-treated animals are shown in Figure 3.11. Two-way ANOVA followed by Tukey's multiple comparison showed that neither treatment nor time has a significant effect on testosterone secretion (Figure 3.11). NPY1R antagonist (BIBO 3304) failed to induce change in testosterone release in juvenile monkeys. Moreover, no significant difference in overall mean testosterone levels was observed between vehicle or antagonist administered animals (Figure 3.12). A similar non-significant difference in mean testosterone release was also observed when AUC for testosterone (ng/ml.4.5hr) was

compared between vehicle and antagonist-treated animals during time lapse 0-270 min (Figure 3.13).

To check the effect of NPY1R receptor antagonism on hypothalamic-pituitary-testicular axis in juvenile monkeys indirectly through testosterone response, comparison between pre (-60, -30, 0 min) and post (30 - 270 min) levels of testosterone in vehicle and antagonist-treated animals was also performed. No significant difference in pre and post-testosterone secretion was observed in either vehicle or antagonist-treated monkeys (Figure 3.14).

Affirmation about the patency of the HPG axis and to delineate the effectiveness of the BIBO 3304 treatment, animals received a single *iv bolus* injection of KP-10 after 1.5 hr of sequential bleeding in vehicle and antagonist-treated animals. This *bolus* injection of KP-10 at 360 min produced significant elevation ($p<0.05$ and $p<0.01$) in testosterone release at 30 min after injection in vehicle and antagonist-treated monkeys, respectively (Figure 3.15A and 3.15B).

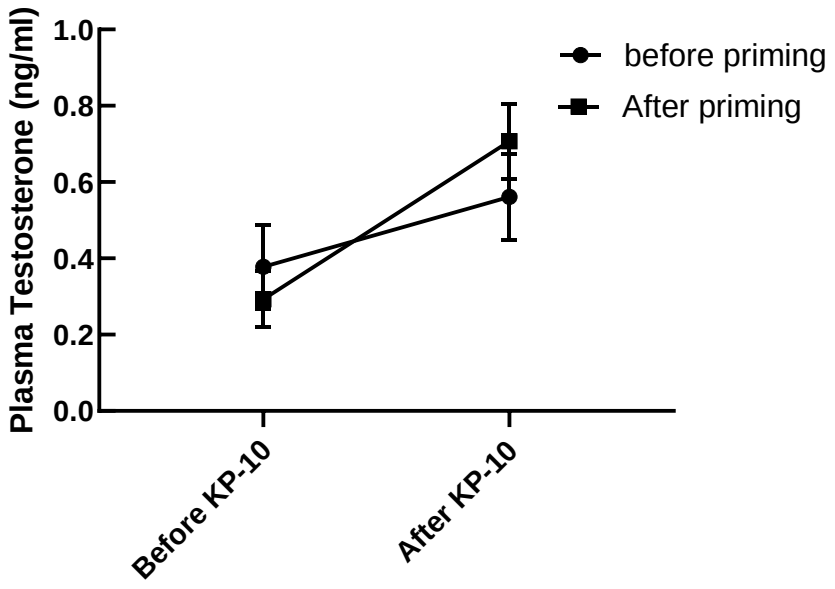

Figure 3.3. Effect of *iv* administration of KP-10 (25µg/800µl per animal) on plasma testosterone (mean±SEM) secretion in pre-primed and post-primed intact juvenile male rhesus monkeys (n=4). The response of testosterone secretion in post-primed animals was greater as compared to pre-primed animals ensuring the efficiency of the priming protocol.

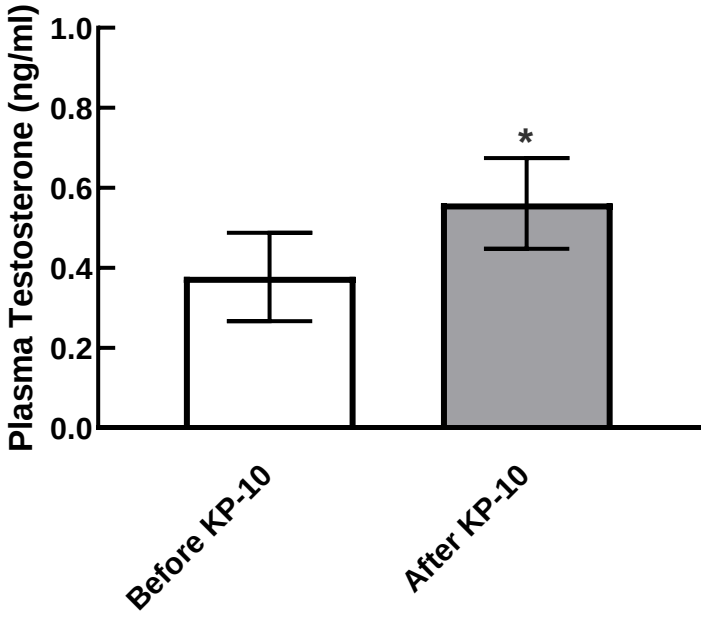

Figure 3.4. A single *iv bolus* injection of KP-10 (25µg/800µl per animal) in intact juvenile male monkeys (n=4) was administered to observe the response of the HPG axis before the start of GnRH priming protocol. The data showed significant increase ($p<0.05$) in plasma testosterone (mean±SEM) secretion prior to initiation of GnRH priming protocol potentiating the role of KP-10 as a potent secretagogue for GnRH.

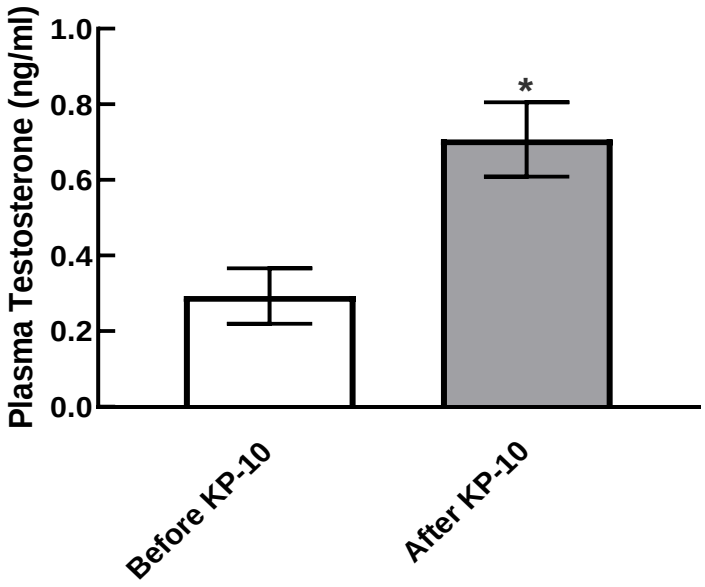

Figure 3.5. A single *iv bolus* injection of KP-10 (25µg/800µl per animal) in intact juvenile male monkeys (n=4) was administered to observe the response of the HPG axis at the end of GnRH priming protocol. A significant increase ($p<0.05$) in plasma testosterone (mean±SEM) secretion after the termination of GnRH priming protocol was observed potentiating the role of KP-10 as a potent secretagogue for GnRH and affirmation about the validity of priming.

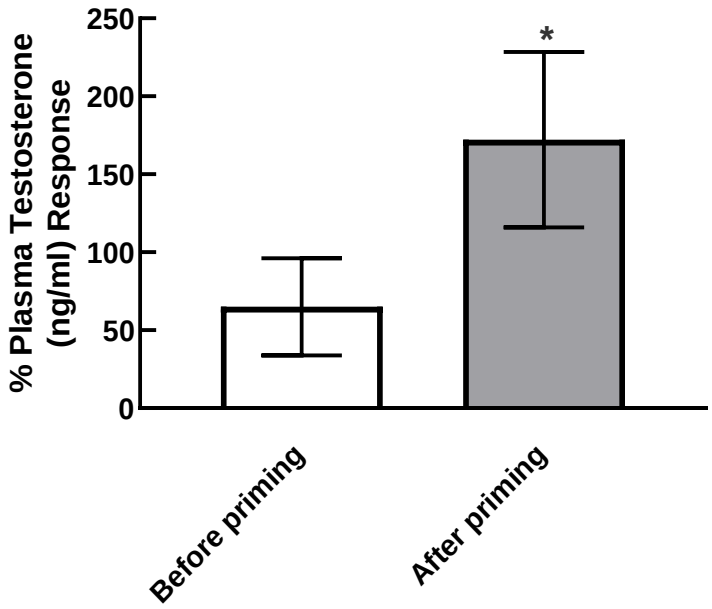

Figure 3.6. Comparison of percentage response of plasma testosterone (mean±SEM) secretion after KP-10 administration in pre-primed and post-primed intact juvenile male rhesus monkeys (n=4). Percentage response of plasma testosterone after KP-10 administration was more pronounced and substantially significant ($p<0.05$) in post-GnRH-primed animals as compared to plasma testosterone response against KP-10 administration in pre-GnRH-primed animals ensuring patency of the HPG axis.

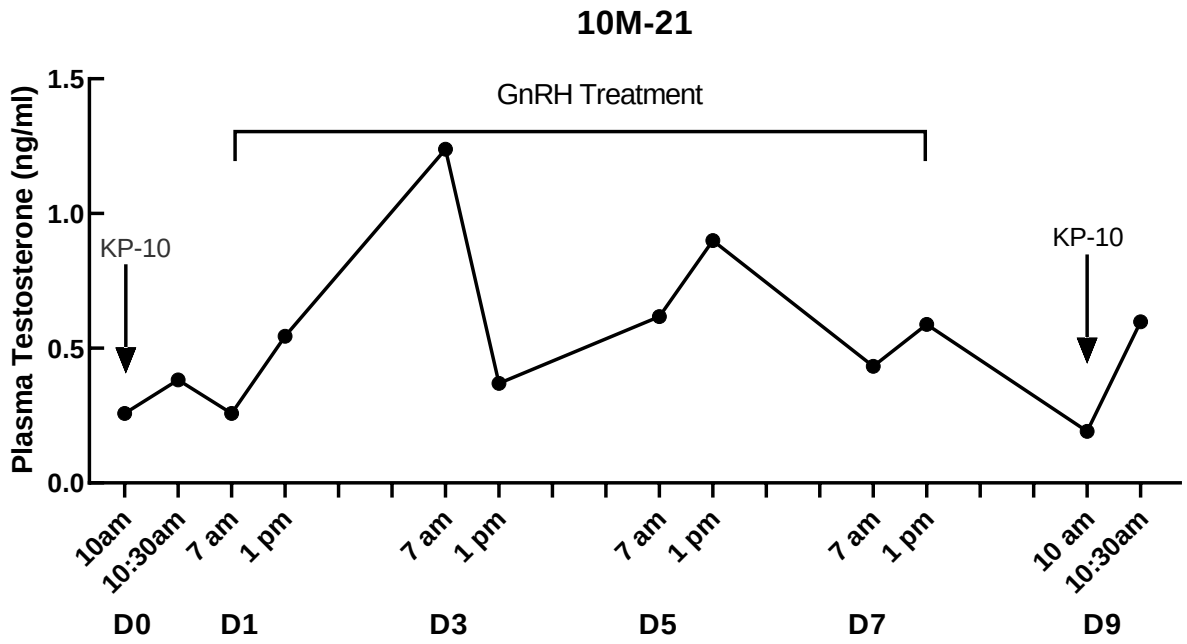

Figure 3.7. Individual variations in plasma testosterone secretion against GnRH agonist and KP-10 administration. To heighten pituitary responsiveness by increasing gonadotropins content, *im* injection of GnRH agonist (1.8µg at 6 hr interval) was given for one week from day 1 (D1) up to day 7 (D7). Testosterone response during GnRH agonist treatment from D1 to D7 is shown in the middle of the graph. During the priming regime, two blood samples at 07:00 am and 01:30 pm were collected on alternative days to observe the pituitary response in terms of testosterone secretion. In addition, single blood sample before and after KP-10 (25µg/800µl *iv bolus* per animal) was collected before the start (day 0) of and at the completion (day 9) of the priming protocol to ensure the validity of priming by comparing testosterone secretion.

