Study of the hypothalamic GnIH pathways in association with neurobiological mechanisms regulating the initiation of puberty in rodents and primates

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Declaration

I hereby declare that the material contained in this dissertation is my original work. I have not previously presented any part of this work elsewhere for any other degree.

HIRA ZUBAIR

Dedication


Dedicated
To
My Grandparents
For their everlasting affection and prayers
My Father
For supporting and encouraging me to believe in myself
My Mother
My first teacher, who taught me to trust Allah and believe in hard work
My Brother
For always being there for me through thick and thin


بسم الله الرحمن الرحيم

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Finally, as is customary the errors that remain are mine alone.

Hira Zubair

List of Abbreviations

AC	Adenylate cyclase
AGD	Anogenital distance
AH	Anterior hypothalamus
ARC	Arcuate center
AVPV	Anteroventral periventricular nucleus
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxy-ribo nucleic acid
DEPC	Diethyl pyrocarbonate
DMH	Dorsomedial hypothalamus
DMN	Dorsomedial nucleus
DNA	Deoxy-ribo nucleic acid
E	Embryonic day
E_2	Estradiol
ERK	Extracellular signal-regulated kinase
FSH	Follicle stimulating hormone
GnIH	Gonadotropin inhibitory hormone
GnRH	Gonadotropin releasing hormone
GPR54	G-protein coupled receptor 54
HPG-Axis	Hypothalamic-pituitary gonadal axis
HRP	Horse radish peroxidase

IACUC	Institutional Animal Care and Use Committee
iHH	Idiopathic hypogonadotropic hypogonadism
IP_e	Inferior parietal lobule
ir	Immunoreactivity
K_{ir}	Inwardly rectifying potassium channels
Kp	Kisspeptin
LH	Luteinizing hormone
MBH	Mediobasal hypothalamus
ME	Median eminence
mRNA	Messenger ribonucleic acid
NDS	Normal donkey serum
NGS	Normal goat serum
NKB	Neurokinin B
NPAF	Neuropeptide AF
NPFF	Neuropeptide FF
NPFF1R	Neuropeptide FF receptor
NPVF	Neuropeptide VF
NPY	Neuropeptide Y
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PND	Postnatal day
POA	Preoptic area

PVN	Paraventricular nucleus
RFa	RF amide
RFRPs	RF amide related peptides
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse Transcriptase-Polymerase chain reaction
TMB	Tetramethyl benzidine
VMN	Ventromedial nucleus

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GENERAL ABSTRACT

1. General Abstract

1.1. Background

A hike in pulsatile discharge of GnRH is mandatory for puberty onset. However, the mechanisms governing the pubertal rise in GnRH secretion are yet ambiguous. During neonatal life, GnRH neuroendocrine system is active in rodents and primates, but shows subsequent dormancy during the juvenile/prepubertal period. Annihilation of GnRH release through prepubertal age is suggested to be carried out by an inhibitory neuronal system and resumption of pulsatile GnRH release at puberty onset is thought to be due to removal of this inhibition. Recently a new neuronal system, named GnIH secreting neurons, was detected in the brain of quail bird. GnIH, the peptide product of these neurons is an inhibitor of reproductive function, acting within the brain and at the level of the pituitary gonadotrophs. In the last years, a few studies have suspected GnIH as an imperative negative regulator of the reproductive function in mammals including rodents and primates. As a result, the purpose of this study was to investigate the GnIH regulation of reproductive plexus activity in rodents and higher primates at various developmental stages using gene and protein expression analyses. Additionally, interaction of GnIH neurons with GnRH neuronal elements and correlative changes between *GnIH*, *GnRH* and *Kiss1* expression at various stages of pubertal development are also assessed.

1.2. Materials and Methods

In rodent study, sixty-day old male (n=8) and female (n=16) C57BL/6J mice were used. One-night timed breeding protocol was used to collect fetuses and pups at specific embryonic (E15.5, E17.5 and E18.5) and postnatal (PND12, PND18, PND40) days (n=5-9/time point). Whole brains were collected from male and female fetuses and pups and every fourth 30µm thick coronal section was used for dual label fluorescence immunocytochemistry by using primary antibodies

against GnIH and GnRH. Variation in GnIH cell number, its nerve terminals' expression/density in various hypothalamic regions and their interaction with GnRH neuronal system across prenatal and postnatal development was analyzed. In the primate study, POA and MBH containing hypothalamic blocks were collected from fifteen male rhesus macaques (*Macaca mulatta*). Monkeys were categorized into distinct developmental groups i.e., infant, juvenile, prepubertal and adult/pubertal based on somatometric and hormonal parameters. All animals' hemi-hypothalamic blocks were fixed in paraformaldehyde solution for immunofluorescence and liquid nitrogen was used for flash freezing the other half of the hypothalamus which was then kept at -80˚C until RNA isolation. For plasma testosterone measurement, blood was collected before euthanization from every monkey. From each animal, three randomly selected 20µm thick, horizontal, hemi-hypothalamic sections were processed for single label fluorescence immunohistochemistry using specific antibodies directed against GnIH to elucidate the alteration in GnIH neuronal elements' expression in MBH of male rhesus monkeys at various developmental stages. Real time polymerase chain reaction was utilized for quantification of the *GnRH*, *Kiss1* and *GnIH* mRNA during different pubertal stages, using specific primers.

1.3. Results

Our results delineated that the expression of GnIH varied significantly across development in male and female mice and male rhesus monkeys. In mice, DMH expression of GnIH cells showed a significant main effect of age ($P<0.0001$), sex ($P<0.0001$) and their interaction ($P<0.05$) during *in utero* and postnatal growth. In both male and female mice, GnIH cells increased significantly just before term ($P<0.001$) and declined after birth. Again, a significant rise was noted in GnIH cells number at PND18 ($P<0.001$) followed by a precipitous decline ($P<0.01$) at PND40.

Number/density of GnIH immunoreactive nerve terminals expressed in ARC, PVN and in whole brain also increased analogously till PND18 ($P<0.0001$), followed by a precipitous decrease at PND40 ($P<0.0001$). Number of GnIH-GnRH axosomatic contacts increased significantly at PND18 compared to all other ages ($P<0.0001$), falling significantly at PND40 ($P<0.001$). Number of GnIH fibers contacting GnRH fibers also increased significantly at PND18 ($P<0.0001$) compared to other ages, followed by a significant decline at PND40 ($P<0.0001$). Bodyweight, testicular volume, testosterone level and testicular histological parameters of monkeys correlated with the reproductive state of the animal, being significantly higher in adults compared to all other stages. GnIH immunoreactivity in the MBH and ARC region was seen to be significantly higher ($P<0.01$) at prepubertal stage which then declined significantly ($P<0.001$) in the adult animals. Similarly, number of GnIH-ir fibers also increased significantly ($P<0.001$) at the prepubertal stage, declining significantly ($P<0.001$) in adult animals. *GnIH* mRNA levels were statistically ($P<0.01$) higher in monkeys of the prepubertal age whereas *GnRH and Kiss1* mRNA levels were significantly ($P<0.05$) lower at this stage. Inverse situation was noticed at the adult stage, where *GnIH* mRNA level was significantly reduced ($P<0.001$). A significant inverse correlation between *GnIH-GnRH* ($P<0.01$) and *GnIH-Kiss1* ($P<0.001$) mRNA was also noticed.

1.4. Conclusions

In summary, the findings of this research work propose that reproductive axis activity during prenatal and pubertal development in rodents and primates is regulated by GnIH signaling. Present work reinforces our understanding about the inhibitory neuropeptide (GnIH) that might be of assistance in the imposition of the prepubertal brake on the reproductive axis activity. In addition, present study also suggests that fading away of this prepubertal brake might activate the GnRH pulse generation

system, triggering onset of puberty in rodents and primates. Foregoing research envisages to generate developmental maturation of a hypothalamic neuropeptide function with respect to its role on key substrates driving the reproductive system and provides a strong rationale for GnIH in therapeutics as a contraceptive tool and as a remedy for the fertility related disorders in human.

GENERAL INTRODUCTION

2. General Introduction

2.1. Puberty

Puberty can be defined as a complex phenomenon advancing an individual from an immature to a sexually mature phase. It is an advanced multifactorial process comprising of hereditary, socioeconomic, nutritional, and environmental influences to mediate hormonal and psychological phenomena in an organized manner resulting in reproductive maturation (Brook, 1999). It is a crucial stage defined by many hormonal fluctuations within the body after the activation of reproductive axis consequent to accretion in sex steroid levels (Terasawa *et al*., 2001). Though, puberty onset is a diverse phenomenon involving a plethora of developmental transformations, full restoration of the reproductive axis is considered to be its tell-tale sign (Avendano *et al*., 2017) because of the succeeding reawakening of the divergent hypothalamic-pituitary-gonadal (HPG) axis components.

2.2. Hypothalamic-Pituitary-Gonadal Axis

Interaction between three divergent neural signals drives the activity of this neurohormonal system. These signals arise from 1) the hypothalamus, where GnRH is produced by a specific set of neuronal cells; 2) the pituitary gonadotrophs, LH and FSH released by the adenohypophysis; and 3) the gonads, that produce testosterone and estradiol and various peptide hormones like activin and inhibin and carry out gametogenesis (Fink, 2000; Schwartz, 2000, Tena-Sempere and Huhtaniemi, 2003). Under varying physiological and physical conditions, these constitutional entities of the HPG-axis are linked via feedback and feedforward loops for homeostatic purposes. GnRH acts as the last neural signal from the hypothalamus for modulation of the reproductive axis activity. GnRH neural plexus is assertive in the reproductive axis hierarchy, and changes in its neural activity are required for puberty to begin. (Foster, 1994; Plant, 1994).

2.2.1. Gonadotropin Releasing Hormone

GnRH, a 10-amino acid peptide discovered by Matsuo and colleagues (Schally *et al*., 1971), is released from the hypothalamic neuronal terminals directly into the pituitary-portal blood circulation (Conn and Crowley, 1991). GnRH secretion from neuronal elements in hypothalamus conforms to a categorical developmental pattern in higher primates, showing transient activity in the neonates, then inactivates in infants and during childhood (Plant and Witchel, 2006). This quiescence is induced by the reduced pituitary gonadotropins secretion and disruption of the pulsatile GnRH secretion that prevails until the true puberty onset in these species (Plant *et al*., 1988). Then, at the time of pubertal onset GnRH neurons attain the adult level of activity (Ross *et al*., 1983). Hypothalamic GnRH secreting neurons do not develop in the central nervous system (CNS), they migrate to the nervous system during early fetal development along the olfactory placode (Schwanzel-Fukuda *et al*., 1989). GnRH neuronal cells penetrate the nervous system by the olfactory tract and settle in the pre-optic area (POA) and arcuate nucleus (ARC) of the hypothalamus, during early gestation. Erroneous migration of these neurons gives rise to pernicious GnRH release and resultant dysfunctioning of the reproductive system.

For appropriate development and preservation of the reproductive activity, adequate pulsatile GnRH release is imperative (Knobil *et al*., 1980; Belchetz *et al*., 1987). The concept of a GnRH pulse generator was proposed by Knobil, (1980). According to him, it is a network of hypothalamic neurons comprising of excitatory and inhibitory elements, that govern the pulsatile release of GnRH. Data obtained from various animal species have indicated that GnRH release pattern is not guided by the innate activation of the GnRH neuronal cells only. It is proclaimed that other neural innervations of the hypothalamus also have a hand in this activity (Terasawa *et al*., 2010). Dendro-dendritic clustering and synapses of the GnRH neurons with other hypothalamic elements have

been highlighted by many studies (Cambell *et al*., 2009), which are likely to aid in the synchronization of the GnRH secretion. Data collected over the last three decades have confirmed that a dynamic interplay of a plenty of transsynaptic signals governs the GnRH neuronal activity (Herbison, 2006; Ojeda *et al*., 2006). Increasing evidence accumulated over last decades has demonstrated that the kisspeptin, neurokinin B, dynorphin and their receptors referred to as the KNDy neurons, serve as the central mechanism regulating the GnRH pulse generator activity (Uenoyama *et al*., 2021). Since the GnRH pulse generator concept was proposed, its framework and major components have been actively researched; however, full disclosure of its components remains unknown.