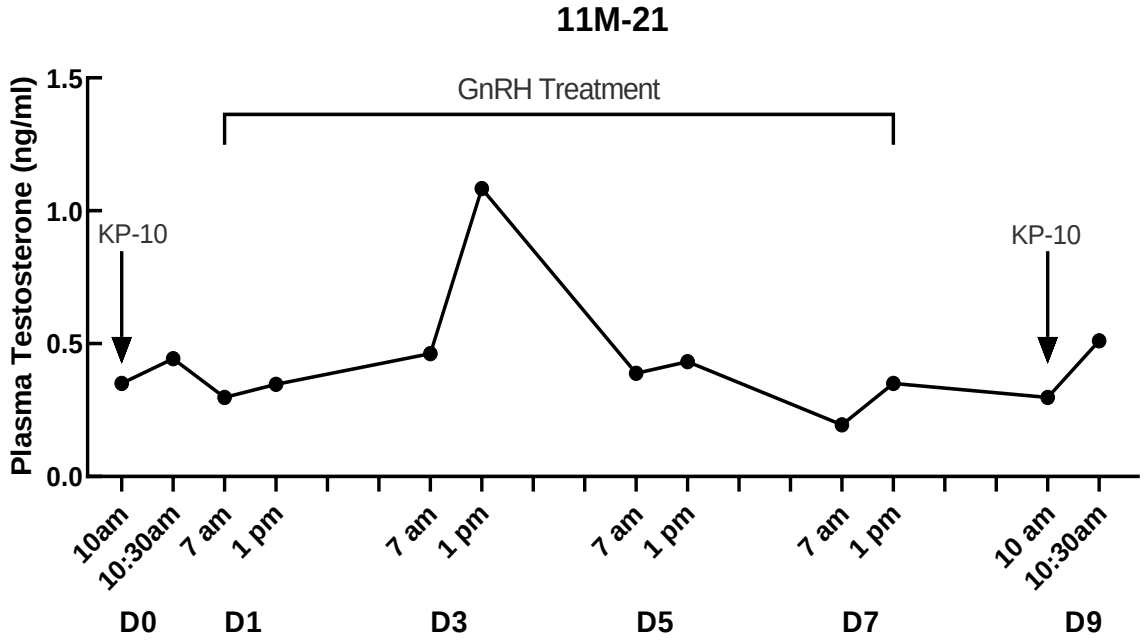

Figure 3.8. Individual variations in plasma testosterone secretion against GnRH agonist and KP-10 administration. To heighten pituitary responsiveness by increasing gonadotropins content, *im* injection of GnRH agonist (1.8µg at 6 hr interval) was given for one week from day 1 (D1) up to day 7 (D7). Testosterone response during GnRH agonist treatment from D1 to D7 is shown in the middle of the graph. During the priming regime, two blood samples at 07:00 am and 01:30 pm were collected on alternative days to observe the pituitary response in terms of testosterone secretion. In addition, single blood sample before and after KP-10 (25µg/800µl *iv bolus* per animal) was collected before the start (day 0) of and at the completion (day 9) of the priming protocol to ensure the validity of priming by comparing testosterone secretion.

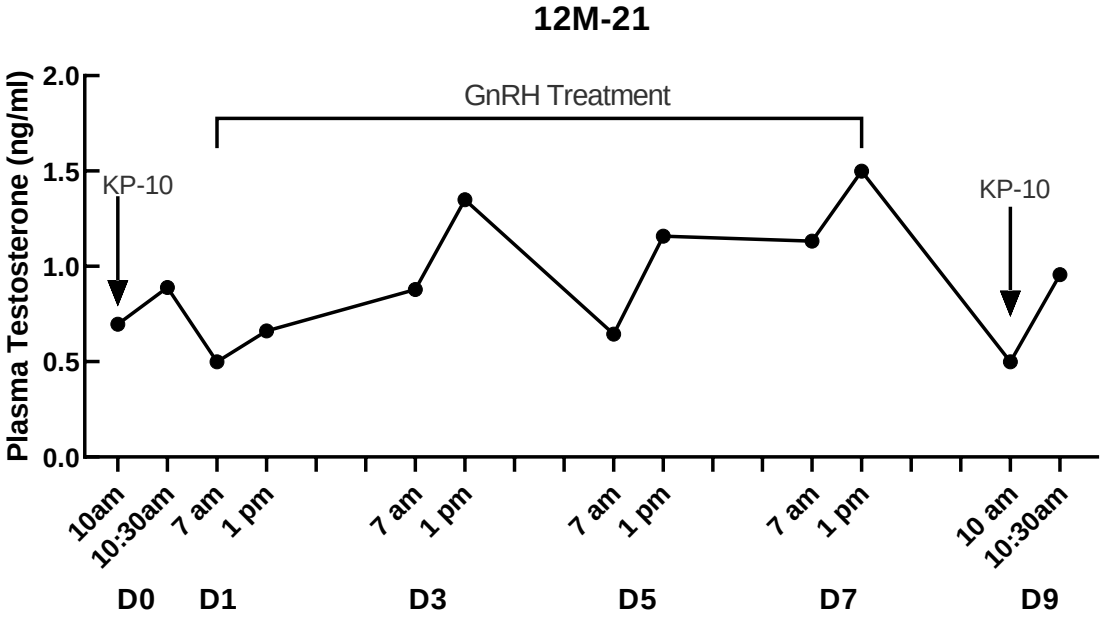

Figure 3.9. Individual variations in plasma testosterone secretion against GnRH agonist and KP-10 administration. To heighten pituitary responsiveness by increasing gonadotropins content, *im* injection of GnRH agonist (1.8µg at 6 hr interval) was given for one week from day 1 (D1) up to day 7 (D7). Testosterone response during GnRH agonist treatment from D1 to D7 is shown in the middle of the graph. During the priming regime, two blood samples at 07:00 am and 01:30 pm were collected on alternative days to observe the pituitary response in terms of testosterone secretion. In addition, single blood sample before and after KP-10 (25µg/800µl *iv bolus* per animal) was collected before the start (day 0) of and at the completion (day 9) of the priming protocol to ensure the validity of priming by comparing testosterone secretion.

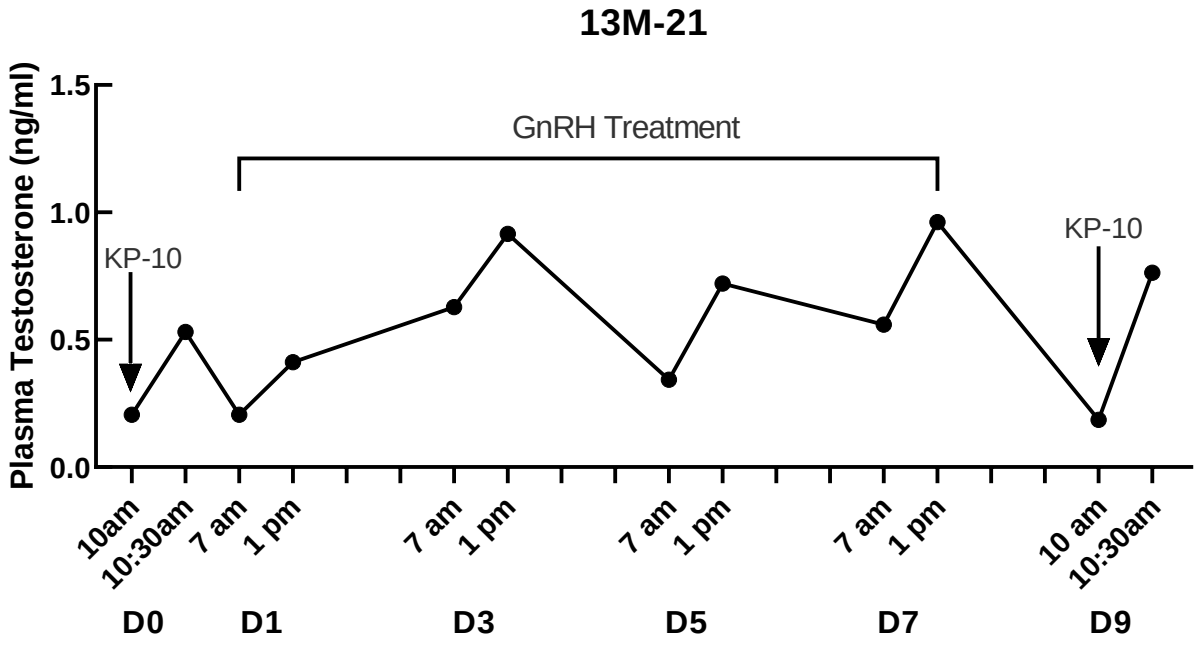

Figure 3.10. Individual variations in plasma testosterone secretion against GnRH agonist and KP-10 administration. To heighten pituitary responsiveness by increasing gonadotropins content, *im* injection of GnRH agonist (1.8µg at 6 hr interval) was given for one week from day 1 (D1) up to day 7 (D7). Testosterone response during GnRH agonist treatment from D1 to D7 is shown in the middle of the graph. During the priming regime, two blood samples at 07:00 am and 01:30 pm were collected on alternative days to observe the pituitary response in terms of testosterone secretion. In addition, single blood sample before and after KP-10 (25µg/800µl *iv bolus* per animal) was collected before the start (day 0) of and at the completion (day 9) of the priming protocol to ensure the validity of priming by comparing testosterone secretion.

Table 3.1. Individual and mean±SEM changes in plasma testosterone concentration (ng/ml) in juvenile male rhesus monkeys (n=4) before and after vehicle administration (at 0 min).

Time (min)	Plasma Testosterone Concentration (ng/ml)				
	Animal Number				mean±SEM
	10M-21	11M-21	12M-21	13M-21	
-60	0.247	0.031	0.298	0.059	0.158±0.066
-30	0.080	0.031	0.392	0.482	0.246±0.112
0	0.031	0.253	0.466	0.031	0.195±0.104
30	0.344	0.031	0.682	0.031	0.272±0.155
60	0.239	0.031	0.466	0.272	0.252±0.089
90	0.031	0.031	0.811	1.340	0.553±0.320
120	0.025	0.031	0.635	1.837	0.632±0.426
150	0.146	0.031	0.490	0.897	0.391±0.194
180	0.031	0.031	0.729	1.225	0.504±0.291
210	0.031	0.031	0.457	0.195	0.178±0.100
240	0.031	0.188	0.031	1.346	0.399±0.317
270	0.031	0.054	0.368	0.763	0.304±0.171

Table 3.2. Individual and mean±SEM changes in plasma testosterone concentration (ng/ml) in juvenile male rhesus monkeys (n=4) before and after antagonist administration (at 0 min).

Time (min)	Plasma Testosterone Concentration (ng/ml)				
	Animal Number				mean±SEM
	10M-21	11M-21	12M-21	13M-21	
-60	0.168	0.031	0.482	0.031	0.178±0.106
-30	0.224	0.031	0.320	0.501	0.269±0.097
0	0.052	0.031	0.623	0.235	0.235±0.137
30	0.201	0.031	0.614	0.138	0.246±0.127
60	0.031	0.031	0.281	0.299	0.160±0.074
90	0.098	0.031	0.484	0.409	0.255±0.112
120	0.031	0.031	0.923	1.128	0.528±0.290
150	0.031	0.031		0.473	0.178±0.147
180	0.031	0.031		0.752	0.271±0.240
210	0.031	0.031		0.273	0.111±0.080
240	0.031	0.031		0.087	0.049±0.018
270	0.037	0.031		0.283	0.117±0.083

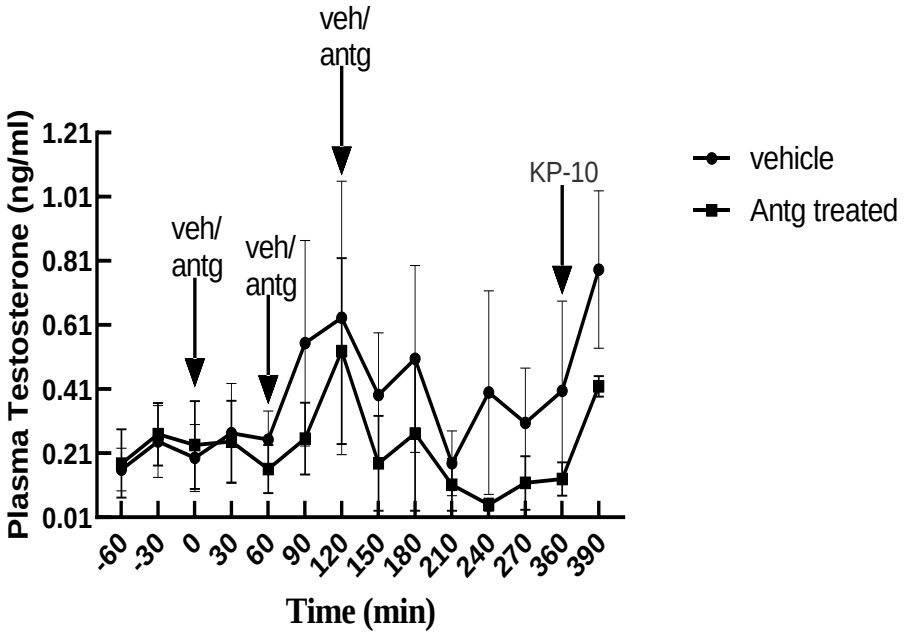

Figure 3.11. Comparison of *iv* administration of vehicle (veh) and NPY1R antagonist (antg) (BIBO 3304) on plasma testosterone concentrations (mean±SEM) in intact juvenile male monkeys in which pituitary responsivity to GnRH was heightened by GnRH agonist. Round and square points show plasma testosterone levels in response to vehicle and antagonist, respectively. Three blood samples at 30 min interval for 60 min (at -60, -30, 0 min) were withdrawn before antagonist administration while nine samples (at 30, 60, 90, 120, 150, 180, 210, 240, 270 min) were taken after *iv* antagonist administration. Three *boli* of antagonist **(**each 1ml *bolus*, 2mg/animal) were administered immediately after taking 0-, 60- and 120-min samples at 1 hr interval indicated by arrows. Following 270 min sample, 1:30 hr rest was given; after that, two additional blood samples (at 360 and 390 min) were obtained before and after KP-10 administration, respectively. KP-10 was given immediately after 360 min sample as indicated by arrow. KP-10 (25µg/800µl) was used to check the patency of the HPG axis and to interrogate the effect of NPY1R antagonist in inducing premature activation of the HPG axis. ANOVA (Two-way) followed by post-hoc test (Tukey's multiple comparison) showed that neither treatment nor time has significant effect on testosterone secretion. A significant increase in plasma testosterone release after *iv* injection of KP-10 1:30 hr after vehicle or antagonist administration confirmed the patency of the HPG axis.

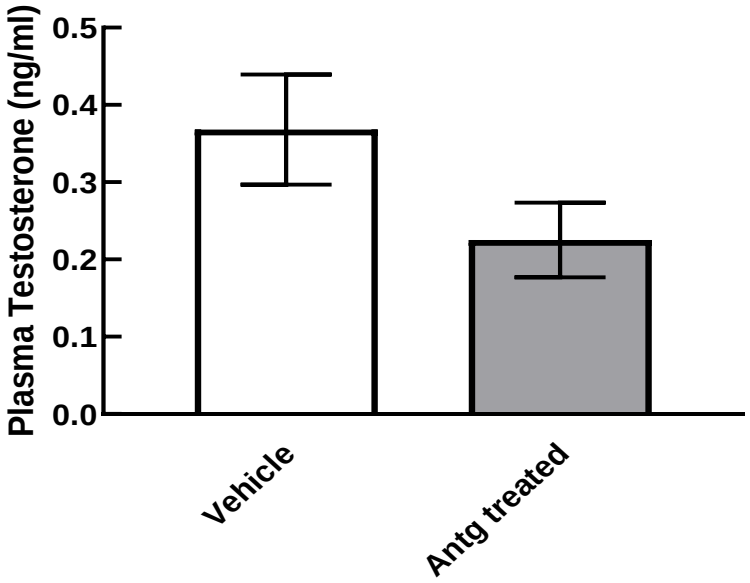

Figure 3.12. Comparison of overall mean±SEM plasma testosterone concentrations in vehicle and antagonist treated intact juvenile male monkeys in which pituitary responsivity to GnRH was heightened by GnRH agonist. No significant difference in overall mean plasma testosterone levels was observed between vehicle or antagonist administered animals.

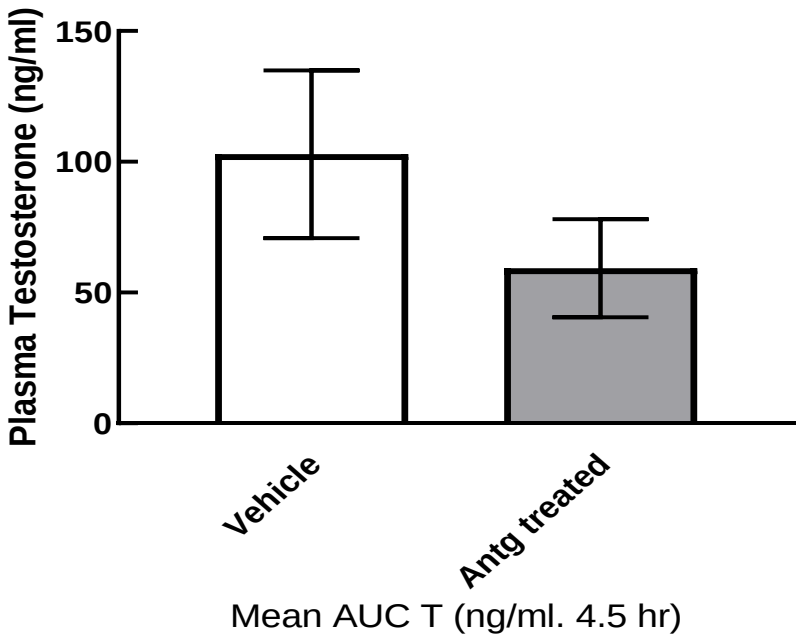

Figure 3.13. Comparison of mean±SEM area under the curve (AUC) for plasma testosterone during time lapse 0-270 min between vehicle and antagonist treated animals. No significant difference in mean plasma testosterone release was observed.

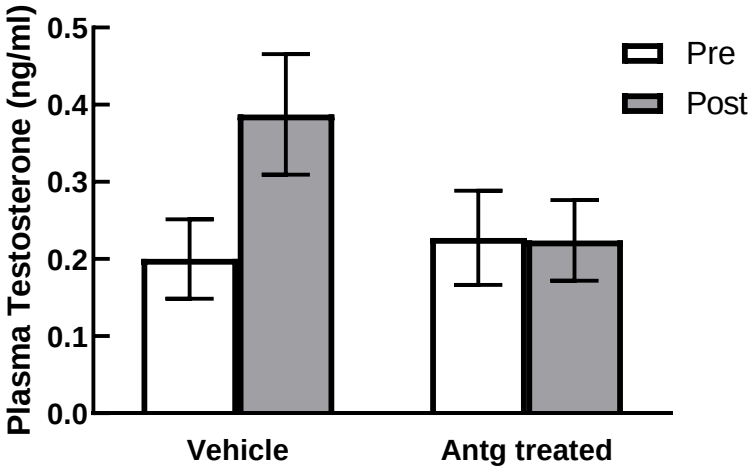

Figure 3.14. Comparison of mean±SEM plasma testosterone concentrations in the 1 hr pre- and 4.5 hr post- vehicle/antagonist administration in intact juvenile male monkeys. No significant difference in pre and post-plasma testosterone secretion was observed in either vehicle or antagonist treated monkeys.

Figure 3.15. Comparison of plasma testosterone concentrations (mean±SEM) in intact juvenile male monkeys before (at 360 min) and after (at 390 min) KP-10 injection in vehicle (A) and antagonist (B) treated animals. A *bolus* KP-10 showed significant elevation ($p<0.05$ and $p<0.01$) in testosterone release at 30 min after the injection in vehicle and antagonist treated animals, respectively.