The escalation in the neuroendocrine activity of GnRH neuronal elements documented during the pubertal onset (Lomniczi and Ojeda, 2016) is assumed to be a consequence of the shift in the equilibrium between inhibitory and excitatory signals projecting to the GnRH pulse generator. Concomitant rise in stimulatory afferents and timely removal of inhibitory inputs to GnRH cells is believed to be a prerequisite for their complete adult like activity (Lomniczi *et al*., 2015). Several signals in this administrative neuroplexus have been discovered as a result of critical experimental findings. These are, 1) RF-amide family members; kisspeptins, gonadotropin inhibitory hormone (GnIH) and orthologs, 26/43 RFamides (Navarro *et al*., 2006; Smith and Clarke, 2010); 2) metabolic neuropeptides; NPY (Garcia-Galiano *et al*., 2010); and 3) tachykinins such as neurokinin B (NKB) (Lehman *et al*., 2010). Among them, kisspeptin has been regarded as the arbiter of puberty onset (Tena-Sempere, 2005; Pinilla *et al*., 2012; Clarke and Caraty, 2013).

2.2.2. Kisspeptin

Kisspeptins are peptides that are part of the RF-amide group of neuropeptides and are structurally related to each other. These proteins are transcribed from the *Kiss1* gene

(West *et al*., 1998). Kisspeptins were discovered in melanoma cells as metastasis suppressors (Lee *et al*., 1996). In the beginning, kisspeptin gene was named KiSS-1, to honor Hershey Pennsylvania, hometown of the researchers responsible for the discovery of this peptide and the famous Hershey's kisses chocolates. But this nomenclature has been successively abandoned. Globally today, peptide products of the *Kiss1* gene are known as kisspeptins (kp), named by Kotani *et al*., (2001) at first. A numeric extension is added at the end to mention the amino acid chain length. Post-translational modifications of one prepro-kisspeptin gives rise to multiple peptides. Kisspeptin precursor in humans consists of 145 amino acids, with characteristic C-terminal Arg-Phe-NH_2 sequence (Kotani *et al*., 2001). The modification and severance of prepro-kisspeptin results in a 54 amino acid long peptide fragment (Kp-54) that was initially named metastin due to its tumor metastasis suppressing capacity (Ohtaki *et al*., 2001). Kp-14, Kp-13, and Kp-10 are the other peptides produced by prepro-kisspeptin cleavage (Bilban *et al*., 2004).

2.2.2.1. Kisspeptin Receptor and Signaling

All kisspeptin peptide fragments can bind with and switch on GPR54. It is a classic seven pass transmembrane receptor coupled to $G_{q/11}$ protein and brings about its action by augmenting the intracellular Ca^{2+} levels in GnRH cells (Kotani *et al*., 2001). G-protein and phospholipase C are activated because of the binding of the kisspeptin to its receptor, which in turn results in the breakage of phosphatidylinositol bisphosphate (PIP_2) producing diacylglycerol (DAG) and inositol triphosphate (IP_3). Stimulation of non-selective TRPC cation channels causes the cell membrane to depolarize possibly involving DAG and diminution of inwardly rectifying potassium channels (K_{ir}). Activation of calmodulin-dependent protein kinase by virtue of variation in intracellular Ca^{2+} or PIP_2 might govern the functioning of these channels (Colledge, 2009). In 2003, two independent studies reported that genetic inactivation of *GPR54* causes

hypogonadotropic hypogonadism and a lack of pubertal progression in humans and mice, respectively (de Roux *et al*., 2003; Seminara *et al*., 2003). Later, *Kiss1* inactivation also reported analogous outputs (d'Anglemont de Tassigny *et al*., 2007). Since then, compelling genetic and pharmacological evidence has accumulated that testifies the pertinence of the *Kiss1* system in *in-utero* developmental events (e.g., sexual differentiation), but also in the reactivation series of events leading to puberty onset (Pineda *et al*., 2010a; Guerriero *et al*., 2012a; Avendano *et al*., 2017).

2.2.2.2. Kisspeptin Expression

In the year 2001, *Kiss1* mRNA presence in various regions of the human brain was unfolded. Most prominent expression was reported in basal ganglia and hypothalamus (Muir *et al*., 2001; Ohtaki *et al*., 2001). GPR54 expression was observed in various brain regions, including the substantia nigra, basal ganglia, amygdala, hippocampus, and hypothalamus (Muir *et al*., 2001; Ohtaki *et al*., 2001). In mouse brain, kisspeptin presence was noticed in hypothalamic anteroventral periventricular (AVPV) nucleus (Smith *et al*., 2005a; 2005b; Clarkson and Herbison, 2006; Clarkson *et al*., 2009b), dorsomedial nucleus (DMN), arcuate area (ARC), ventromedial nucleus (VMN), caudal ventrolateral medulla and nucleus of the solitary tract (Brailoiu *et al*., 2005). All-inclusive neuroanatomical analyses of the mouse brain have shown that kisspeptin neural elements of the ARC nucleus innervate the POA, raising the possibility of their direct interaction with the GnRH cells (Yeo and Herbison, 2011). In female and male rhesus macaques, *Kiss1* gene is reported to be expressed in the POA and ARC regions (Shahab *et al*., 2005; Trujillo *et al*., 2017).

To decipher the position of kisspeptin cells in GnRH pulse generation system, many efforts have been made. Many anatomical and functional studies in rodents and primate species altering hypothalamic kisspeptinergic signals in a regulated manner during

developmental progression have bolstered such an action of kisspeptin neuronal elements in regulation of GnRH secretion. The use of a specific antagonist to block kisspeptin receptor signaling delayed the pubertal onset in female rats (Pineda *et al*., 2010b) and blockade of kisspeptin receptor in monkeys caused decreased GnRH release in pre/peri-pubertal females (Guerriero *et al*., 2012b). Whereas acute or chronic treatment of female rats with kisspeptins triggered gonadotropin release, a process dependent on GnRH release (Avendano *et al*., 2017) and induced various phenotypic markers of puberty onset (Navarro *et al*., 2004b). Intriguingly, the kisspeptin system is reported to undergo considerable changes during the pubertal transition. A statistically significant rise in *Kiss1* mRNA expression (Shahab *et al*., 2005), kisspeptin positive cell number and their innervation of the GnRH cells (Clarkson and Herbison, 2006; Castellano *et al*., 2006) is documented. During pubertal development, the sensitivity of the GnRH/LH system to kisspeptin's releasing actions is also reported to increase (Castellano *et al*., 2006). All these characteristics maximally activate the kisspeptin neuronal signaling within hypothalamus mastering the reproductive activity through the pubertal progression. Altogether, the current notion is that the onset of puberty is governed not by a specific trigger, but by the dynamic reciprocation of various excitatory and inhibitory elements, that ultimately impinge upon the GnRH pulse generation system. These elements have the capability to squarely transmit the fine-tuning effect of diverse signals regulating the puberty onset.

In search of a neurohormone that negatively regulates gonadotropin production and release, Tsutsui and colleagues identified gonadotropin-inhibitory hormone in the hypothalamus of Japanese quail.

2.3. Gonadotropin Inhibitory Hormone

It is a dodecapeptide (Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH2) that falls in RFamide category of neuropeptides (SIKPSAYLPXRFamide) with characteristic C-terminal Arg-Phe-NH2 motif (Tsutsui *et al*., 2000). In 1977, Price and Greenberg first described the RF-amide neuropeptide family in the bivalve mollusk having cardioexcitatory action. GnIH peptide in mammals is called RFamide related peptide (RFRP) (Tachibana *et al*., 2005) while in various vertebrates it is known as LPXRFamide (Tsutsui *et al*., 2000). In mammals, GnIH orthologous gene was known as RFRP (Iwasa *et al*., 2017). In many vertebrates like mammals, reptiles, birds and amphibians, gonadotropin inhibitory hormone orthologs are reported (Tobari *et al*., 2010; Chartrel *et al*., 2002).

Based on molecular structure, GnIH molecules of mammals are named LPXRFamide peptides as they possess the common LPXRFamide (X=L or Q) sequence at C terminal (Khan and Kauffman, 2012; Hu *et al*., 2018). On the recorded database of DNA sequences, NPVF gene of humans encodes three RF-amide-related peptides, among which RFRP-1 and RFRP-3 are functionally active (Hinuma *et al*., 2000). Among different mammalian species such as Siberian hamster, bovine and humans, hypothalamic RFRP-1 and RFRP-3 were isolated as functional peptides, while in rats and macaques RFRP-3 is the only neuropeptide that is characterized biochemically (Koda *et al*., 2002; Ubuka *et al*., 2018). Although, RFRP-1 has structural homology to GnIH of birds, but several studies have highlighted that RFRP-3, is more active form of GnIH (term used in lieu of RFRP-3 in this document) molecule directing release of gonadotropins in mammalian species (Ancel *et al*., 2012).

Chromosomal location of *GnIH* gene varies among different vertebrate species. In humans, *GnIH* gene is located on chromosome 7, in rhesus monkeys on chromosome 3,

in mouse on chromosome 6 and in rabbits on chromosome 10 (Osugi *et al*., 2014). *GnIH* gene in chicken was found on chromosome 2 (Ikemoto and Park, 2003). In zebra fish it is present on chromosome 19 (Osugi *et al*., 2014). PCR amplification shows the structure of chicken RFRP gene being composed of 3 exons separated by 2 introns. The exons of the gene encode a long polypeptide chain precursor composed of 173 amino acids that further generates twelve amino acids peptide cGnIH, thirty-seven amino acids cGnIH-RP-1 and thirteen amino acids cGnIH RP-3 (Ikemoto and Park, 2003). Amino acids present in human and macaque GnIH share the same C-terminal, but amino acid number is doubled in macaque GnIH than that of human GnIH. The cDNA of GnIH precursor in higher primates consists of 864 base pairs (Ubuka *et al*., 2012). The translated region of cDNA contains only a single unwrap reading structure which consists of 58 nucleotides while 4 to 229 nucleotides are untranslated region having a *5'* to *3'* direction. The open reading frame is approximately 588 nucleotides long and encodes a 196 amino acid long precursor polypeptide. GnIH mature peptide in macaque was isolated by immunoaffinity purification procedure and with the help of mass spectrometry, its arrangement was shown to be SGRNMEVSLVRQVLNLPQRF-NH2 (Fukusumi *et al*., 2001).

2.3.1. GnIH Expression

Samples taken from different parts of the quail brain show that diencephalon was the only part of the brain that expresses gonadotropin inhibitory hormone messenger RNA precursor (Herde *et al*., 2011). With the use of *in situ* hybridization, it was further revealed that within hypothalamic cells GnIH mRNA was expressed in cells clustered in paraventricular nucleus (PVN) (Poling and Kauffman, 2015). Many significant studies using immunohistochemistry technique revealed the expression of bundles of GnIH-ir nerve elements in quail paraventricular nucleus (Tsutsui *et al*., 2000). While GnIH-ir

nerve fibers were found in abundance in the mesencephalic and diencephalic areas in white crowned sparrows and European starlings (Tsutsui *et al*., 2006). Bentley and colleagues. (2006b) further identified the neuronal fibers of GnIH that project from hypothalamic PVN into the median eminence, brain stem and spinal cord. These fibers were found to be limited to the fasciculus longitudinalis medialis (FLM). There were some dispersed neuronal cell bodies in the lateral and medial-septal regions, and the presence of its precursor mRNA was reported in the paraventricular region (Ubuka *et al*., 2012). In addition to that, they also reported dispersed but relatively smaller volume of GnIH immune-positive cells located in posterior region of ventral area of PVN (Ubuka *et al*., 2014). It was also recognized that male quail's mesencephalic and diencephalic regions had higher GnIH expression than females but in other parts of the brain such as cerebellum and cerebrum, expression of GnIH was barely detectable (Ubuka *et al*., 2012).

The dispersion of GnIH neuronal cells has been described in the hypothalamic areas of many mammals including higher primates (Hu *et al*., 2018). Specific brain areas where GnIH expression is reported in various species include, Rat; DMN, ARC and lateral hypothalamus (Yano *et al*., 2004; Kriegsfeld *et al*., 2006; Johnson *et al*., 2007; Legagneux *et al*., 2009; Peragine *et al*., 2017). Hamsters; DMN, AH, and pre-mammillary nucleus (Ubuka *et al*., 2005; Kriegsfeld *et al*., 2006; Gibson *et al*., 2008; Ubuka *et al*., 2012; Henningsen *et al*., 2016), in mice DMN (Ukena and Tsutsui, 2001; Kriegsfeld *et al*., 2006). Sheep; DMN, PVN (Clarke *et al*., 2008; Dardente *et al*., 2008; Clarke *et al*., 2009), in rhesus monkeys PVN, DMN, IPe (Ubuka *et al*., 2009a) and humans DMN (Ubuka *et al*., 2009b).