DISCUSSION

The results of the present study that peripheral (*iv*) administration of NPY1R antagonist (BIBO 3304) to juvenile intact male rhesus monkey failed to elicit any change in testosterone secretion indicates that NPY dependent inhibition of GnRH pulse generator during juvenile stage mediated by NPY1R in non- human higher primates remains elusive. To the best of our knowledge, this is the first report where selective NPY1R antagonist was employed intravenously in juvenile non-human higher primates. These results contrast with the previous findings where GnRH dependent stimulation of LH after *icv* administration of NPY antagonist has been observed in juvenile castrated monkeys (EI Majdoubi *et al*., 2000a). Since the antagonist 1229U91, which El Majdoubi and colleagues employed, exhibits both antagonistic and agonistic properties for NPY1R and NPY4R respectively, therefore, to characterize exclusive involvement of NPY1R to mediate NPY dependent inhibition of GnRH pulse generator during juvenile stage, highly specific NPY1R antagonist BIBO 3304 (Wieland *et al*., 1998) was used in the current study. Higher affinity and selectivity of BIBO 3304 for NPY1R has been documented by Weiland *et al*. 1998.

It is quite intriguing that LH releasing action of 1229U91 was only observed after central administration; however, in the same study, *iv* injection of the 1229U91 antagonist was unable to induce LH release (El Majdoubi *et al*., 2000a). Moreover, *icv* administration of two more antagonists (VD-11 and Compound A), highly specific for NPY1R, failed to stimulate LH levels in castrated male juvenile monkeys (Shahab *et al*., 2003). However, in rodents, chronic *icv* infusion of another highly specific NPY1R antagonist BIBP 3226 caused advancement in pubertal development in pre-pubertal female rats (Pralong *et al*., 2000). Both BIBO 3304 and BIBP 3226 are selective antagonist for NPY1R but higher efficacy of BIBO 3304 was likely to be expected. This is because BIBP 3226 showed low solubility and toxic nature while ten times more potency of BIBO 3304 than BIBP 3226 was observed. In addition, BIBO 3304 also showed high solubility (Rudolf *et al*., 1994) and less toxicity in vivo (Weiland *et al*., 1998). In this regard, the present study is the first

report about the effect of highly specific NPY1R antagonist on testosterone secretion in the juvenile male rhesus monkeys.

In the present study, the incompetency of BIBO 3304 to evoke testosterone release after peripheral administration is likely to raise concerns about the potency of the drug at the level of the monkey hypothalamic NPY1R. In this context, it should also be noted that in the previous study intravenous administration of 1229U91 (a mixed antagonist and agonist for NPY1R and NPY4R, respectively) was also unable to induce LH secretion (El Majdoubi *et al*., 2000a). Although the dose of the antagonist used in the current study was almost five times higher than in the study of El Majdoubi *et al*. 2000a (7910nmol vs. 1480nmol *iv*), therefore, failure of the antagonist to elicit any change in testosterone secretion also shows concern about the accessibility of the drug to the hypothalamus. As the intravenous administration of BIBO 3304 in the current study was unable to trigger testosterone release in juvenile animals therefore it can also be speculated that cryptic pool of NPY1R would have been unmasked for which quantity of the dose was not enough. However, 1229U91 effectively caused robust release of LH after *icv* administration (El Majdoubi *et al* 2000a). Central blockade of NPY1R by BIBO 3304 still needs to be observed. Keeping in view the paradoxical actions of NPY1R antagonists' further explanation of these results, however, is adjourned until additional experimental paradigm with different doses of antagonist would have been tested.

It is quite intriguing that peripheral pharmacological inhibition of NPY1R has never been able to clearly offset the inhibitory action of NPY on the HPG axis. The results of the current study also showed that BIBO 3304 was impotent to trigger the HPG axis as reflected by no change in testosterone secretion in juvenile monkeys. The impotency of the antagonist indicates that BIBO 3304 could not cross blood brain barrier. Notwithstanding, two areas ARC and ME are devoid of blood brain barrier making them highly penetrable (Weindle and Joynt, 1973). Hence, systemic administration of antagonist was expected to work. This is because abundant concentration of NPY1R mRNA was evident in the ARC (Mikkelsen and Larsen, 1992) and close contact of NPY1R containing fibers with GnRH fibers in ME (Li *et al.,* 1999) has been observed. Furthermore, elevated expression of NPY1R gene in juvenile male monkeys within MBH has recently been documented (Bano

et al., 2022) which also supports that peripherally injected antagonist could reach to these areas and physiological action of antagonist was likely to occur at least at the level of ME.

Although *iv* infusion of the antagonist did not work to uplift plausible neurobiological brake imposed by endogenous NPY tone on GnRH pulse generator, as reflected by no change in testosterone secretion, nevertheless, possible direct action of antagonist on GnRH neurons cannot be precluded. Intimate association between NPY fibers and GnRH neurons in different animal models (Turi *et al*., 2003; Plant and Shahab, 2002; True *et al*., 2017), and juxtaposition of NPY1R stained fibers with GnRH cell bodies and GnRH neuro terminals at ME (Li *et al*., 1999) provides the morphological basis for the physiological function of NPY via NPY1R either on GnRH cell bodies or at ME. In vitro electrophysiological data from mice (Klenke *et al*., 2010) and immunostaining data from monkey (El Majdoubi *et al*., 2000a; Bano *et al*., 2022) foster the idea about direct action of NPY on GnRH neuronal activity through NPY1R.

Parenthetically, the presence of NPY4R on GnRH neurons (Roa and Herbison, 2012) and the involvement of NPY5R in inhibiting GnRH neuronal activity (Verma *et al*., 2014) has also been observed. Therefore, for the non-functionality of NPY1R antagonist, it is also possible that commutative collaboration between different NPY receptors may modulate GnRH driven pituitary testicular axis. It is interesting to note that BIBO 3304 also failed to modulate GnRH neuronal activity in retrochaismatic GnRH neurons of pre-pubertal male rats (Lebrethon *et al*., 2000) which agrees with the current study regarding the non-potency of the used antagonist. It has also been shown that central administration of NPY prior to 1229U91 administration did not offset the stimulatory action of the antagonist therefore it has been suggested that NPY1R may not be involved in 1229U91 dependent GnRH secretion (Shahab *et al*., 2003). Hence, the involvement of other subtype of receptors of NPY in addition to NPY1R in suppressing GnRH neuronal activity during the pre-pubertal period cannot be ruled out.

Considering the diverse expression of NPY neurons within hypothalamus and heterogeneity of its receptors, it is important to segregate subpopulation of NPY neurons with specific receptors to further clarify the role of NPY in controlling reproductive axis

activity. Inefficiency of the antagonist in inhibiting NPY tone in juvenile monkeys in the current study also supports the idea proposed by El Majdoubi and colleagues, 2000a. According to that two subpopulations of NPY neurons regulating GnRH pulse generator distinctly may be present. According to this idea, the subset of NPY neurons that keep the pulse generator in check during the juvenile phase is likely to project farther from ME while the second subpopulation establishes an association with GnRH axons and terminals in ME becomes functional at the time of puberty. Current data where no effect of NPY1R antagonism on the HPG axis was observed also supports the above cited hypothesis. Albeit the idea seems good, however, the anatomical and physiological attributes of these two subpopulations remain to be addressed.

The intimate association between NPY and kisspeptin neurons in sheep (Backholer *et al*., 2010; Polkowska *et al*., 2014) and reduction in neuronal activity of kisspeptin neurons by NPY was demonstrated by in vitro voltage gated cell potentials (Padilla *et al*., 2017; Hessler *et al*., 2020) can be interpreted to thrive the rationale that NPY dependent suppression of the HPG axis may be mediated through kisspeptin neurons. Imperatively, the presence of NPY1R on arcuate kisspeptin neurons in rats (unpublished data as cited in Hessler *et al*., 2020) and inverse correlation between *KISS1* and *NPY1R* relative mRNA in male monkeys across pubertal development (Bano *et al*., 2022) suggest that NPY1R is responsible for translating inhibitory effect of NPY on kisspeptin neurons. Immunocytochemical data from previous study where higher percentage of double label kisspeptin neurons with NPY1R during juvenile and pre-pubertal stage as compared to infant stage was observed, also supports NPY inhibition of kisspeptin neurons via NPY1R. It is interesting to note that direct inhibition of kisspeptin neuronal activity imposed by NPY can be reversed partially by applying NPY1R antagonist BIBO 3304 (Hessler *et al*., 2020). However, this ex vivo observation is inconsistent with our finding that *iv* administration of the same antagonist failed to elicit testosterone secretion in juvenile monkeys. The discrepancy between the two studies may be attributed by the experimental paradigm, i.e., ex vivo and in vivo. In the former study, fluorescent-tagged arcuate kisspeptin neurons in acute brain slices were used to record firing rate and calcium imaging by direct application of antagonist in CSF. In contrast, the current study employed

intravenous administration of the antagonist. In addition, this may also be attributed to specie difference. Furthermore, no effect of NPY1R antagonism on HPG axis activity may also be pertained to the difference in developmental stage i.e., adult animals were used in the former study while juvenile animals were used in the current study.

Albeit the fact that LH could not be measured in the present study due to the non-availability of rhesus monkey LH assay, it is accentuated that strict coherency in secreting pattern of LH and testosterone has been (Plant, 1981). Hence, plasma testosterone concentrations can reliably be interpreted as bioassay to measure LH levels under experimental paradigm employed in the study. In this context, GnRH priming was done to substantiate the gonadotropins content within pituitary gonadotrophs to incite testosterone secretion from testicular Leydig cells. It has been documented that one-week *iv* infusion of GnRH caused testosterone secretion (Abeyawardene *et al*., 1989); therefore, pituitary-Leydig cell response was first heightened by priming with GnRH agonist for one week. The fidelity of the priming regime was discerned by the difference in testosterone secretion in pre-primed and post-primed animals following KP-10 administration. It has been clearly manifested that the percentage response of testosterone after KP-10 administration was more pronounced and substantially significant in post-primed animals as compared to testosterone response against KP-10 administration in pre-primed animals. It showed the patency of the HPG axis. Thereby any effect of antagonist treatment on testosterone secretion could have been possibly interpreted in terms of hypothalamic GnRH and pituitary gonadotrophs activity.

Direct regulation of testosterone secretion by the testes through NPY1R antagonism cannot be perceived owing to the absence of NPY1R on Leydig cells (Allen *et al*., 2011). No significant difference in overall mean testosterone levels and by comparing AUC for testosterone (ng/ml.4.5hr) between vehicle and antagonist treated animals suggests that NPY1R may not be involved in regulating testosterone secretion locally.

The current data also demonstrate that no difference in pre- and post-testosterone secretion was present in NPY1R antagonist treated animals. These results in non-human primates complement two medical observations in humans. Genetic analysis in two medical reports

demonstrated no dynamic involvement of NPY1R related mutation as plausible cause for gonadotropin-dependent precocious puberty (Barker-Gibb *et al*., 2004; Freitas *et al*., 2007). However, further investigations are necessary to clarify the definitive role of NPY1R during pubertal development before any conclusion about the non-indulgence of NPY1R in regulating the HPG axis activity during pubertal development is drawn.

Current data did not show any fluctuations in testosterone secretion after pharmacological manipulation by NPY1R antagonism. This can be explained by considering two arbitrary neuroendocrine mechanisms. First, different subpopulations of NPY neurons regulating GnRH pulse generator may be distinctly present and the possible role of different subclass of NPY receptors in mediating inhibition of HPG axis may also be considered. The second plausible notion about the inactivity of the HPG axis in terms of testosterone secretion after antagonist administration lends credence that in addition to NPY inhibition, other neuroinhibitory factors such as GnIH or GABA may also participate as a component of a neurobiological brake.

In conclusion, current data contrast with the proposed hypothesis that pharmacological manipulation/blockage of NPY1R with highly specific antagonist in the juvenile monkeys may instigate premature activation of the HPG axis. Present findings implicate that the putative inhibitory action of NPY on the HPG axis mediated by NPY1R, if any, posit concerns about the potency of the drug at the level of the hypothalamic monkey NPY1R or its accessibility to the monkey hypothalamus. However, an extensive exploration about the functionality of NPY1R in mediating NPY based inhibition of GnRH release during the juvenile period remains to be substantiated by different experimental paradigms to unwind twists and turns in the pubertal development story.

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

GENERAL DISCUSSION

GENERAL DISCUSSION

In the current research work potential involvement of NPY and NPY1R in regulating kisspeptin dependent GnRH pulse generator activity from infant to adult stage in male rhesus monkey was envisaged. Three different experimental paradigms were employed to delineate the possible interaction between NPY and kisspeptin neuronal system during pubertal development and to decipher NPY role as a plausible neurobiological brake for kisspeptin-GnRH pulse generator during juvenile phase in male monkeys. To achieve these objectives, first study was conducted to quantify and compare hypothalamic expression of *KISS1, NPY, KISS1R,* and *NPY1R* mRNA across different pubertal stages. Second experiment was undertaken to find out possible coherency between gene and protein expression profile. For this purpose, double label immunofluorescence for NPY and kisspeptin, and NPY1R and kisspeptin on hypothalamic sections of infant, juvenile, pre-pubertal, and adult animals was done. Lastly, in the third experiment pharmacological approach was taken up to observe the role of NPY1R in mediating the putative inhibitory action of NPY on the HPG axis by testosterone measurement as a surrogate of LH secretion after intravenous (*iv*) administration of highly selective NPY1R antagonist BIBO 3304 in the juvenile male rhesus monkey.

Puberty is an intricate process involving interplay among plethora of neurobiological components within hypothalamus to decide its timing of onset (Plant, 2019). However, the final conduit for pubertal onset relay on pulsatile GnRH secretion in all species (Uenoyama *et al*., 2019). The pulsatile GnRH secretory activity seemed to be at its peak during infant and adulthood period characterizing mini and true puberty intervened by quiescent state where GnRH activity enters dormant mode identifying juvenile stage (Plant, 2019). The mystery behind this on-off-on mode of GnRH pulse generator has been subjected to extensive research from the last forty years. However, neurobiological mechanism associated with reactivation of pulse generator and central neuroinhibitory mechanism which brought into check this generator activity during juvenile/pre-pubertal stage has not been clearly elucidated yet. Among multiple central neurobiological factors describing activity of GnRH pulse generator, the role of kisspeptin and NPY, being stimulatory and

inhibitory respectively, is worth to mention. Thus, it was imperative to study NPY dependent regulation of kisspeptin neurons across different developmental stages. Therefore, present research work was conducted for the dissertation to study interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*) and the potential involvement of NPY1R in mediating NPY based suppression of the HPG axis during juvenile phase.

Fifteen animals were characterized into four different developmental stages i.e., infant, adult, (when GnRH release is heightened), juvenile and pre-pubertal (when GnRH release is quiescent) to conduct first two experiments, a) quantitative comparison of mRNA expression of *NPY* and kisspeptin along with their receptors *KISS1R* and *NPY1R* and b) double label immunofluorescence of kisspeptin and NPY, and NPY1R and kisspeptin. Somatometric parameters like body weight and testicular volume and hormonal parameter (testosterone) was gauged for characterization of animals into developmental stages. The first reliable hormonal sign for pubertal initiation is the rise in nocturnal LH levels (Plant, 2015) but due to non-availability of the facility to measure monkey LH, above mentioned parameters were used to characterize pubertal stage of each animal. Since LH diurnal variations are tightly correlated with diurnal plasma testosterone levels (Plant, 1982), therefore plasma testosterone was used as reliable representative hormonal index to gauge developmental stages. It has also been observed that diurnal variations of testosterone and LH in infant animals mimic the diurnal variations in adult animals (Plant, 1982). However, testosterone levels observed in the infants were, unexpectedly, no higher than those in the juveniles. In this regard, plasma testosterone concentrations were observed to be 3- to 6-fold higher at night than in the morning in infantile male monkeys during first 6 weeks of life (Plant, 1982). Since the samples from infant animals were taken during daytime therefore plasma testosterone concentrations were possibly no higher than juveniles. Moreover, after 6 weeks the diurnal variations of plasma testosterone progressively decline, becoming apparent by the second or third month of life (Plant, 2015). The age for the animals used in the current study for infant stage was between 4-7 months hence testosterone levels were supposed to become nadir at this time. In addition, morphometric data of testicular histology and spermatogenic status through standard differential staining

technique was also used to gauge a particular developmental stage as represented in earlier studies (Plant *et al*., 2005; Simorangkir *et al*., 2012).

Puberty is a serpentine physiological process and central mechanism governing GnRH pulse generation at mini and true puberty is much uncovered but neuronal substrate keeping pre-pubertal dormant state of pulse generator in between the two active phases is not fully defined. Thus, it was of considerable interest to find out gene and protein expression analysis of critical neuropeptides i.e., NPY and kisspeptin along with potential immunocytochemical interactions between these two neuropeptidergic populations during pubertal development in the male rhesus monkeys (*Macaca mulatta*).

Gene and protein expression profile demonstrated inverse correlative age associated changes between NPY and kisspeptin during sexual development in intact male rhesus monkeys. More precisely, mRNA and kisspeptin-like immunoreactive neuronal expression gradually decline from infant to pre-pubertal stage while an uplift in expression was evident in the adult stage as compared to juvenile and pre-pubertal stage. These results are consonant with previous observations in rodents (Navarro *et al.,* 2004a; Takumi *et al*., 2011), non-human primates (Shahab *et al.,* 2005; Terasawa *et al*., 2013) and humans (de Vries *et al*., 2009; Bano *et al*., 2009). The likeliness of the current data with the previous literature strengthening the concept that developmental maturation of kisspeptin and sensitivity of its receptor progressively approaches at maximum during pubertal transition.

Furthermore, Han *et al.,* 2005 found that developmental maturity and sensitivity of GnRH neurons is achieved with the passage of time. As it has been observed that response of GnRH neuronal population against 100nmol kisspeptin is more evident in adult mice as compared to mice at juvenile or pre-pubertal phase. Commutatively, From the above-mentioned data coherency with present results suggest that lack of activation of HPG axis resurgence during pubertal progression in juvenile/prepubertal period in non-human primates which parallels to stage I-III in humans may be due to neurobiological brake imposed by increased activity of NPY and NPY1R on kisspeptin neuronal circuitry in conjunction with insufficient expression of KISS1R.

Nevertheless, equal intense co-staining of kisspeptin and GnRH between infant and juvenile male (Ramaswamy *et al*., 2013) and between prepubertal and gonadectomized adult female (True *et al*., 2017) monkeys was observed. Moreover, response of GnRH neurons against kisspeptin administration is more pronounced in pubertal monkeys in both male and female as compared to pre-pubertal animals (Guerriero *et al*., 2012; Garcia *et al*., 2017). These observations touted to reconsider the role of kisspeptin in triggering pubertal onset and raised the possibility that some neurobiological factor like NPY may involve in inhibition of kisspeptin mediated GnRH secretion in higher primates during juvenile phase.

As far as NPY gene expression is concerned, higher expression during juvenile and pre-pubertal stages while lower levels during infant and adult stages was observed. A similar expression profile of NPY mRNA was also observed in pre-pubertal female monkeys compared to gonadectomized adult animals (True et al., 2017). Besides primates, pre-pubertal lamb also showed increased NPY mRNA expression suggesting high transcriptional activity with quick release of the peptide to inhibit the HPG axis (Tillet et al., 2010). These findings provide strong evidence to consider NPY as prime candidate to act as neurobiological brake for the HPG axis. However, the idea was authenticated by observing inverse association between NPY gene and peptide expression with respect to LH release from neonatal to pubertal transition in agonadal male monkeys (EI Majdoubi *et al*., 2000a). The idea was further nurtured on morphological basis in rodents (Li *et al*., 1999; Turi *et al*., 2003; Ward *et al*., 2009) and non-human primates (Plant and Shahab, 2002; True *et al*., 2017). In highly evolved non-human old-world primates, the intimate contacts of NPY/AgRP onto GnRH neurons were more in both male and female pre-pubertal monkeys as compared to adult/pubertal monkeys (Plant and Shahab, 2002; True *et al*., 2017). This morphological association between NPY and GnRH warranted the physiological basis of NPY suppression of GnRH/LH release in different animal models including non-human primates (McDonald *et al*., 1989; Kaynard *et al*., 1990; Malven *et al*., 1992; Pau *et al*., 1995; Pierroz *et al*., 1996; Shahab *et al*., 2003). Although the above cited literature corresponds to the current observations regarding NPY as potential neurobiological brake in restraining GnRH release directly, curiously enough, kisspeptin labelled neurons connection with GnRH neurons showed no significant difference between

pre-pubertal and OVX adult female rhesus macaque (True *et al.,* 2017), suggesting another upstream layer of morpho-functional mechanism i.e., plausibly NPY driven kisspeptin mediated GnRH release during sexual maturation. In this regard, the existence of reciprocal connections between NPY and kisspeptin neurons has been demonstrated (Backholer *et al*., 2010). Close apposition between kisspeptin-ir cell bodies and NPY-ir fibers observed under confocal microscope in the ARC nucleus of lamb (Polkowska *et al*., 2014) is suggestive of suppressive role of NPY in regulating the HPG axis via inhibiting kisspeptin dependent GnRH pulse generator activity. The current double label immunofluorescent cytochemical data of kisspeptin neurons with NPY showed significantly increased expression in juvenile and pre-pubertal animals as compared to infant animals. Additionally, a significant decrease in percentage of kisspeptin neurons with NPY was also observed in adult group as compared to juvenile group. These results indicate that NPY may be the potential factor in restraining kisspeptin neuronal activity at minimum during these developmental stages. Recent in vivo physiological and in vitro electrophysiological data showed that NPY predominantly inhibits kisspeptin neuronal activity thus inhibiting GnRH/LH secretion (Padilla *et al*., 2017; Hessler *et al.* 2020), favors NPY regulation of kisspeptin neurons. These observations predominantly foster the idea that NPY may potentially act as neurobiological brake to inhibit HPG axis activity by diminishing kisspeptin drive during juvenile period.