In the brain of rhesus macaque, innervations of GnIH neuron fibers were exclusively scrutinized. GnIH positive nerve fibers were probed in the PVN, POA, habenular

nucleus, nucleus of the stria terminalis, IPe, ARC area, median eminence and dorsal hypothalamic region, medial superior colliculus, central gray substance, and the parabrachial nucleus in the pons (Ubuka *et al*., 2009a). In several areas of brain such as central gray substance, ARC of hypothalamus, IPe, and POA, GnIH positive nerve fibers were localized narrowly in proximity with neurons of dopamine, pro-opiomelanocortin (POMC), GnRH-I and GnRH-II, respectively (Ubuka *et al*., 2009a). Clarke and colleagues. (2009) have reported that GnIH cells of mammals project to different neural systems such as POMC neurons and neuropeptide Y (NPY) in the region of ARC, lateral hypothalamic neurons of melanin-concentrating hormone and orexin, paraventricular corticotrophin-releasing hormone, and oxytocic cells, and preoptic GnRH cells in sheep. It is postulated that GnIH neurons may synchronize the mentioned neural systems to regulate anterior pituitary gonadotropins secretion (Tsutsui *et al*., 2013).

GnIH hormone expression was also documented in reproductive system of various bird species like songbirds, quail, and chicken. GnIH expression was noticed in tissues of gonads and reproductive accessory organs such as vas deferens, epididymis and oviduct (Tsutsui *et al*., 2007). In different avian species, like Japanese quail, house sparrow, which was caught but fed and European starling, *GnIH* mRNA was also reported in gonads (Tsutsui *et al*., 2010a). Predominantly, a marked GnIH expression was shown in the testicular interstitium. Leydig cells, spermatogonia and spermatocytes of rhesus monkey testis are reported to express gonadotropin inhibitory hormone and its receptor (Tsutsui *et al*., 2006). These two are expressed in the granulosa cells and oocytes of preantral follicles. Thus, GnIH can potentially function as a modulator of steroidogenic and gametogenic cells in female and male macaques (Tsutsui *et al*., 2007).

2.3.2. GnIH Receptor and Signaling

At the beginning of 20th century, just like the finding of RFamides many orphan receptors coupled to G proteins were found as a usual target of their native mammalian RFamides. In one study, researchers cloned two GPCR orthologs for neuropeptide FF (NPFF), naming them NPFF1R (similar to GPR-147) and NPFF2R (similar to GPR-74) (Bonini *et al*., 2000). At the same time, another study reported a specific RFRP receptor, OT7T022, which only responds to artificial hRFRP-1 and hRFRP-3 (Hinuma *et al*., 2000). In a similar study, another receptor called HLWAR77 was discovered to have a higher affinity for neuropeptides AF (NPAF) and NPFF (Laemmle *et al*., 2003). In addition to these studies, Parker and colleagues cloned one more presumptive receptor termed GPR74 (Parker *et al*., 2000). After comparing the functional activity of all these receptors, GPR147 (NPFF1R, OT7T022) was declared to be the putative receptor for mammalian RFRP-3 (Bonini *et al*., 2000).

Receptor for GnIH is extensively dispersed in different regions of nervous system, in particular brain areas like medulla, PVN, septal areas, amygdala, DMN, bed nucleus of stria terminalis (Bentley *et al*., 2008; McGuire and Bentley, 2010). While GPR147 is reported to be distributed throughout hypothalamus in rats and mice by autoradiographic analysis (Jadhao *et al*., 2017). *In situ* hybridization studies revealed that GPR147 is expressed by 25-30% of GnRH positive cells in different rodents. Taken together, above mentioned data strengthen the idea that GnIH may expend its actions on reproductive axis directly via modulation of GnRH neuronal activity (Tsutsui *et al*., 2010b). In fact, in rat GnIH like fibers are seen to be contacting kisspeptin cells and GPR147 was expressed by a subset of kisspeptin cells (Tsutsui, 2009).

For further investigation of the intracellular signaling mechanism liable for the effects of gonadotropin inhibitory hormone and its probable interaction with gonadotropin releasing hormone, Son and colleagues. (2012) employed a rodent gonadotroph cell line, LβT2. Findings of this study helped them establish the fact that GnRH-induced cAMP signaling is effectively blocked by the mouse GnIHs (mRFRPs). This also indicates that mouse RFRP-3 inhibits the activation of adenylate cyclase (AC). The results of this study further indicated that ERK phosphorylation stimulated by the GnRH and the LH/FSH gene transcription is blocked by the mouse RFRP-3 (Figure 1). Mouse GnIH inhibits gonadotropin gene transcription by interfering with AC/AMP/PKA-dependent ERK activation (Son *et al*., 2012). Similarly, an electrophysiological study in adult mice revealed that GnIH had a rapid downregulatory effect on the firing of GnRH neuronal cells (Ducret *et al*., 2009).

Recently, modulation of the reproductive function in various mammalian species by RFRPs in particular RFRP-3, has been investigated. Central administration of RFRP-3/GnIH represses all features of reproductive behavior and significantly decreases plasma LH concentrations in male rats (Tsutsui *et al*., 2006). Chronically administered GnIH to female rats inhibited GnRH neuronal excitation dose dependently, at the LH surge peak. AVPV neuronal elements that provide stimulatory drive to the GnRH neurons, were also found to be suppressed by GnIH administration to rats (Tsutsui *et al*., 2010a). GnIH intravenous administration to ovariectomized rats reduced plasma LH concentration. In ovine and bovine species, gonadotropin release is inhibited by GnIH, mainly at the pituitary gonadotropins level (Tsutsui *et al*., 2006). Further investigations have revealed that mammalian GnIH inhibits gonadotropin synthesis and release in mammals like in birds (Tsutsui, 2009; Tsutsui *et al.*, 2010a; 2013; Kriegsfeld *et al*., 2015; Tsutsui *et al.*, 2016). For instance, avian and hamster GnIH

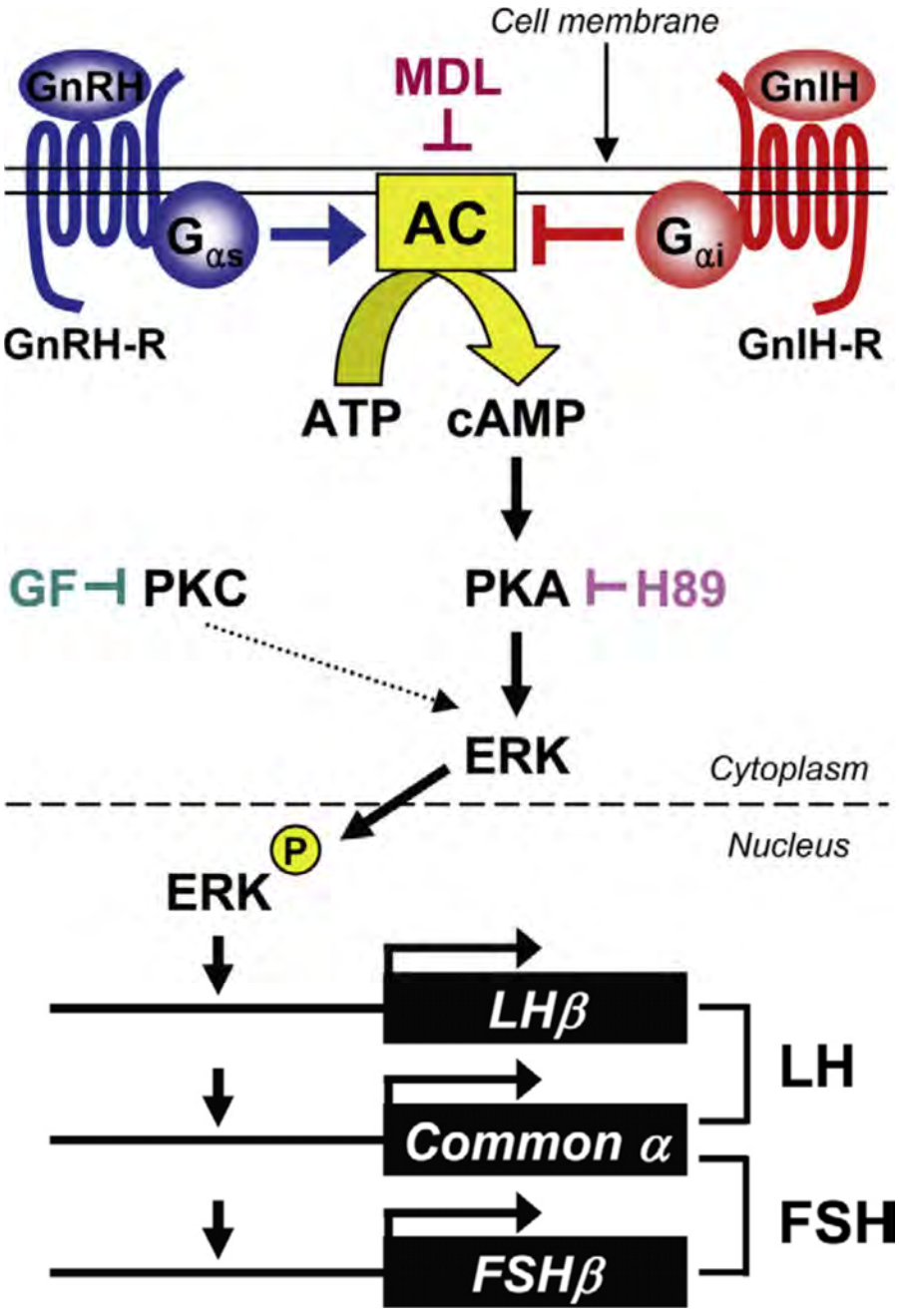


Figure 1: A proposed model demonstrating GnIH's inhibitory effect on GnRH-stimulated gonadotropin synthesis (Ubuka *et al*., 2013).

inhibit luteinizing hormone secretion in Syrian hamsters (Kriegsfeld *et al*., 2006) and Siberian hamsters (Ubuka *et al*., 2012). Likewise, rat GnIH decreases LH secretion (Johnson *et al*., 2007) and LH/FSH secretion (Murakami *et al*., 2008) in rats. Furthermore, mammalian GnIH reduces the amplitude of the LH pulse and interferes with GnRH-induced gonadotropin release in sheep (Clarke *et al*., 2008; Sari *et al*., 2009) and cows (Kadokawa *et al*., 2009). Moreover, human/ovine GnIH reduces GnRH-stimulated gonadotropin release in ovine (Clarke *et al*., 2008; Ubuka *et al*., 2009b). These findings imply that avian and mammalian GnIH reduce pituitary gonadotropin production and secretion, as well as GnRH-induced LH/FSH release.

2.4. Puberty Onset and Components of HPG-axis

From birth to adolescence, different developmental stages have been characterized in mammals including higher primates depending upon somatometric and hormonal variations. Infancy is the duration when the young one obtains the nutriment *via* suckling, extending from birth until 6 months approximately. Juvenile period is the second stage of development, a time frame when the offspring does not rely on parents for survival (Plant *et al*., 1994). In higher primates, gonadotropin release from neonacy to adolescence displays a distinctive pattern with higher FSH and LH secretion in infants and adults, separated by an extended hiatus during juvenile development in the activity of gonadotrophs (Plant *et al*., 1994). But what controls the timing of reactivation of GnRH pulse generation at pubertal onset is still not known. Developmental variations in expression of genes, consequent protein synthesis, nerve cell activation, and release of reproductive hormones are probably the crucial prospects governing puberty onset. In monkeys and humans, GnRH pulsatile release is robust during infantile phase and shows gradual decrease during juvenile period of development (Ojeda *et al*., 2006; Plant and Witchel., 2006; Guerriero *et al*., 2012a).