Significant elevation in NPY mRNA in arcuate of post-menopausal older females suggest that NPY induced aging may shut off HPG axis activity (Escobar *et al.,* 2004). However, no effect on NPY mRNA expression have been observed after ovariectomy and orchidectomy (Escobar *et al.,* 2004). In addition to the above cited observations, it has also been noted that no difference in NPY gene expression present in intact vs. castrate male monkeys (Goldstone *et al.,* 2002). All this implicated steroid independent dominant role of NPY in central regulation of reproductive aspects of hypothalamus and pituitary. Therefore, pre-pubertal increase in NPY gene expression seems to be intrinsic event because gonadal status does not affect NPY expression (Goldstone *et al.,* 2002; Escobar *et al.,* 2004). Interestingly, insignificant difference in KISS1 expression between young and old age monkeys (Eghlidi *et al.,* 2018) on one side of coin and significant elevation in NPY

mRNA in arcuate of post- menopausal older females (Escobar *et al.,* 2004) on the other side, subjected to question whether Kisspeptin driven reproductive axis may again be brought into check by NPY dependent inhibitory input at aging.

Higher levels of NPY along with low levels of kisspeptin, thrives a notion that NPY may act as upstream regulator for kisspeptin and hence reduction of NPY based inhibitory tone may be involved in establishing kisspeptin-dependent stimulatory drive onto GnRH neurons for the onset of puberty. Although based on current observations the idea seems cogent, however, which receptor subtype from the bunch of receptors for NPY is necessary to mediate NPY signaling is debatable. In this context, based on importance of NPY signaling via NPY1R, it was pertinent to observe developmental variations in NPY1R mRNA expression and to observe changes in dual labeled kisspeptin neurons with NPY1R during different stages of pubertal progression.

Both in vivo and in vitro studies from different animal models showed that NPY inhibition of gonadotropins release is mediated by NPY1R as manifested by pharmacological strategy (Pralong *et al*., 2000; Shahab *et al*., 2003; Hessler *et al*, 2020). Presence of NPY1R on GnRH (Li *et al*., 1999; Roa and Herbison, 2012) and arcuate kisspeptin neurons (unpublished data as cited in Hessler *et al.,* 2020) is revealed by immunocytochemical and RNA sequencing, respectively. These experimental observations led the idea that NPY suppression of HPG axis can be achieved by diminishing GnRH neuronal activity directly or indirectly by diminishing kisspeptin neuronal activity. Since the critical role of kisspeptin in regulating the HPG axis activity has been thoroughly defined, the latter opinion appears to be more reasonable. Thereby, regarding the double label semi-quantitative immunofluorescence observations indicated that significantly higher percentage of kisspeptin neurons with NPY1R expression during juvenile and pre-pubertal stages and vice versa during adult stage perhaps highlights the possibly increased concerted action of NPY signaling which may likely cause subservience in kisspeptin signaling before pubertal onset.

From the first two experiments, it was evident that NPY mediated HPG axis suppression via kisspeptin neurons may involve NPY1R signaling at juvenile/pre-pubertal stage of

development. Therefore, to delineate the exclusive involvement of NPY1R in mediating suppressive effect of NPY in inhibiting the HPG axis, specific NPY1R antagonist was used in third experiment. It was the first study in which highly specific NPY1R antagonist was used in juvenile male monkeys. Regardless of the specificity of the antagonist, it could not provoke testosterone secretion hence premature activation of the HPG axis could not achieve. Earlier studies in monkeys (EI Majdoubi *et al*., 2000a; Shahab *et al*., 2003) in which elevated LH levels were observed after *icv* administration of NPY1R antagonist, it should be noted that the antagonist used in those studies exhibited both antagonistic and agonistic properties for NPY1R and NPY4R respectively. Therefore, by comparing results of juvenile monkeys in previous and current study, characteristic involvement of NPY1R to mediate NPY dependent inhibition of GnRH pulse generator during juvenile stage could not be affirmed. Another concern is to state that the releasing LH action of NPY antagonist used in previous studies in juvenile male monkeys was only evident after central administration, however, almost ten times higher dose was futile to achieve desire results (EI Majdoubi *et al*., 2000a). Peripheral administration of the antagonist was also ineffective in inducing testosterone release in the current study therefore it may be speculated that higher doses of the drug may be helpful to find out functional significance of NPY1R in regulating kisspeptin-GnRH-LH-testosterone secretion. Moreover, it is also interesting to mention that two more highly specific antagonists used earlier were also failed to stimulate LH release in male juvenile monkeys (Shahab *et al*., 2003).

The antagonist used in the current study was failed to uplift the brake imposed on kisspeptin neurons to regulate GnRH driven pituitary gonadal axis, nonetheless, the same antagonist was partially effective to disinhibit kisspeptin neuronal activity imposed by NPY as revealed by in vitro electrophysiological settings (Hessler *et al*., 2020). Considering heterogeneity of NPY receptors, collaborative mechanism between different receptor subtypes like NPY4R and NPY5R (Verma *et al*., 2014) along with NPY1R cannot be ruled out to modulate GnRH driven pituitary testicular axis.

Although the results of the last experiment showed that NPY1R may not be critically involved in mediating the putative inhibitory action of NPY on the HPG axis during the

juvenile period, however, further investigations are required to ascertain obligatory mechanism by which NPY can down regulate HPG axis.

In negative energy situation, higher expression of NPY in the hypothalamus (Pralong, 2010) and its inverse regulation by metabolic hormones (Barroso *et al*., 2019) makes it a plausible candidate to sense metabolic energy status. Thus, NPY neurons may act as barometer to relay energy status information to GnRH neurons via suppression of kisspeptin neurons (Navarro and Tena-Sempere, 2011; Castellano and Tena-Sempere, 2016) during pubertal development allowing to suppress pubertal onset under sub nutritional conditions. Since reproduction is energy demanding process and NPY showed orexigenic property in rodents (Hahn *et al*., 1998) and primates (Larsen *et al*., 1999) so it may act as first order afferent input to convey energy status information to kisspeptin neurons. In this regard, previous studies clearly defined fasting induced increased expression of NPY and decreased expression of kisspeptin in rodents (Kalamatianos *et al.*, 2008; Pralong, 2010) and non- human-primates (Grove *et al.,* 2003; Wahab *et al.,* 2011) also highlights the reciprocal response of two neuronal systems under adverse energy situation. In addition, negative regulation of NPY (Takahashi and Cone, 2005; Mayer and Belsham, 2009) and positive regulation of kisspeptin (Smith *et al.,* 2006; Backholer *et al*., 2010) neurons by leptin suggests that both these neuronal populations are the direct target for leptin. However, ample evidence supports indirect effect of leptin on kisspeptin neurons (Barroso *et al.,* 2019) which might be corroborated via NPY neuron. To support the notion, it was revealed that congenital ablation of leptin receptor in NPY neurons prevented positive effect of leptin resulting in delayed puberty (Egan *et al.,* 2017). Interestingly, leptin receptor ablation from kisspeptin neurons failed to demonstrate such effects (Donato *et al.,* 2011). Thus, foregoing information suggest that under negative energy situation, higher expression of NPY and its inverse regulation by metabolic hormones makes it a plausible candidate to sense metabolic status and relay this energy status information to GnRH neurons via suppression of kisspeptin neurons during pubertal development is considerably important to be further elucidated.

By summarizing the foregoing considerations, it may be proposed that increasing NPY tone in MBH of male monkeys at the end of the infantile period is critical for contributing

hypogonadotropic state in juvenile animals. However, NPY dependent inhibition via NPY1R is not exclusively involved in the dormancy of the HPG axis. Therefore, many other central neural substrates, i-e. GABA and GnIH should be reckoned while interpreting the concept of neurobiological brake in higher primates.

A sequel of findings proposed that GABA is the central component of neurobiological brake ensuring pre-pubertal hiatus of GnRH pulse generator activity (Terasawa and Garcia, 2020). It has been demonstrated that levels of GABA are higher in pre-pubertal monkeys in ME than in pubertal monkeys (Mitsuhima *et al.,* 1994). GABA A antagonist bicuculline administration into ME provoke GnRH (Mitsuhima *et al.,* 1994) and kisspeptin release in pre-pubertal monkeys (Kurian *et al*., 2012), indicating GABA neuron is a part of the central brake. Paradoxically, no fluctuation in GAD 65 and GAD 67 gene expression (Urbanski *et al*., 1998) and no differential change in these two enzymes in male rhesus monkeys (El Majdoubi *et al*., 2000a) during neonatal-juvenile transition may imply contentious role of GABA in considering it as neurobiological brake. The precise mechanism of how upstream neuronal substrates are involved in GABA inhibition (Terasawa *et al*., 2013) is still debatable. It is important to note that GABA inhibition is predominately involved in female monkeys. In contrast, NPY dependent inhibition of pre-pubertal brake is chiefly concerned with male monkeys. Hence, whether sex difference is purely related to the protracted activity of GnRH pulse generator during the juvenile period is still unknown. Nonetheless, colocalization of NPY and GABA (Horvath *et al*., 1997) in ARC neurons and NPY suppression of kisspeptin neurons showed gabaergic nature (Padilla *et al*., 2017) suggest that there is a possible common mechanism may exist between two neuropeptides in central inhibition of GnRH pulse generator during the juvenile period.

Another neuroinhibitory factor that suppresses HPG axis/GnRH release in multiple species including higher primates is GnIH (Tsutsui and Ubuka, 2021). Regarding pubertal development, increased expression of RFRP gene has been documented in pre-pubertal non-human primates (Wahab *et al*., 2017). However, GPR147 deleted mice showed normal awakening of the reproductive axis led to pubertal onset (Leon *et al*., 2014). Contrary reports about GnIH regulation of puberty have been described (Ullah *et al*., 2016). Intravenous RF-9 administration (GnIH antagonist) did not affect testosterone secretion in

adult male monkeys (Wazir *et al*., 2020), while the advancement of puberty after RF-9 *icv* injection was observed in pre-pubertal female rats (Sahin *et al*., 2015). Since RF-9 acts as an antagonist for GnIH receptor and agonist for GPR54 (Min *et al*., 2015), it is pertinent to propose that GnIH may not play an important role in the pre-pubertal hiatus of GnRH pulse generator. Close contact between NPY neurons and GnIH fibers has been also demonstrated in mice (Jacobi *et al*., 2013) which probably contemplate that NPY based inhibition of GnRH pulse generator might be mediated through GnIH dependent intermediatory neuronal network.

The findings of the present studies suggest multifaceted future research ideas. Some of them are discussed below.

Presence of both NPY and GABA within ARC neuronal population confer the idea about common mechanistic interplay between these two neuropeptides, like kisspeptin, neurokinin B, and dynorphin constituting KNDy neurons, in central restraining of GnRH pulses during pre-pubertal phase. The idea needs to be tested in future studies by utilizing different optogenetic and chemo genetic approaches. The integration of NPY neuronal activity with other neurotransmitter/ neuropeptide systems to control characteristic pattern of GnRH pulse generator activity throughout the infant-juvenile-pubertal transitions remains to be established.

Established data revealed NPY as plausible neurobiological brake in restraining GnRH pulse generator functioning during postnatal development, however, what if early developmental ablation of NPY or both NPY and kisspeptin neurons would have occur? Would any compensatory mechanism be operative that will regulate GnRH pulsatility at and after puberty? Prenatal expression profile of NPY and kisspeptin would also be helpful to understand the functional interaction between two neuronal populations. Furthermore, literature has supported the fact that kisspeptin is the most potent secretagogue of LH in juvenile animals. Considering inhibitory role of NPY on HPG axis, secretagogue potential of kisspeptin can be inspected on NPY knockout models. Target deletion of NPY1R on kisspeptin neurons are also required to delineate NPY regulation of kisspeptin activity.

Moreover, maturational changes in NKB and KP signaling has been observed in non-human primates. Keeping in view these maturational signaling shifting of NKB and KP during pubertal development, it can be speculated that NPY may have dual role in regulating components of pulse generator at different time periods. Although studies have been documented about NPY regulation of GnRH/LH release but the involvement of NPY in NKB or kisspeptin release needs to be investigated in different animal models under various paradigms.

Mediobasal hypothalamus (MBH) is not only the hub for dictating reproductive process but also involved in integrating metabolic/food status of the body. This prime position of the MBH is bestowed by the presence of multiple neuronal entities harboring this small but vital area. An interrelationship between energy status/food availability and reproduction has been evident from the earlier era of reproductive biology. Although well-coordinated mechanisms have been proposed in deciphering the impact of metabolism on reproductive events, conclusive evidence has not yet been identified till now. Among different neuronal populations involving metabolic and reproductive schemata, NPY has been considered to play an indispensable role in feeding circuit while kisspeptin has been regarded as pivotal for the reproduction and pubertal onset. Both neuronal populations kisspeptin and NPY residing within the arcuate nucleus of MBH area are thought to be the on-off switch respectively for the reproductive process. Very few studies have addressed the idea about their intimacy leading to culminate physiological impact. These studies have shown observations mostly in ovine females. Recently a high throughput study utilizing transcriptomics technique has revealed the impact of food restriction on key genes involving metabolic and reproductive processes. It has been shown that food restriction leads to higher levels of NPY expression but no changes in kisspeptin expression were observed. The study highlighted the link between metabolic and reproductive processes under the roof of NPY and kisspeptin neurons (Dardente *et al.*, 2022). However, such morphological and physiological interrelationship between these two neuronal populations have not been studied in monkeys (a representative non-human primate), the data from which can easily be interpreted to draw conclusion in humans.

Since NPY has been regarded as orexigenic peptide and reproduction is metabolically expensive process therefore additional studies under different physiological settings such as under fasting will be helpful to observe the role of NPY in regulating kisspeptin neuronal activity during pubertal development. Whether NPY act as a hypothalamic somatometer to regulate kisspeptin-GnRH-LH secretion based on sensing peripheral metabolic factors including leptin, insulin, ghrelin, etc. still needs to be tested. This is because the presence of leptin, insulin, and ghrelin receptors on NPY neurons indicates that NPY neuronal population is sensitive to several metabolic signals, particularly during pubertal initiation. If it is like so whether Frisch hypothesis of critical metabolic rate or body weight is going to be rejuvenated to understand the pubertal mechanism needs to be investigated.

Peripheral administration of the antagonist was failed to elicit pituitary gonadal activity in juvenile animals which prompted to examine alternative mode of antagonist administration that is the effect of central administration of the antagonist or at ME level still are the open loops which are required to be probed. This is because site dependent effect of NPY has been documented in previous studies.

It is interesting to note that the secretion of the two gonadotropins LH and FSH from a single cell type depends on two different frequency patterns of GnRH (rapid GnRH pulse frequency favors LH secretion while slower one favors FSH release). Harmonizing this modality with NPY functioning, it is likely to propose that NPY may also release with two different modulating frequencies at ME site. These modulating frequencies during postnatal development that is from infancy to juvenile and from juvenile to pubertal transition to control changing GnRH frequency may putatively mediate kisspeptin dependent GnRH pulse generator frequency during these developmental transitions. This is because interaction of NPY neuro terminals with GnRH at the level of ME have been documented. However, neither morphological interaction of NPY and kisspeptin at the level of ME nor simultaneous measurement of NPY and kisspeptin release at stalk-ME has been described yet.

Neurophysiological events and their mechanism are essentially the same in monkeys and humans from fetal stages up to pubertal onset. This has been manifested via various

endocrinological, biochemical, and physiological techniques. Recently bioinformatics tools are also helping to decode hidden mysteries about neuroendocrinological mechanisms. After the cumbersome efforts by the scientist from early decades of twentieth century, the question to resolve the key trigger involved in dampening the HPG axis during juvenile phase after its full activation is during infant phase still included in hundred unsolved questions in science. Therefore, such neuroanatomical and physiological studies are warranted to uncover basic biological mechanisms behind pubertal development story.

Although the results of the present thesis demonstrated a critical role played by NPY as neurobiological brake for kisspeptinergic tone to control reactivation of HPG axis for the initiation of puberty, however, it has number of potential limitations that could be considered as caveats. The number of animals in each group was less. In this context, it is regretted that some of the animals had to be excluded from a specific group as the age of those animals surpassed the designated age for that group during COVID-19 pandemic. Moreover, due to ethical consideration involving euthanization of animals, number of animals in each group was consciously chosen to a minimum. Nevertheless, we believe that inference is going to be alike even if n is increased. The results of the thesis, albeit obtained with a limited number of animals, supports the earlier finding of El Majdoubi and colleagues and reinforces the view that the NPY neurons are the paramount player acting as neurobiological brake to restrain GnRH pulse generator capability to induce trigger for the HPG axis in juvenile period of highly evolved primates. However, NPY based inhibition of kisspeptinergic pathway may not be mediated by NPY1R. The latter idea, however, must be reconsider with the view, derived from the classical study by Plant's laboratory. Which states that NPY1R is entailed for NPY dependent inhibition of LH release in highly evolved primates. In order to fully comprehend the relative physiological importance of NPY signaling via NPY1R to inhibit kisspeptin neuronal activity, selective ablation of NPY1R in kisspeptin neurons is necessary to carry out.

These findings can also be extended up to transcriptomics or epi transcriptomics level. Functional study with NPY antagonist could also be performed in combination with kisspeptin antagonist to delineate hierarchical significance of the two neuropeptides during pubertal development. Although, it was the part of the plan but due to shipment restrictions

during COVID-19 pandemic and restrictions mandated by the state of Pakistan, we could not purchase kisspeptin antagonist in due time. Moreover, as the result of the third experiment showed no effect of antagonist in modulating HPG axis activity in juvenile monkeys as demonstrated by testosterone levels, therefore, central administration of the antagonist in this regard would be helpful to implicate significance of NPY based hypothalamic brake. In addition, to enhance penetration of the NPY1R antagonist across blood brain barrier via peripheral route, a penetratin could also be utilized. Immunocytochemical data can be extended to confocal and electron microscopy to further clarify the intimate association between two neuronal populations at axo-somatic, and axo-dendritic level. Morphological plasticity in neuronal interaction between NPY and kisspeptin require further studies at cellular and transcriptomics level to elaborate clear picture about the current findings that NPY projections from MBH are the major afferent inputs to kisspeptin neurons may be subjected to alteration during pubertal process.

Genetic manipulation (gene knock out model) would also be helpful to decode the importance of NPY regulation of kisspeptin system during prenatal period. For the macaque, genetic manipulation is difficult to achieve although it is a good model from which results can be recapitulated on humans. Hence, NPY/NPY receptor based targeted genetic deletions are imperative to observe the influence of NPY on kisspeptin neuronal system during development.

Chronological, site dependent and physiological basis are involved for varied role of NPY in regulating HPG axis, from developmental perspective, it is plausible to propose that collaborative kisspeptin and NPY signaling exists before puberty and after pubertal onset predominant role of kisspeptin signaling may take charge after waning of NPY inhibitory tone.

In addition to hypothalamic nuclei both NPY and kisspeptin neuronal populations are also found in different brain areas generally and in amygdala particularly (Pineda *et al.,* 2021). Kisspeptin neurons particularly play an important role in modulating the activity of hypothalamic kisspeptin neurons. In this regard, optogenetic stimulation of amygdaloid kisspeptin caused enhancement in pulse frequency of luteinizing hormone (Lass *et al.,*

2020). Neurophysiological mechanisms concerning increased pulses of LH under amygdaloid kisspeptin directives may be attributed to other neuronal entities residing in amygdala such as NPY, GABA, and Glutamate (Pineda *et al.,* 2021). It is interesting to note that NPY neurons are residing in the same region of amygdala where kisspeptin population is present. Therefore, it can be expected that amygdaloid kisspeptin and NPY may have some coordination in dictating or modulating the activity of hypothalamic kisspeptin neurons solely or to affect both kisspeptin and NPY neurons of hypothalamic origin (Pineda *et al.,* 2017). The above idea seems good as NPY receptors have also been reported in amygdala (Wood *et al.,* 2016). The role of amygdaloid kisspeptinergic neurons in pubertal onset can be perceived. Interestingly amygdaloid kisspeptin neurons are also sexually dimorphic like hypothalamic population. However, contrary to hypothalamic population amygdaloid kisspeptin population is more abundantly present in males than in females. In addition, scanty kisspeptin population is observed during early postnatal development period while higher during puberty in contrast to hypothalamic population which is high during early post birth period (Kim *et al.,* 2011; Di Giorgio *et al.,* 2014). These observations suggest that amygdaloid kisspeptin population may arise at maturity. One of the functional studies where kisspeptin antagonist was administered directly has shown the decrease levels of LH release (Lass *et al.,* 2020). Hence amygdaloid kisspeptin neuronal population is not only involved in modulating hypothalamic kisspeptin-GnRH circuitry at adulthood but it might also be involved in deciding pubertal timings and sexual behavior. From above points it can be perceived that neurobiological mechanism underpinning interaction between amygdaloid kisspeptin and NPY neurons to regulate hypothalamic NPY-kisspeptin-GnRH circuitry is an engrossing avenue to be addressed.

Detailed analysis of the genes involved in pubertal development and their epigenetic mechanisms is also required to understand complex mechanism of pubertal process. Mutations in different number of genes are also documented to control timing of pubertal onset. In this regard, apart from GnIH and GABA, makorin ring finger protein 3 (MKRN3) is one of the recently discovered genes implicating pubertal development in rodent and primate species and regarded as central inhibitory factor for HPG axis.