During infancy, elevated levels of FSH and LH successively end in elevated levels of testicular hormone secretion (inhibin B and testosterone) (Majumdar *et al*., 1997; Winters *et al*., 1999). Pulsatile GnRH delivery to immature female guinea pigs and male and female monkeys induced precocious puberty (Wildt *et al*., 1980; Loose and Terasawa, 1985; Plant, 1988) and higher secretion of GnRH and/or LH/FSH was seen in many mammals (Grumbach and Styne, 1998; Terasawa and Fernandez, 2001; Terasawa and Kurian, 2012). In males, higher GnRH release in pulsatile manner triggers LH/FSH release, that consequently stimulates testosterone release initiating spermatogenesis. Tonicity in LH release is based upon the recurrency in its secretion, conforming to pattern of pulsatile GnRH secretion (Plant, 1986). In females, estradiol (E_2) secretion and folliculogenesis are triggered by the increased GnRH release driving the tonic gonadotropin release. However, capability to produce a huge upsurge in the GnRH secretion consequent to positive feedback of the high estradiol levels is a prerequisite for ovulation to occur in most mammalian species (Plant, 2012).

Similarly, increased *Kiss1* mRNA expression and GPR54 signaling is reported in male and female monkeys during the pubertal progression while reduced kisspeptin neuronal activity is reported during the juvenile phase (Shahab *et al*., 2005; Plant, 2020). At pubertal onset, a significant elevation in the *Kiss1* mRNA levels was noticed in female and male rats (Navarro *et al*., 2004a). In the arcuate region of female rats, there is unremarkable variation in the kisspeptin cell number from postnatal day 3 (P3) to adulthood (Takumi *et al*., 2011) but expression of *Kiss1* mRNA is reported to be four folds higher at P26 (3-4 days before vaginal opening) than that at P21 (Takase *et al*., 2009). While in arcuate of male rats, kisspeptin expressing cell number progressively enlarges across postnatal growth (Takumi *et al*., 2011) and the number of kisspeptin cells is statistically higher at P45 than that at P15 (Bensten *et al*., 2010).

Additionally, in rats, *Kiss1r* mRNA levels also increase significantly at puberty onset (Navarro *et al*., 2004a). More precisely, *Kiss1r* expression in the AVPV of female rats increases significantly at puberty (Takase *et al*., 2009).

As mentioned earlier, in higher primates pulsatile GnRH release is sturdy in the infants but dampens during the juvenile phase causing hypogonadotropism and consequent dormancy of the gonads (Plant and Witchel, 2006; Terasawa and Kurian, 2012). This discontinuity in the pulsatile release of GnRH during juvenile phase might be due to a neurobiological "brake" reducing GnRH secretion till puberty onset (Plant, 1988). Release from this brake reactivates GnRH release robustly, ending the juvenile age of growth (Plant, 1988; Plant and Barker-Gibbs, 2004). Monkeys castrated during neonatal life (Plant, 1985; Pohl *et al*., 1995) and agonadal humans (Ross *et al*., 1983) do not show sufficient pulsatility in the GnRH release. Also, the finding that already lower LH and GnRH secretion in ovariectomized juvenile female monkeys does not decreases further by replacement of ovarian steroids (Chongthammakun and Terasawa, 1993a), hints at the possibility that the paucity in GnRH release in the juvenile primates does not depend on the testicular or ovarian steroids.

It is observed in most species in which GnRH neuronal system has been studied, that its dispersion pattern is established *in utero,* but the morphology, functionality, biosynthetic activity, and synaptic connectivity of GnRH cells is not fully developed until puberty onset (Terasawa and Fernandez, 2001). The number of GnRH neurons having smooth contour are reported to be decreased while the GnRH neurons with wrinkled contour are seen to be increased in rats hitting puberty (Clarkson and Herbison, 2006). Further, mRNA expression of *GnRH* increases steadily with advancing age in mice and rats, but depending upon experimental conditions and sex, a

significant rise is reported to occur at P15–P30 (Clarkson *et al*., 2009a; Dwarki *et al*., 2011; Yeo and Herbison, 2011; Nestor *et al*., 2012).

These findings signify that GnRH expression matures before puberty in rodents and GnRH cells may receive more intensive projections from other neuronal systems. While primate data manifest that GnRH neuronal activity is fully developed before the puberty but no changes in the number (Mayer *et al*., 2010) or the contour of GnRH cells (Rometo *et al*., 2007) or in the expression of *GnRH* mRNA (Smith *et al*., 2010) between juvenile and adult monkeys are reported. It was suggested by Plant. (Guerriero *et al.*, 2012a) that the HP-axis regulating testicular functionality is fully developed in neonates and levels of pituitary gonadotropins decline after 3 months of age in male macaques (Watanabe and Terasawa, 1989; Chongthammakun *et al*., 1993b).

As described before, GnRH cells tend to be centrally inhibited in primates and rodents during prepubertal phase of development, and the onset of puberty is triggered by curtailment of this central inhibition. Foregoing evidence make GnIH signaling attractive as a potential candidate for occasioning a developmental brake on pubertal activation of GnRH neurons in mammals, rodents and higher primates. Variations in GnIH expression are likely to play a critical role in early-life development given the prominent actions of this neurohormone on the reproductive physiology. Consistent with this possibility, developmental changes in the GnIH system have been observed across fish species, including Zebrafish (Zhang *et al*., 2010), Indian major carp (Biswas *et al*., 2015), European bass (Paullada-Salmerón *et al*., 2017), cichlid (Di Yorio *et al*., 2018), and catla (Kumar *et al*., 2020) as well as European green frogs (Pinelli *et al*., 2020) and Japanese quail (Ubuka *et al*., 2003). Whereas previous studies have evaluated developmental changes in *GnIH* gene expression in rats (Yano *et al*.,

2004; Legagneux *et al*., 2009; Iwasa *et al*., 2012) and peripubertal mice (Poling *et al*., 2012). Sex specific developmental and pubertal changes in the GnIH neuronal system have not been investigated with sufficient spatiotemporal detail in rodents and higher primates. The observation that GnIH expression changes broadly across species during development suggests an evolutionarily conserved mechanism of reproductive axis regulation participating in sexual differentiation and the timing of puberty.

Most studies examining changes in GnIH expression in rodents across development have focused on peripubertal postnatal development (Poling and Kauffman, 2015). Furthermore, studies on the development of the mammalian GnIH system have not explored interaction of the GnIH system with GnRH neuronal system. In this dissertation, we present the first comprehensive investigation of the GnIH immunoreactivity distribution in female and male mice (*Mus musculus*) and male rhesus monkeys (*Macaca mulatta*) across development. Communication of GnIH neuronal elements with GnRH cells in the brains of male and female mice from late embryonic developmental stages (during which sexual differentiation occurs) to adulthood and in MBH of male rhesus monkeys from infantile to pubertal stages is delineated here. The present study is carried out to examine the ontogenetic trends in GnIH expression in mammals and lend insight into the potential roles of GnIH in development of the reproductive axis.

2.5. Aims and Objectives

Prime objective of the present study is to describe the patterns of GnIH expression in hypothalamus of mammals across development and to elucidate the interaction of GnIH neurons with GnRH system through pre- and postnatal development in mice and across pubertal development in monkeys using protein and gene expression analyses. As a corollary objective, we have chosen an animal model where these developmental

stages have been studied before, with addition of more stages corresponding to activation of the HPG-axis.

2.5.1. Global Objectives

Through this research work we will understand and generate knowledge on neurobiological mechanisms controlling initiation of puberty and adult fertility. This knowledge will be translational and can lead to better treatment of reproductive disorders and will reduce the disease burden on the population. Therefore, this will be instrumental in ensuring better health.

Secondly, in the long run the present research will provide strong rationale for developing, testing, and manufacturing GnIH based drugs for clinical and preventive (contraception) use.

2.5.2. Specific Objectives

This research work was aimed at studying the ontogenetic trends in development of GnIH neuronal system with spatiotemporal details in rodents and primates. In male and female mice development of GnIH neuronal system is studied from late embryonic stages till pubertal development while in male rhesus monkeys GnIH expression is studied from infantile to adult stages. Presently, there are no data available about GnIH mRNA and peptide expression with sufficient spatiotemporal details. To the best of information we have, till date no research has been conducted to see morphological interaction between GnIH and GnRH expressing neuronal elements during pre- and postnatal development in mice and monkeys. Similarly, correlative changes between *GnIH*, *Kiss1* and *GnRH* genes' expression during pubertal development have not been established in higher primates. This interaction has been assessed by examining GnIH positive fibers and nerve terminals' appositions on GnRH somas and fibers in male and

female mice. While in monkeys, interactions between GnIH, GnRH and kisspeptin systems have been evaluated by probing the corelative alterations in the *GnIH*, *GnRH* and *Kiss1* genes' expression using quantitative real time PCR.

Following experiments were performed to achieve these objectives:

A. Study of developmental alteration in expression of GnIH and its crosstalk with GnRH in mice (*Mus musculus*)

a) Examining the ontogenetic trends in GnIH expression through prenatal and postnatal development

b) Elucidation of the role of GnIH in sexual differentiation in mice

c) Assessing the direct innervations of GnRH cells/fibers by GnIH afferent pathways in female and male mice from late embryonic to early pubertal stages

B. Study of hypothalamic pathways governing pubertal progression in rhesus monkey (*Macaca mulatta*)

a) Determination of variation in the hypothalamic expression of GnIH through postnatal development in male rhesus macaques

b) Establishment of correlative changes in *GnIH-GnRH* and *GnIH-Kiss1* expression across development in male macaques

GENERAL MATERIALS AND METHODS

3. General Materials and Methods

3.1. Animals

Twenty-four wild type, adult, intact C57/BL6 mice and fifteen intact, wild male macaques were used for this study. Developmental expression of GnIH and its interaction with GnRH during pre- and postnatal development was studied in mice at specific time points, corresponding to activation of the HPG-axis. While modulation in GnIH immunoreactivity and its correlation with *GnRH* and *Kiss1* mRNA expression during pubertal development was analyzed in male rhesus macaques.

3.1.1. Mice

Sixty day old wild type male (n=8) and female (n=16) C57/BL6 mice (*Mus musculus*) were purchased from Jackson's Laboratories, CA, USA. Animals were kept under a 12 h light and dark cycle and fed a standard rat diet. Water was available *ad libitum*. One-night timed breeding paradigm was used to collect the fetuses on specific gestational ages. Breeder pairs were set up for one night, and females were examined for presence of vaginal plugs the next day. If one was seen, the day was marked as embryonic day zero (E0.5). The breeder male was removed from the cage and the bred female was housed individually for the remainder of the gestation period. Body weight of the female was tracked for confirmation of pregnancy. If no vaginal plug was seen, then the mice were paired again for one night, and the female was checked for the vaginal plug the next morning. Pregnant mice were kept under standard conditions and were provided with a standard rodent diet for the duration of the pregnancy. For fetus collection, pregnant mice were killed on specific gestational days. For pups' collection, pregnant mice were monitored for the duration of the pregnancy, and pups were collected on specific postnatal days (PND) (day of birth marked as day 1). Both male

and female fetuses and pups were collected at specific ages (n=5-9/age group) that correspond to specific developmental phases i.e., E15.5, E17.5, E18.5 (~ one day before parturition normally occurs), PND12, PND18 and PND40. Litters from at least 3 different females for each specific day were collected to avoid litter effects. All the experimental procedures were allowed by the Institutional Animal Care and Use Committee (IACUC), University of California Berkeley, CA, USA.

3.1.2. Monkeys

Fifteen male rhesus monkeys (*Macaca mulatta*), weighing 0.7-11.6 kg, were used for the present study. These monkeys were trapped from Ayubia National Park, KPK, Pakistan; 33.86˚N, 73.13˚E and the Margalla Hills National Park, Islamabad, Pakistan; 33.73˚N, 72.93˚E. The animals were grouped according to age in four groups; 1: infant (n=3; 4–7 months), 2: juvenile (n=4; 10–14 months), 3: pre-pubertal (n=4; 21–24 months) and 4: adult (n=4; 7–10 years). This characterization was based upon a previous study by Garcia and colleagues. (2018) who used somatometric parameters like body weight, plasma testosterone, testicular volume, and morphology to classify the animal's age. Further, testicular morphometric data (seminiferous tubular diameter and epithelial height) were also used to ascertain a particular developmental stage of the animals, as described earlier (Plant, 2005). The monkeys were kept in separate cages for about 2-3 weeks under semi-ambient conditions, at the Primate Facility of the Department of Zoology, Quaid-i-Azam University, Islamabad before euthanization. They were given peanuts, fresh fruits (0900-0930 h), boiled eggs (1100 h), and bread (1300-1330 h) every day, as well as unlimited access to water. All experimental protocols were carried out with the Departmental Ethical Committee for Care and Use of Animals, Quaid I Azam University, Islamabad's prior approval. These animals were

used to study GnIH developmental expression and its relationship with mRNA expression of *GnRH* and *Kiss1* during different phases of postnatal life.

3.2. Tissue Collection

Pregnant mice were dissected on specific day of gestation i.e., E15.5, E17.5 or E18.5. Internal gonads and anogenital distance (AGD) were examined for the sexing of the fetus under a dissecting microscope. All procedures were performed during the daytime to maintain uniformity of the study conditions. Pregnant females were anesthetized with euthanasia solution (Euthasol, Med-Pharmex, Pomona, CA, USA NDC# 54925-045-10) and were given transcardial perfusion of ice cold 0.1M PBS and of 4% paraformaldehyde (PFA) in 0.1M PBS. Their uterine horns were removed, and the collected fetuses were quickly decapitated. Whole heads were postfixed in 4% PFA at 4°C, overnight. The same procedure was performed for postnatal brains collection, where individual pups received 4% PFA transcardially. Their brains were extracted from their skulls and post-fixed in 4% PFA overnight.

For monkeys' brains collection, ketamine hydrochloride (Ketamax, Rotex Medica, Trittau, Germany; 10-20 mg/kg BW, im) was used to deeply sedate the monkeys. All surgical equipment was autoclaved before use. All monkeys were dissected in daytime (0010h-0014h) to maintain uniformity in the conditions for the study. For hormonal measurement a single blood sample (approx. 2.5 ml) was collected from saphenous vein of each animal before euthanizing it. Blood sample was collected using a syringe and was quickly transferred into cold EDTA coated vacutainer. Samples were centrifuged for 15 minutes at 3000 rpm using Kokusan-H-103RS refrigerated centrifuge machine (Tokyo, Japan). The separated plasma was kept at -20°C for testosterone quantification. Their body weight and testicular volume were also noted.

To ascertain the reproductive status of the animals, tissue from one testicle of each animal was collected to examine its morphology.

All the hairs on the monkey's head were pruned while he was sedated, and the skin was scrubbed with 70% ethanol. The skin of the head and underlying muscle was then ripped off with a scalpel. The cranium was then cut in a circle from the top of the skull using a bone cutter. When the cranium was completely open, the animal was injected intravenously with a high dose of ketamine hydrochloride for euthanization. The brain was removed carefully and immediately placed over a chilled plate of glass keeping the ventral side upwards and was washed with normal saline.

Then, the hypothalamic block was excised out. Following landmarks were used to take hypothalamic blocks as already described in literature (Shibata *et al*., 2007). Briefly, keeping median eminence in the center, mammillary bodies towards the posterior side and optic chiasm at the anterior side, a coronal cut was made next to the mamillary bodies and rostral to the anterior commissure. Likewise, two parasagittal cuts were made on each side of the midline. The optic chiasm's caudal boundary was then cut, and a square block of hypothalamus was collected. Lastly, this hypothalamic block was cut into two equal halves by making a cut at the midline and normal saline was used to wash each hemi-hypothalamic block. Liquid nitrogen was used for flash freezing of one of the hemi-hypothalamic blocks from each animal and later was kept at -80°C for qPCR analyses while other block was processed for immunohistochemistry protocol.

All animals' testicular tissues were also collected for histological analysis. Following the cleaning of the testis, a sharp scalpel was used to make a first cut into the skin over the testis, followed by a second cut within the fascia and a third within the peritoneum. The spermatic cord was tied at that point, and both testes were removed. Testicular tissue was obtained from the central portion of the testicle after the tunica albuginea

was removed. After washing with normal saline solution, the tissue was immediately transferred to sera for hematoxylin and eosin staining.

3.3. Tissue Fixation and Processing

Paraformaldehyde (Mice: Electron Microscopy Sciences, Hatfield, PA, USA; Cat# 19202. Monkey: Merck Darmstadt, Germany; Cat# 818715) in phosphate buffer saline (PBS) was used for hypothalamus' fixation. Brains of mice and hemi-hypothalami of monkeys were kept in 4% PFA solution at 4°C overnight. Then, all the tissues were cryoprotected via sequential treatment with sucrose solution. First, tissues were shifted to 10% sucrose solution (Mice: Fischer Chemical, Ottawa, Ontario, Canada; Cat # L-12686. Monkey: Fischer Chemical, Leicestershire, UK). After sinking to bottom, tissues were shifted to 20% sucrose until sunken again. Then, tissues were moved to 30% sucrose solution. Monkey hypothalami were treated with 20% and 30% sucrose solutions only.

Testicular tissues of monkeys were fixed in sera. Sera was prepared by mixing 10% of glacial acetic acid (Merck, Darmstadt, Germany), 30% formaldehyde (Merck, Darmstadt, Germany) and 60% of absolute alcohol (Sigma-Aldrich Chemie GmbH, Steinem, Germany). Ascending grades of alcohol (70%, 80%, 90%, 100%; 1 hour each) were used to dehydrate the tissues. The tissues were then placed in cedar wood oil to become clear. For 1 hour each, two xylene washes were given to all the tissues. Then the tissues were impregnated in liquid paraffin wax. The wax was liquefied at 62°C so that tissues were fixed and embedded in it.

3.4. Plasma Testosterone Measurement

A commercially available human testosterone ELISA kit (Astra Biotech GmBH, Luckenwalde, Germany) was employed for the measurement of plasma testosterone

levels of all the monkeys. The protocol supplied with the kit was followed for this purpose. The assay's sensitivity was 0.05 ng/ml, and the intra- and inter assay coefficients of variation were less than 10% and 9%, respectively.

3.4.1. Principle of the Assay

This was a competitive assay, with testosterone in the samples competing with testosterone conjugated with horseradish peroxidase (testosterone-HRP) for a limited number of antibodies fixed on micro titer plate wells. In the bound fraction, the HRP enzyme reacts with the tetramethylbenzidine (TMB) substrate. When the stop solution is added, the reaction produces a blue color that turns yellow. The concentration of testosterone in the sample correlates negatively with the color intensity and is measured using a calibration curve.

3.4.2. Assay Procedure

This assay was carried out in a 96-well plate. Each well was designated as a sample, standard, or quality control well. Initially, 10µl of standards, samples, and quality controls were poured into the designated wells. Following that, each well received 100µl of testosterone-HRP conjugate and 50µl of rabbit anti testosterone reagent. Plate was then incubated for 90 min at 37ºC in an incubator. After incubation, the reaction mixture was removed from the plate and the plate was washed with autoclaved distilled water, three times. The plate was then incubated at room temperature (18-25°C) for 20 minutes with 100µl TMB substrate added to each well. To stop the reaction, 100µl of stop solution was added to each well. For 30 seconds, the microtiter plate was thoroughly mixed. Within 15-20 minutes of the entire blue color turning yellow, the absorbance was calculated at 450 nm on a microtiter plate reader (Model 680XR, BIO RAD, Tokyo, Japan).

3.4.3. Result Calculation

The concentration of testosterone in the samples was determined using a standard curve created by plotting absorbance on the y-axis and testosterone concentration on the x-axis. The plasma testosterone levels in the samples were determined using the Bio Rad microplate manager/PC software's point-to-point method (Version 5.2.1; Bio-Rad Laboratories, Hercules, CA, USA).

3.5. Measurement of Testicular Volume

The volume of both the right and left testis was calculated using the formula $V=(\pi w^2 l)/6$, where 'w' represents the width, 'l' represents the length of each testicle, and V is the volume in milliliters (Steiner and Bremner, 1981). The total volume of the testes was calculated by adding the volumes of the right and left testis.

3.6. Tissue Sectioning

Serial sectioning of mice brains on cryostat yielded thirty µm thick coronal sections. On a cryostat, hemi-hypothalamic blocks from monkeys were cut horizontally at a thickness of 20µm (Bright OTF 5000, A-M Systems, Sequim, Washington, USA). Sections were stored at -20°C in an anti-freeze solution (30% ethylene glycol, 1% polyvinylpyrrolidone, and 30% sucrose). Testicular tissues were sectioned at a thickness of 20µm on a vibrating microtome (Thermo Electron Corporation, Cheshire, UK) and fixed onto glass slides for histological staining.

3.7. Fluorescence Immunohistochemistry

Every fourth coronal section of mice brains, already fixed on glass slides, was double labelled for simultaneous visualization of GnIH and GnRH cells and fibers. First, sections were washed 6 times with 0.1M PBS for 5 (6x5) minutes each. The sections were then incubated at room temperature for 10 minutes with 30% H_2O_2 (Fisher

Chemicals, Fair Lawn, NJ, USA; Cat # S-15828) to block endogenous peroxidase, after which they were washed three times with PBS. At room temperature, sections were blocked with normal goat serum (NGS; 1:50, diluted in phosphate buffer saline+1% TritonX-100 (PBS-T)). After that, the sections were washed with PBS-T for 6x5 minutes. The sections were then placed in a humidified chamber for 48 hours in a primary antibody-containing solution (rabbit anti-white crown sparrow; PAC 123,124 antigen sequence SIKPFSNLPLRF, gift of Dr. George Bentley, University of California, Berkeley, CA, USA, used at dilution 1:5000 in PBS-T with 1% NGS). After that, the sections were washed with PBS-T for 6x5 minutes.

The sections were then incubated for one hour in biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA, USA; Cat # BA-1000, diluted 1:300 in PBS-T), followed by three 5-minute PBS-T washes. The sections were then incubated with ABC (diluted 1:500 in PBS-T) for 3x5 minutes before being rinsed with PBS-T. The sections were then incubated for 30 minutes with biotinylated tyramide (diluted to 0.6% in PBS with a 1:1000 H_2O_2 concentration). After washing with PBS for 3x5 minutes, sections were incubated for one hour at room temperature in the dark with secondary antibody (Cy-3 conjugated streptavidin, Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA; Cat# 016-160-084, used at dilution 1:500 in PBS-T). After washing with PBS for 3x5 minutes, sections were blocked for one hour at room temperature in the dark with normal donkey serum (NDS, diluted 1:50 in PBS-T). The sections were then incubated for 48 hours at 4°C in a humidified chamber with a monoclonal primary antibody for GnRH (QED Bioscience Inc, San Diego, CA, USA, Cat# 19304, used at dilution 1:2500 in PBS-T with 1% NDS). The sections were then rinsed with PBS-T six times for five minutes each. The sections were then incubated for one hour at room temperature in the dark with secondary antibody (Cy-2

anti-mouse, Jackson Immunoresearch Laboratories Inc, Cat# 715-225-150, used at dilution 1:200 in PBS-T), followed by three washes. The sections were then dried overnight at room temperature before being coverslipped the next day. Slides containing sections were passed through a series of alcohols (dH2O, 70%, 95%, 100%, 100% ethanol) for two minutes each, followed by three two-minute washes in xylenes. The slide was then covered with a small amount of mounting medium (Krystalon mounting medium, EMD Millipore Corp, Darmstadt, Germany, Cat# 64969-71) and a cover slip (Microscope cover glass, Fisher Scientific, Pittsburgh, PA, USA, Cat# 12545M). The slides were then kept horizontal in the dark for nearly a week before being examined under the microscope.

The GnIH immunoreactivity on free floating 20µm thick monkey hypothalamic sections was examined using a standard single label IHC protocol. Three sections of each monkey hypothalamus containing the mediobasal hypothalamus were randomly selected and processed for immunofluorescence, while one section of each animal's hypothalamus served as the primary antibody omitted control. First, the sections were rinsed with PBS for 8x15 minutes to remove the anti-freeze solution completely. To inhibit nonspecific binding, the sections were slowly shaken at room temperature for one hour in a blocking solution containing 10% NGS, 0.05% TritonX-100, and 0.05% bovine serum albumin (BSA) in PBS. The sections were then washed three times for 15 minutes with PBS. The sections were then incubated for 48 hours at 4°C on shaker with primary antibody against GnIH (rabbit anti-white crown sparrow; PAC 123,124 antigen sequence SIKPFSNLPLRF, gift of Dr. George Bentley, University of California, Berkeley, CA, USA, used at dilution 1:5000 in PBS with 10% NGS, 0.05% TritonX-100, 0.05% BSA). The sections were then washed 3 times for 15 minutes with PBS. After that, the sections were incubated for two hours at room temperature in the

dark, on shaker, with secondary antibody (Cy-3 goat anti-rabbit, Jackson Immonoresearch Laboratories Inc, Cat# 111-165-003, used at dilution 1:200 in PBS containing 10% NGS, 0.05% TritonX-100, 0.05% BSA), followed by three 15-minute washes.