Frame shift loss of function mutation in *MKRN3* gene encoding makorin RING-finger protein 3 have been reported in patients with central precocious puberty (Abreu *et al.,* 2013). Number of studies have documented the reciprocal relation of MKRN3 and precocious pubertal onset in human male and females. For instance, circulating levels of MKRN3 decline both before and during puberty and inversely correlates with increase in luteinizing hormone (LH) and testosterone in boys (Busch *et al.,* 2016; Varimo *et al.,* 2016) while circulating MKRN3 levels are lower in girls with central precocious puberty than healthy controls (Hagen *et al.,* 2015). Importantly, the mutation in MKRN3 occurs at the zinc-finger domain, and zinc-finger protein-mediated transcriptional repression of GnRH neurons considering as the central inhibition has also been reported in pre-pubertal monkeys (Lomniczi *et al.,* 2015). Both sexes of mice showed a decreased in expression of MKRN3 before sexual maturation and this developmental decline of expression is not dependent on gonadal steroids as evident by the similar pattern of expression in gonadectomized mice (Abreu *et al.,* 2015). Intriguingly, in this striking research both the hypothalamic nuclei ARC and AVPV (known as the negative and positive regulatory centers) have been shown to exhibit developmental decrease of MKRN3 mRNA (Abreu *et al.,* 2015). Presence of MKRN3 in ARC and AVPV area in mice encouraged Abreu and his colleagues to observe colocalization between MKRN3 and kisspeptin. As per expectations, they have observed that colocalization between MKRN3 mRNA and kisspeptin. It was observed that repression in transcription of kisspeptin and neurokinin B was due to inhibition of Kiss1 and Tac3 promoter activity by MKRN3 (Abreu *et al.,* 2013). In case of non-human highly evolved primates, female monkeys not older than six months have shown highest mRNA expression of MKRN3 which gradually decline between six to twelve months and reached to a minimum up to thirty months. Taking into consideration about the reciprocal connection between MKRN3 and precocious puberty, the data from female monkeys is quite ambiguous which raises the following questions. First, the ages at which MKRN3 expression observed corresponding to a particular pubertal developmental stage was not clearly defined. For example, highest MKRN3 mRNA expression observed during first six months of life consider as mini puberty period when GnRH release is high while lowest expression observed during 12-30 months corresponds to pre-pubertal period where GnRH secretion is nadir. Considering age and a particular developmental phase

based on GnRH releasing pattern, the role of MKRN3 as central inhibitor is cryptic. Therefore, additional study with age defined developmental groups based on gonadotropins levels is needed to be conducted. Second, it is consistently observed that NPY is the most plausible neurobiological candidate for central inhibition of HPG axis in human and non-human primates. Therefore, how the developmental changes of MKRN3 in regulating hypothalamic-kisspeptin dependent pituitary gonadal axis relate through NPYergic pathway needs to be fully investigated.

Above cited discussion although give a detailed analysis about the neurobiological basis of pubertal development in general and particularly about central restraint, however, the exact nature of the molecular switch which keeps GnRH pulse generator halt at juvenile phase needs to be substantiated.

In summary gene and protein expression analyses demonstrated that suppression of the kisspeptin neurons may be attributed due to enhanced activity of NPY neurons. Increased expression of NPY and NPY1R and decreased expression of kisspeptin and kisspeptin receptor during juvenile/pre-pubertal stage may be interpreted that enhanced NPY signaling may downregulate GnRH release by diminishing KISS1-KISS1R signaling. Developmentally increased expression of NPY1R on kisspeptin neurons is also suggestive of NPY based inhibition of kisspeptin neurons. However, non-exclusive involvement of NPY1R in mediating the putative inhibitory action of NPY on the HPG axis during the juvenile period as evident from the pharmacological data is suggestive to reappraise the role of NPY in occasioning pre-pubertal suppression of GnRH pulse generator in rhesus monkey.

The results of the present data corroborated the notion that suppression of the HPG axis during pre-pubertal stage after transient stimulation in infant stage may be contributed, at least in part, by NPY mediated suppression of kisspeptinergic drive to GnRH neurons. Nevertheless, NPY mediated inhibition of the kisspeptin neurons may involve different receptor subtype other than NPY1R. Moreover, NPY may act in conjunction with some other neurobiological components, and the collaborative message of these may be translated to repress GnRH pulse generator activity before pubertal onset. Nature of this

component that regulate NPY-kisspeptin-GnRH pathway needs to be defined explicitly. Nonetheless, current findings amplify the role of NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in non-human higher primates. These findings hitherto may be helpful to open new avenues in the development of novel clinical, pharmacological, and therapeutic strategies to cater fertility problems in humans.

Based on the findings of the present work a model about NPY regulation of kisspeptin neurons across pubertal development can be posited, which is shown in Figure vii.

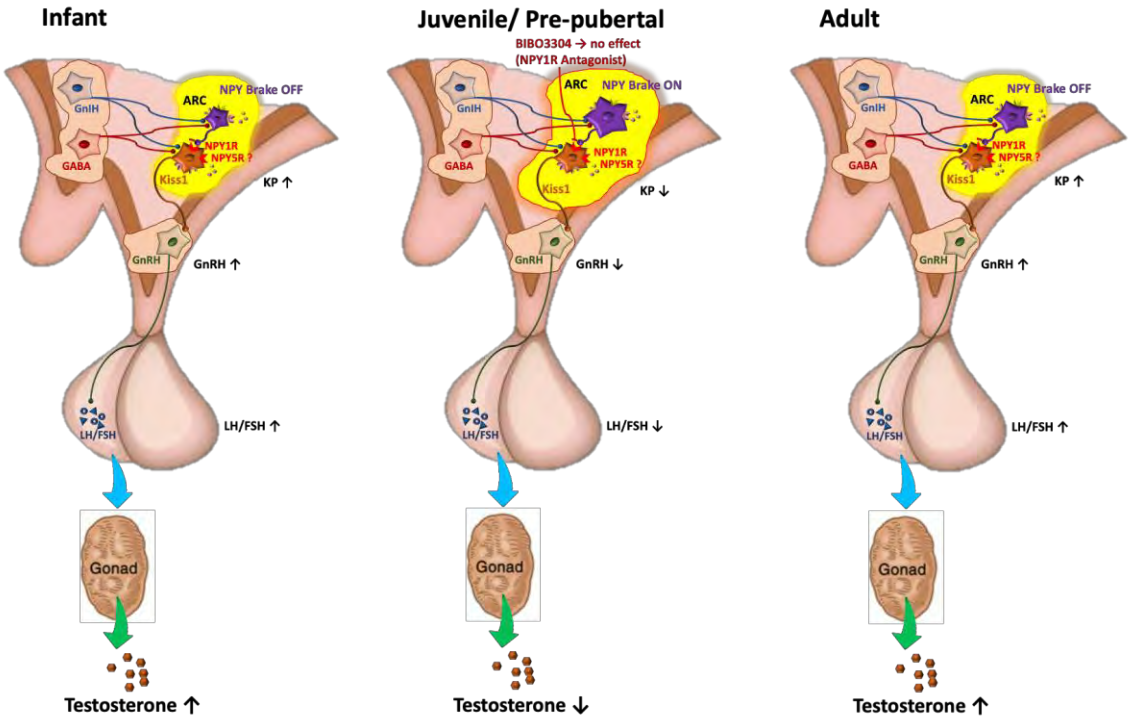

Figure xii. A suggestive model posits NPY regulation of kisspeptin neurons across pubertal development. Both central and peripheral factors may regulate NPY regulation of kisspeptin neurons across pubertal development.

Study of interaction between NPY and kisspeptin neuronal system in the hypothalamus during pubertal development in the male rhesus monkey (*Macaca mulatta*)

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Original article

Inverse age-related changes between hypothalamic NPY and KISS1 gene expression during pubertal initiation in male rhesus monkey

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ARTICLE INFO

Article history:
Received 1 September 2021
Received in revised form 30 November 2021
Accepted 26 December 2021
Available online xxx

Keywords:
Hypothalamus
Kisspeptin
Monkey
Neuropeptide Y
Puberty

ABSTRACT

The neuroendocrine mechanism underlying the sinusoidal wave nature of gonadotropin–releasing hormone pulse generator activity from infantile to adult age still needs to be meticulously defined. Direct inhibition of kisspeptin neurons by neuropeptide Y (NPY) and close intimacy between the two rekindle the importance of these two neuropeptides controlling reproductive axis activity. Thus, the present study was undertaken to decipher simultaneous fluctuations and to profile correlative changes in the relative expression of *KISS1*, *NPY*, and their receptor genes from the mediobasal hypothalamus of infant (n = 3), juvenile, pre-pubertal, and adult (n = 4 in each stage) male rhesus monkey (*Macaca mulatta*) by RT-qPCR. Significant elevation ($p < 0.05$--0.01) in *KISS1* and *KISS1R* and low ($p < 0.05$) expression in *NPY* and *NPY1R* mRNA in the adult group as compared to the pre-pubertal group was observed. Moreover, significantly high ($p < 0.05$) expression of *NPY* and *NPY1R* mRNA with non-significant ($p > 0.05$) decline in *KISS1* and *KISS1R* in pre-pubertal animals in comparison to infants describe inverse correlative age-associated changes during pubertal development. Current findings imply that NPY may contribute as a neurobiological brake for the dormancy of kisspeptin neurons before pubertal onset, while dwindling of this brake is likely to occasion kisspeptin dependent hypothalamic–pituitary–gonadal axis activation at puberty. These findings may help in the development of clinical and therapeutic strategies to regulate fertility in humans.

1. Introduction

Puberty is an intricate process that is essential for the initiation and maintenance of reproductive phenomena in mammals. Maturation of the reproductive neuroendocrine axis is the prerequisite for this fascinating event to set up. The commanding position of this hierarchical axis is bestowed to gonadotropin–releasing hormone (GnRH) neurons releasing decapeptide from the hypothalamus, which directs pituitary gonadotrophs to release gonadotropins, "luteinizing hormone (LH)" and "follicle–stimulating hormone (FSH)". These gonadotropins reach the distant target sites (testes and ovaries) to trigger steroidogenesis and gametogenesis, ensuring the reproductive capability to be achieved [1]. The prologue of the pubertal process is GnRH which is released because of synchronous activity of hypothalamic GnRH neurons controlled by the GnRH pulse generator, which remains in a dormant state during the juvenile period after brief stimulation in the infant stage, thus, opting for on-off-on mode characterizing infant (mini puberty), juvenile (hypogonadotropic state), and pubertal state (adolescent puberty), respectively [2]. Several lines of evidence try to manifest multiple players, both stimulatory [kisspeptin, glutamate, etc.] and inhibitory [neuropeptide Y (NPY), gamma aminobutyric acid (GABA), etc.] to uncover disguise neuroendocrine mechanism controlling pubertal onset [3]. The prime agent involved in the pre-pubertal hiatus of the GnRH pulse generator is still vague [4]; hence the puberty question is included in unsolved enigma [5].

Among stimulatory regulators, kisspeptin has been identified as a potent secretagogue of GnRH secretion in mammals, including rodents, ruminants, and primates, by applying a plethora of experimental strategies, both pharmacological and physiological [6,7]. From the last two decades, the dynamic role of kisspeptin in regulating the reproductive axis has been well defined [1,2,7]. The critical role of kisspeptin and its receptor in pubertal onset has been implicated by observing inactivating mutation in *KISS1*- or

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https://doi.org/10.1016/j.repbio.2021.100589