Then the sections were mounted on the glass slides (CrystalCruzR, Santa Cruz Biotechnology Inc, Dallas, TX, USA, Cat # Sc-363562) and were kept at 4°C overnight in dark for drying. Next day slides were cover slipped (Microscope Cover Glass, MAS GmbH, Leonberg, Germany, 24x50mm) using mounting medium (Immu-Mount, Thermo Shandon Limited, Cheshire, UK, Cat# 238402) and were kept at 4°C for complete drying, after which they were viewed under a microscope. Primary and secondary antibodies used for immunofluorescence in mice and monkey hypothalamus are mentioned in Table 1.

3.8. Testicular Morphology

For delineation of the variation in morphological features across ages, sections from each animal's testis were stained with hematoxylin and eosin.

3.8.1. Hematoxylin and Eosin (H&E) Staining

Sections were deparaffinized by washing them twice with xylol for five minutes each. The sections were rehydrated using descending alcohol grades (100%, 90%, and 70% for one minute each). The sections were then stained with hematoxylin for 5 minutes before being washed with freshwater for 2 minutes. The sections were then dipped in 1% acid alcohol 2-3 times. The sections were then stained with eosin for 2 minutes after a 2-minute tap water wash, followed by another 2-minute tap water wash. Finally, sections were dehydrated and given two 1-minute xylol washes. After that, the sections were coverslipped.

Table 1: List of antibodies used for immunofluorescence

Peptide	Host	Catalogue No.	Company	Dilution Used
GnRH	Mouse	19304	QED Bioscience Inc, San Diego, CA, USA	1:2500
GnIH	White Crown Sparrow	PAC 123,124	Gift from Dr. George Bentley. University of California, Berkeley, CA, USA	1:5000
Biotinylated Goat anti Rabbit	Goat	BA-1000	Vector Laboratories, Burlingame, CA, USA	1:300
Cy-3 Conjugated Streptavidin	-	016-160-084	Jackson Immonoresearch Laboratories Inc, West Grove, PA, USA	1:500
Cy-2 anti-mouse	Donkey	715-225-150	Jackson Immonoresearch Laboratories Inc, West Grove, PA, USA	1:200
Cy-3 anti-rabbit	Goat	111-165-003	Jackson Immonoresearch Laboratories Inc, West Grove, PA, USA	1:200

3.9. Microscopy

The Axioimager A1 slide scanner was used to examine GnIH and GnRH immunoreactive cells in mouse brains (Zeiss, Jena, Germany). The standard wavelength for Cy2 (488nm) and Cy3 (568nm) was used to examine all sections. Every fourth section from the medial septum to the caudal aspect of the anterior hypothalamus was manually counted for GnIH-ir cells and presumptive nerve terminals. The arcuate nucleus, paraventricular nucleus, dorsomedial hypothalamic area, and ventral hypothalamus were specifically studied. GnIH-ir terminal fiber axosomatic contacts onto GnRH soma were recorded. Only a GnIH-ir bouton-like structure close to a GnRH cell body was deemed a contact (with both the bouton and cell body being in the same plane of focus). These points of contact were then confirmed by viewing under a confocal microscope (Zeiss, LSM 710).

GnIH immunoreactivity in monkey hypothalamus was captured by using fluorescent microscope (Olympus BX51, Tokyo, Japan). GnIH immunoreactivity was visualized using the standard wavelength for Cy3 (568nm). GnIH-ir nerve terminals and fibers were manually counted in four random sections containing the MBH area. Photographs were taken at different magnifications using the digital camera linked directly to the microscope. The whole MBH area was scanned and mean±SEM immunoreactivity was calculated.

A light microscope (Olympus CX51) was employed to study the testicular histological features of monkeys of different ages. Testicular epithelial height and seminiferous tubular diameter were measured by using a micrometer. Photomicrographs were taken at different magnifications by a digital camera directly linked with the microscope.

Image J (https://imagej.nih.gov/ij/) was also used to measure the density of GnIH fibers in various regions, such as the lateral septum, PVN, POA and arcuate nucleus. In a nutshell, digital images were converted to 8-bit format, and regions of interest (ROI) were drawn in areas with higher GnIH fiber density. To estimate the background signal, one ROI (with the same dimensions) was drawn in any other region (with no GnIH immunoreactivity), which was then subtracted from the density measured in that specific region.

3.10. Real Time-quantitative PCR

Real-time quantitative PCR (RT-qPCR) was used to determine the relative fold change in expression of *GnIH*, *GnRH*, and *Kiss1* from hemi-hypothalamic tissues of male rhesus monkeys from four different age groups.

3.10.1. RNA Extraction

Wizol™ Reagent (Wizbiosolutions, Seongnam, South Korea) was used to extract total RNA from the hemi-hypothalamic block of each animal, according to manufacturer's instructions. Briefly, 1 ml of Wizol™ Reagent was added to 50 ~ 100mg of tissue sample and grinded in a pestle and mortar by using liquid nitrogen for homogenization. The homogenate was collected and transferred to RNase free tube. Then, 200µl of chloroform was added to each tube, shaken vigorously for 15 seconds by hand, and incubated at room temperature for 3 minutes. The homogenate was then centrifuged at 12,000g for about 15 minutes at 4°C in an Eppendorf Centrifuge 5415R (Hamburg, Germany). The aqueous phase was then isolated and 500µl of isopropanol was added. It was then incubated at room temperature for 10 minutes before being centrifuged at 12,000g for 10 minutes at 4°C. Later, the pellet was collected, washed with 75% ethanol, and briefly vortexed. It was then centrifuged at 7500g for 5 minutes at 4°C,

and the pellet obtained was dried to remove all traces of ethanol. The obtained RNA was then resuspended in DEPC water and was stored at -80°C.

3.10.2. RNA Quantification

After RNA extraction and purification, the quality of RNA was determined by using nanodrop. Briefly, 1µl of RNase free water was used as blank and 1µl of each RNA sample was put on the center of the nanodrop machine (Colibri Spectrophotometer, Berthold Detection System GmbH 75173 Pforzheim, Germany). The OD and the absorbance ratio of A260/280, A260/230 respectively were recorded.

3.10.3. Complimentary DNA (cDNA) Synthesis

After RNA isolation and quality assessment, WizScript^{TM} First Strand cDNA Synthesis kit (Wizbiosolutions) was used for the synthesis of cDNA, from 1µg of total purified RNA in a 20µl reaction. Total RNA was reverse transcribed in a thermocycler using random hexamer primers and the manufacturer's protocol (T100 Bio-Rad Thermocycler, Hercules, CA, USA). For cDNA synthesis, the kit's components were thawed, mixed, and briefly centrifuged. The reagents were then placed on ice in sterile, nuclease-free tubes. In brief, the RNA/primer mixture was incubated for 5 minutes at 65°C before being incubated at 37°C for 60 minutes after the buffer/enzyme components were added. The reaction was then stopped by incubating it for 10 minutes at 70°C. The cDNA prepared was kept at -20°C until analysis.

3.10.4. Primer Designing for RT-qPCR

For present study, specific primers of GnIH were designed by using NCBI (National Centre for Biotechnology Information) site at https://www.ncbi.nlm.nih.gov and In-Silico PCR at https://genome.ucsc.edu/cgi-bin/hgPcr as primers for given gene (*GnIH*) in rhesus monkeys were not reported in previous literature. However, specific primers

of *GnRH*, *Kiss1* and *GAPDH* were already reported (Wahab *et al*., 2019; Bano *et al*., 2022). Primer details are mentioned in Table 2. After the specific nucleotide sequence was analyzed, primer parameters were filled as per primer requirements at NCBI. After designing primers, Tm of both forward and reverse primers was adjusted at UCSC Genome Browser In-Silico PCR. Different properties of primers including GC content, GC clamp, self-annealing and hairpin formation were accessed at Sequence manipulation site; PCR primer stats (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). Primers were synthesized by the Macrogen Company (Seoul, South Korea).

3.10.5. Real-time qPCR

Wizpure^{TM} qPCR Master (SYBR) kit was used for RT-qPCR (Wizbiosolutions). A 10µl reaction was run in duplicate for each gene, with 5 µl SYBR Green, 0.45 µl of primer set, 2.5 µl of cDNA (1:4 dilution), and RNase free water to bring the total volume to 10 µl. Table 2 shows the sequence of all primers. The efficiency of primers was tested in reactions with 3 serial 1:5 dilutions of cDNA as a template to form a calibration curve. 100 µM of the primers were diluted to 1:10 working dilutions. For efficiency of primers 20µl reaction mixture was taken into the PCR tubes (0.1ml 8-tube strips) containing 10µl SYBR green, 5µl pooled cDNA, 0.9µl of forward primer, 0.9µl of reverse primer and 3.2µl RNase free water. For relative mRNA expression *GAPDH* was used as housekeeping gene for normalization. To amplify desired targets in 45 cycles, the following reaction conditions were used: pre-denaturation temperature of 95°C for 180s, denaturation temperature of 95°C for 10s, annealing temperature of 60°C for 15s, and elongation temperature of 72°C for 20s. The endogenous housekeeping gene GAPDH was used to normalize each gene in each sample. Cycle

Table 2: Sequence, accession number and product length of the primers used for RT-qPCR

Gene	Accession No.	Primer sequence (5′ to 3′)	Product length
GnRH	S- 75918	Rev: TTTCCAGAGCTCCTTTCAGG For: AGATGCCGAAAATTTGATGG	134
Kiss1	XM-028852143.1	Rev: TGACTCCTCTGGGGTCTGAA For: GGACCTGCCGAACTACAACT	141
GnIH	NM-001033115.2	Rev: ATTGGCACATGGTGAATGC For: CCTCGTGAGACGGGTTCTTA	118
GAPDH	NM-001195426.1	Rev: TTGATGACGAGCTTCCCGTT For: TGTTGCCATCAATGACCCCT	119

threshold (Ct) values were calculated using CFX Maestro software version 2.3 (Biorad, Hercules, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative fold change in mRNA expression of the target genes (Livak and Schmittgen., 2001; Thayil *et al*., 2020), with the infant group serving as a calibrator.

3.11. Statistical Analyses

Data analysis was performed using GraphPad Prism Version 8 (GraphPad Software Inc, La Jolla, CA, USA), and data are expressed as means±standard error of the mean (SEM). To compare the number of GnIH cells in the DMH of mice of different developmental ages and GnIH-ir in various hypothalamic regions of mice across development, one-way ANOVA was used, followed by Tukey's multiple comparison post hoc tests. Same analysis was used to compare body weights, plasma testosterone levels, testicular morphological parameters, GnIH-ir in hypothalamus and *GnIH* expression relative to *GnRH* and *Kiss1* at different developmental stages in rhesus monkeys.

The number of GnIH cells in the DMH of male and female mice was compared using multiple t-tests and two-way ANOVA. GnIH-GnRH contacts in mice were assessed using non-parametric one-way ANOVAs. When $P \leq 0.05$, the results were considered statistically significant.

CHAPTER A

Study of developmental variation in GnIH expression and its crosstalk with GnRH in mice (*Mus musculus*)

4.1. Abstract

4.1.1. Background

GnIH signalling in the hypothalamus has been reported to negatively correlate with the hypothalamic pituitary gonadal axis activity in many species. Recent evidence hints at involvement of ontogenetic variation in GnIH expression in the sexual differentiation and activity of the reproductive axis during prenatal and postnatal development in varied species.

4.1.2. Objectives

Present study concentrated on analysing the expression of GnIH neuronal elements and the interplay of GnIH neurons with hypothalamic gonadotropic releasing hormone neurons in the female and male mice brain at various time points across development.

4.1.3. Materials and Methods

Sixty-day old male (n=8) and female (n=16) mice (C57/bl6) were timed-bred to collect male and female fetuses and pups at various time points across prenatal and postnatal development (n=5-9/time point). Whole brains were collected from fetuses and pups and processed for immunofluorescence. Every fourth coronal section was double labelled for GnIH and GnRH using specific antibodies. The number of GnIH-immunoreactive cells, fibers and number of GnIH-GnRH contacts were manually counted. Image J was used to measure the GnIH fiber density in various hypothalamic nuclei. One-way and two-way analyses of variance were employed to compare the expression of GnIH neuronal elements across development and between sexes.

4.1.4. Results

The number of GnIH positive cells was seen to significantly vary with age ($P<0.0001$) and sex ($P<0.0001$) with females exhibiting higher number throughout development. Highest expression of GnIH positive neuronal bodies in the dorsomedial

hypothalamus and GnIH-immunoreactive nerve terminals and fibers in both sexes was noted at post-natal day (PND) 18 which then declined significantly at PND40 ($P<0.01$). The number of GnIH-GnRH axosomatic and fiber to fiber contacts were also found to be the highest at the PND18 stage, decreasing significantly at PND40.

4.1.5. Conclusion

Based on *in utero* and postnatal variation in GnIH expression in the hypothalamus of mice, we suggest that increased GnIH signalling near term and at PND18 correlates with the reproductive axis' suppression at these time points and activation of the GnRH pulse generator system in adult animals is occasioned by a reduction in the GnIH expression and signaling, leading to onset of puberty in this rodent species. We also suggest that GnIH and GnRH function in concordance, with divergent outcomes, to regulate reproductive axis activity during pubertal development.

4.2. Introduction

The reproductive axis awakens in two stages before puberty, the first during *in utero* development and the next during early postnatal life (Kuiri-Hänninen *et al*., 2014). Following early postnatal life, the reproductive axis is quieted for the juvenile period by pathways that remain elusive (Kuiri-Hänninen *et al*., 2014). Initiation of puberty and adult reproduction depends on a dynamic interaction between hormones of the hypothalamus, pituitary, and gonads (Sherwood, 1994). GnRH, a hypothalamic decapeptide (pEHWSYGLRPGamide), is a well-established master regulator of reproduction (Foster, 1994; Sherwood, 1994). Increased pulsatile GnRH secretion is crucial for the release of LH and FSH from the pituitary, both of which are required for gonadal maturation and ultimately, puberty (Terasawa and Fernandez, 2001). Several neuronal substrates including gamma amino butyric acid (GABA), melatonin, neuropeptide Y (NPY), endogenous opioids, kisspeptin, RFamide peptides and glutamate regulate GnRH secretion prior to puberty (Terasawa *et al*., 2010), but when and how these neuronal circuitries modulate during pubertal progression to modulate GnRH secretion is poorly understood (Tena-Sempere, 2012, Terasawa *et al*., 2013).

Although pituitary hormone release is regulated by many specific agonists and antagonistic neuropeptides, until recently, endogenous neuropeptide antagonizing the synthesis and release of pituitary gonadotropins was not identified. Some gonadal hormones, like testosterone, estradiol, and inhibin, are capable of modulating the gonadotropin release, yet none of these has the strong neuroendocrine inhibitory consequence (Bentley *et al*., 2003). In the year 2000, a neuropeptide was discovered with a potential of directly inhibiting the pituitary gonadotropin synthesis and release (Tsutsui *et al*., 2000). This provided a new insight into the modulation of LH/FSH release and helped us understand the mechanisms governing the timing of

activation/reactivation of the reproductive axis.

This neuropeptide named GnIH was identified in the brain of Japanese quail (*Coturnix japonica*) by Tsutsui and colleagues in the year 2000 (Tsutsui *et al*., 2000) and is a part of the RFamide peptide family, (a group of peptides possessing the characteristic terminal arginine-phenyl-alanine motif; Arg-Phe-NH_2). The discovery of a cardioexcitatory neuropeptide in the bi-valve mollusk, *Macrocallista nimbosa* with the C-terminal Phe–Met–Arg–Phe–NH2 (FMRFamide) booted this family (Price and Greenberg, 1977). These proteins regulate multiple physiological systems like pain perception, food intake and the endocrine system (Tsutsui, 2009; Tsutsui *et al*., 2010a; 2013).

Based on molecular structure, GnIH molecules of mammals are termed as LPXRFamide peptides because of the common LPXRF (X=L or Q) at C-terminal (Hu *et al*., 2018). In several mammalian species including humans, RFamide related peptide-1 (RFRP-1) and RFRP-3 are present as mature peptides, but in rodents RFRP-3 (mammalian orthologue of GnIH) is the only peptide that is characterized biochemically (Koda *et al*., 2002; Ubuka *et al*., 2018). Ancel *et al*. (2012) have reported that RFRP-3 a dodecapeptide, (SIKPSAYLPLRFamide) is the most potent bioactive form of this neuropeptide.

Following the discovery of GnIH, a gene database search found cDNAs encoding GnIH orthologs, LPXRF amide peptides, in mammals (Hinuma *et al*., 2000). Human, macaque, and bovine LPXRF amide precursor DNAs encode three RFamide related peptides (RFRP-1, 2 and 3). (Bentley *et al*., 2010). As seen in birds, the mammalian homologs of GnIH, RFRP-1 and RFRP-3, inhibit gonadotropin release and synthesis (Tsutsui *et al*., 2010a). GnIH cells are found in the hypothalamic dorsomedial nucleus (DMN) of rodents, with fibers projecting broadly to midline hypothalamic loci (Ukena

and Tsutsui, 2001, Kriegsfeld *et al*., 2006). Similarly, GnIH cells project from the DMH to GnRH cells and the median eminence region (Bentley *et al*., 2003; 2006a; 2006b), with GnIH application inhibiting GnRH neuronal activity and gonadotropin release across species (Bentley *et al*., 2006a; 2006b) (Tsutsui *et al*., 2000; Bentley *et al*., 2006a; 2006b).

Variations in GnIH expression are likely to be a critical part of the early-life development. However, sex specific developmental and pubertal changes in the GnIH neuronal system have not been investigated with sufficient spatiotemporal detail in mice. Expression of *GnIH* gene during *in utero* development has been reported earlier in Japanese quail (Ubuka *et al*., 2003) and rats (Yano *et al*., 2004; Legagneux *et al*., 2009) and peripubertal development in mice (Poling *et al*., 2012). Likewise, the expression pattern of GnIH neurons during development in various fish species, including Zebrafish (Zhang *et al*., 2010), Indian major carp (Biswas *et al*., 2015), European bass (Paullada-Salmerón *et al*., 2017), cichlid (Di Yorio *et al*., 2018), and catla (Kumar *et al*., 2020) has been reported. Additionally, immunohistochemical findings of the expression pattern of GnIH during embryonic development were described in European green frog (Pinelli *et al*., 2020). The observation that GnIH expression changes broadly across species during development suggests an evolutionarily conserved mechanism of reproductive axis regulation participating in sexual differentiation and the timing of puberty.

Most of the studies examining changes in GnIH across development have focused on peripubertal postnatal development (Poling and Kauffman, 2015). Furthermore, studies on the development of the mammalian GnIH system have not explored interactions between the GnIH and GnRH neuronal systems. Therefore, this study was aimed at detailed investigation of the GnIH immunoreactivity distribution and its

communication with GnRH neurons in the brains of male and female mice from late embryonic developmental stages during which sexual differentiation occurs to adulthood to examine ontogenetic trends and lend insight into the potential roles of GnIH in development of the HPG-axis.

We hypothesized that transient inactivation of the reproductive axis near term and during prepubertal period is due to an increase in GnIH tone and its contacts onto GnRH neuronal elements. GnIH signaling and GnIH-GnRH crosstalk were tracked by dual label immunofluorescence. If this is the case, one would predict that GnIH tone would be higher at the late embryonic stage and during prepubertal period of postnatal development and there would be higher number of contacts from GnIH neuronal elements onto GnRH soma. Similarly, it can also be predicted that fading away of this tone at pubertal stage and lesser number of axosomatic contacts between GnIH and GnRH cause reactivation of the HPG-axis leading to steroidogenesis and gametogenesis. In addition to age, the objective was to find out the effect of sex on the GnIH cell expression in the DMH.

4.3. Materials and Methods

4.3.1. Animals

Sixty day old male (n=8) and female (n=16) C57/BL6 mice (*Mus musculus*) were housed in ventilated cages with a 12 h light/dark cycle and free access to food and water. Female mice were paired with an age-matched male and were checked for a vaginal plug every morning. The morning when a vaginal plug was discovered was designated as embryonic day zero (E0.5). At the start of pregnancy, the breeder male was removed from the cage and females were housed individually. For fetal collection, pregnant mice were sacrificed on E15.5, 17.5, and 18.5. Tissues from postnatal animals were collected at post-natal day (PND) 12, 18 and 40. Both male and female animals (n=5-9/time point) were collected at each time point (Figure A1.).

4.3.1.1. Sexing of Fetuses

Sexing of the collected fetuses was done by comparing their anogenital distance (AGD) under a dissecting microscope, where AGD in male fetuses was found to be higher than females (Figure A2.). Additionally, fetuses were dissected under a microscope to see their internal gonads, for further confirmation of the sex of the fetus.

4.3.2. Immunofluorescence

4.3.2.1. Tissue Collection

Pregnant females were deeply anesthetized with 0.5cc euthanasia solution (Euthasol, Med-Pharmex, Pomona, CA, USA NDC# 54925-045-10) and were perfused transcardially with chilled 0.1M PBS followed by 4% paraformaldehyde (PFA). Uterine horns were removed, and entire fetal heads were postfixed in 4% PFA at 4°C, overnight. Individual pups were perfused with 4% PFA for postnatal brain collection, and brains were excised from the cranium and post-fixed in 4% PFA overnight; all

brains were then cryoprotected via sequential treatment with increasing concentrations of 10, 20, and 30% sucrose in 0.1M PBS.

4.3.2.2. Fluorescence Immunohistochemistry

Every fourth coronal section was double labelled for simultaneous visualization of GnIH and GnRH using a rabbit anti-, white-crowned sparrow GnIH antibody (PAC123,124, antigen sequence SIKPFSNLPLRF, generous gift of Prof. George Bentley, used at dilution 1:5000) and a mouse anti-GnRH antibody (QED Bioscience Inc. Cat# 19304, used at dilution 1:2500). Immunostaining was done on a series of 30µm thick sections that were fixed onto slides. After rinsing, the sections were incubated in 0.3% H_2O_2. After rinsing, the sections were blocked with 2% normal goat serum, followed by incubation in a solution containing the GnIH antibody for 48 hours in a humidified chamber at 4°C. After incubation in the primary antibody, the sections were washed and incubated for 1 hour at room temperature in a biotinylated secondary antibody (biotinylated goat anti-rabbit 1:300, Vector Laboratories, CA, USA. Cat#BA-100). Sections were then washed in PBS-T before being placed in ABC (1:500, Vector Laboratories) and amplified for 30 minutes with biotinylated tyramide. After rinsing, sections were incubated in Cy3-streptavidin (1:500 in PBS-T, Jackson Immunoresearch Laboratories Inc. PA, USA) for 1 hour at room temperature. Cat# 016-160-084). After blocking with 2% normal donkey serum for 1 hour, the sections were incubated in a primary antibody against GnRH for 48 hours at 4°C. Slides were rinsed with PBS-T before being incubated in a secondary antibody solution (Cy2-anti mouse, 1:200, Jackson Immunoresearch laboratories Inc. Cat# 715-225-150). Finally, slides were cover slipped using antifading medium (Krystalon mounting medium, EMD Millipore Corporation, Darmstadt, Germany. Cat# 64969-71) and stored at 4°C in the dark

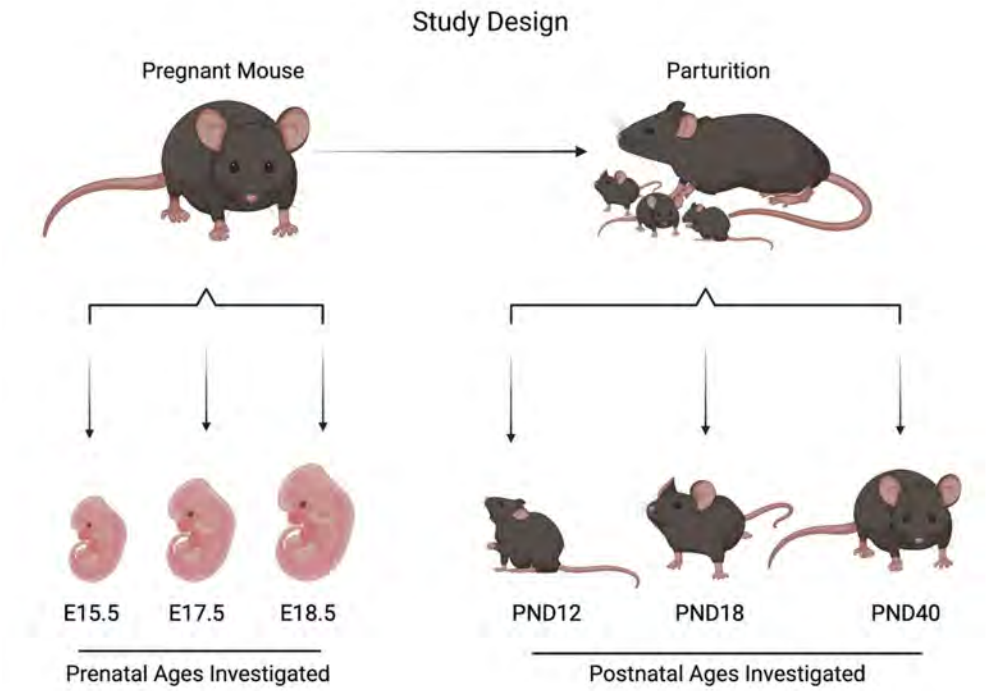


Figure A1. Brain sampling timeline of mice across prenatal and postnatal development

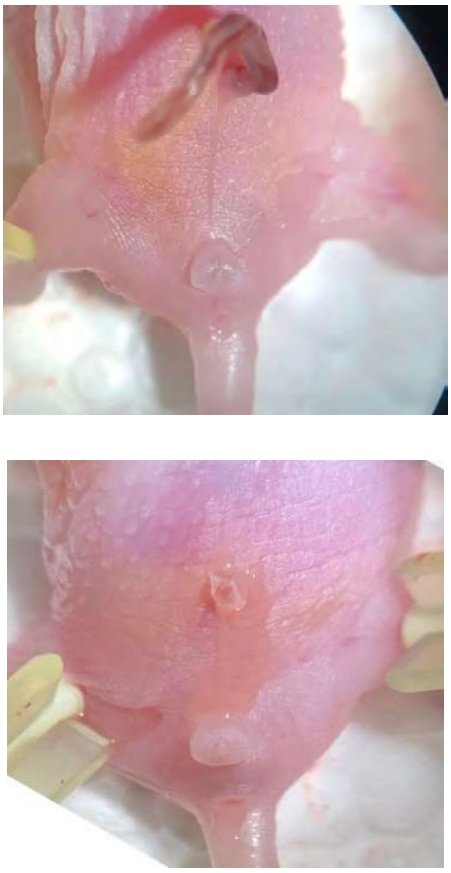


Figure A2. Comparison of the anogenital distance (AGD) in female (top) and male (bottom) mice under a dissecting microscope.

4.3.2.3. Fluorescence Microscopy

GnIH and GnRH immunoreactive cells and their contacts were counted in whole brains using an Axioimager A1 slide scanner (Zeiss, Jena, Germany). Specifically, the arcuate nucleus, paraventricular nucleus, dorsomedial hypothalamic area, and ventral hypothalamus were investigated. GnIH-ir nerve terminals in the ARC and PVN were counted two ways; manually and using image J. Presumed GnIH like projections onto GnRH soma were recorded. The presence of a putative GnIH-ir bouton like structure near a GnRH cell was mandatory for a contact to be scored, later confirmed by confocal microscopy.

4.3.3. Statistical Analyses

Data analysis was performed using GraphPad Prism Version 8 (GraphPad Software Inc., GraphPad software, CA, USA), and data are expressed as means±standard error of the mean (SEM). To examine findings when collapsed over sex across embryonic and postnatal days, one-way analyses of variance (ANOVA) were used, followed by Tukey's multiple comparison post hoc tests. GnIH cells in the DMH of female and male mice were compared using multiple t-tests and two-way ANOVA. GnIH-GnRH contacts were evaluated using non-parametric one-way ANOVAs. When $P \leq 0.05$, the results were considered statistically significant.

4.4. Results

4.4.1. GnIH Cell Numbers and Developmental Pattern of Expression

Individual and mean number of GnIH-ir cells in male and female mice across development are given in Table A1. GnIH-ir cells were localized to the DMH across prenatal (Figure A3.) and postnatal (Figure A4.) development in both male and female mice. There was a significant main effect of age ($F_{5,28}$=54.85, P<0.0001) and sex ($F_{1,28}$=106.4, P<0.0001) and a significant age x sex interaction ($F_{5,28}$=3.083, P<0.05) for GnIH-ir cell counts. GnIH-ir cells were found to be significantly higher in females than in males throughout development (Figure A5.).

In males, a significant reduction in GnIH-ir cell numbers was seen at E17.5 relative to E15.5 (P<0.01; Figure A6.), rising significantly at E18.5 relative to E17.5 (P<0.001). During male postnatal development, GnIH cell numbers at PND12 and PND18 were comparable to values at E18.5 (P>0.05 in each case), with a precipitous reduction at PND40 relative to all other developmental timepoints (P<0.01 in all cases).

In females, GnIH immunoreactivity at E17.5 was comparable to cell numbers at E15.5 (P>0.05; Figure A7.). As seen in males, a significant elevation in the number of GnIH cells was seen at E18.5 relative to E17.5 (P<0.05), remaining. During female postnatal development, PND12 and PND18 GnIH cell numbers remained at E18.5 values, falling below all other groups at PND40 as seen in males (P<0.01 in all cases).

When collapsed by sex, a similar pattern was observed ($F_{5,34}$=9.5, P<0.0001; Figure A8.). Where, GnIH-ir cell number was comparable between E15.5 and E17.5 (P>0.05) while a significant elevation in the cell count was seen at E18.5 (P<0.05). After birth, GnIH cell counts remained at preterm values, declining significantly at PND40

(P<0.01).

4.4.2. RFRP-3 Fiber Projections and Developmental Pattern of Expression

GnIH-ir nerve terminal boutons and fibres extended broadly to the medial septum/diagonal band of Broca (dBB), pre-optic area (POA), anterior hypothalamus, and PVN at all developmental ages. No sex differences were seen in GnIH-ir nerve terminals ($F_{1,28}$=0.7910, P>0.05) and data were collapsed over sex for analysis and presentation.

Individual and mean number of GnIH-ir nerve terminals manually counted in the arcuate region of male and female mice brain are given in Table A2. In the ARC, minimal GnIH-ir terminals and fibres were seen during prenatal stages of development, while considerable variation in the number of GnIH-ir nerve terminals was seen across postnatal development ($F_{5,34}$=29.74, P<0.0001). More specifically, a significant increase in presumptive ARC GnIH-ir nerve terminal boutons occurred on PND18 relative to PND12 (P<0.001), with a significant reduction at PND40 compared to PND18 (P<0.001 relative to all other developmental time points) (Figure A9.). Individual and mean fiber density measured in the ARC is presented in Table A3. Very low GnIH fiber density was seen in the ARC of male and female mice during prenatal development, while postnatal stages showed a substantial variation in the density measures ($F_{5,34}$=22.21, P<0.0001). More precisely, there was a gradual increase in the fiber density measured in the ARC of male and female mice till PND 18 (P<0.001), followed by a significant reduction at PND40 (P<0.0001) (Figure A10.).

Individual and mean number of GnIH-ir manually counted nerve terminals present in the paraventricular nucleus of male and female mice are given in Table A4. There was a substantial variation in the number of GnIH-ir nerve terminals observed in PVN in males and females across development ($F_{5,34}$=52.90, P<0.0001), with the number of terminals increasing from E17.5 to E18.5 (P<0.05) and declining significantly after

birth (PND12) ($P<0.01$). As in the ARC, at PND18, a sharp increase was seen ($P<0.0001$ relative to all other time points) followed by a significant reduction at PND40 ($P<0.0001$; Figure A11.). Individual and mean fiber density measured in the PVN is presented in Table 1.5. GnIH fiber density was found to vary considerably in the PVN of male and female mice across development ($F_{5,34}=19.46$, $P<0.0001$). Specifically, there was a significant increase noted in the fiber density at E18.5 compared to E15.5 and E17.5 ($P<0.001$) followed by a significant decline at PND12 ($P<0.05$). Then, a second significant rise in the fiber density in PVN was seen at PND18 ($P<0.0001$), declining significantly at PND40 ($P<0.0001$; Figure A12.).

Individual and mean GnIH fiber density measured in the POA is given in Table A6. GnIH fiber density in the POA varied significantly across ages ($F_{5,34}=25.13$, $P<0.0001$). There was a significant increase seen at PND18 compared to PND12 ($P<0.0001$), followed by a significant decline at PND 40 ($P<0.0001$; Figure A13.). Fiber density measured in the lateral septum is presented in Table A7. Mean fiber density in the lateral septum varied significantly through development ($F_{5,34}=9.44$, $P<0.0001$). Precisely, there was a significant increase in the fiber density at PND18 compared to PND12 ($P<0.001$), followed by a significant decline at PND40 ($P<0.01$; Figure A14.).

Individual and mean number of GnIH-ir nerve terminals manually counted in brain of male and female mice are given in Table A8. Presumptive GnIH-ir nerve terminal boutons observed across the brain (medial septum/(dBB), POA, anterior hypothalamus, ARC and PVN) in males and females varied significantly across developmental stages ($F_{5,34}=41.35$, $P<0.0001$). The total number of GnIH-ir nerve terminals increased significantly at E18.5 relative to E15.5 ($P<0.05$) and declined significantly after birth ($P<0.05$). Fiber terminals then increased significantly relative

to all ages at PND18 ($P<0.0001$), declining significantly at PND40 ($P<0.0001$; Figure A15.).

4.4.3. GnIH-GnRH Crosstalk

Very few contacts were observed between GnIH-ir nerve terminals and GnRH neuronal somas (Table A9.) and between GnIH-ir and GnRH-ir fibers across developmental stages (Table A10.). No sex differences ($F_{1,28}=1.087$, $P>0.05$) were observed in the number of GnIH-GnRH axosomatic contacts or GnIH-ir fibres contacting GnRH-ir fibres ($F_{1,28}=0.1243$, $P>0.05$) and data were collapsed over sex for analysis and presentation.

Figure A16. contains representative confocal images of the arcuate and the preoptic areas, showing GnIH- and GnRH-like immunoreactivity. Figure A17. shows a representative contact between GnIH-ir terminal and GnRH-ir soma while Figure A19. presents a representative fiber to fiber contact. The number of GnIH fibre terminals contacting GnRH somata ($F_{5,34}=18.92$, $P<0.0001$) and fibre to fibre ($F_{5,34}=292.7$, $P<0.0001$) contacts differed across developmental stages when collapsed by sex. A small number of GnIH-GnRH axosomatic contacts were seen early postnatally from E15.5 to PND12. A significant rise in GnIH contacts onto GnRH-ir cell bodies was observed at PND18 ($P<0.0001$ relative to all earlier developmental stages) falling significantly at PND40 ($P<0.001$, Figure A18.**)**. Contacts between GnIH-ir fibres and GnRH-ir fibres were analogously low until PND12, rising significantly at PND18 relative to all earlier developmental stages ($P<0.0001$ in all cases), and falling significantly at PND40 ($P<0.0001$) compared to PND18 ($P<0.0001$; Figure A20.).

Table A1. Individual and mean number of GnIH immunoreactive cells observed in DMH of male and female mice of different developmental ages

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	212	126	239	162	169	38
	192	115	233	150	213	40
	146	114	222		197	52
	213	79	203			
		122	150			
mean±SEM	**191±15.70**	**111±8.35**	**209±16.10**	**156±6.00**	**193±12.90**	**43.3±4.37**
Female	355	208	332	254	285	84
	297	208	340	225	272	83
	311	308	361	210		61
			287			
mean±SEM	**321±17.50**	**241±33.30**	**330±15.60**	**230±12.90**	**279±6.50**	**76±7.51**
mean±SEM (Collapsed by Sex)	**247±28.40**	**160±26.70**	**264±23.50**	**200±19.50**	**227±22.20**	**59.70±8.27**

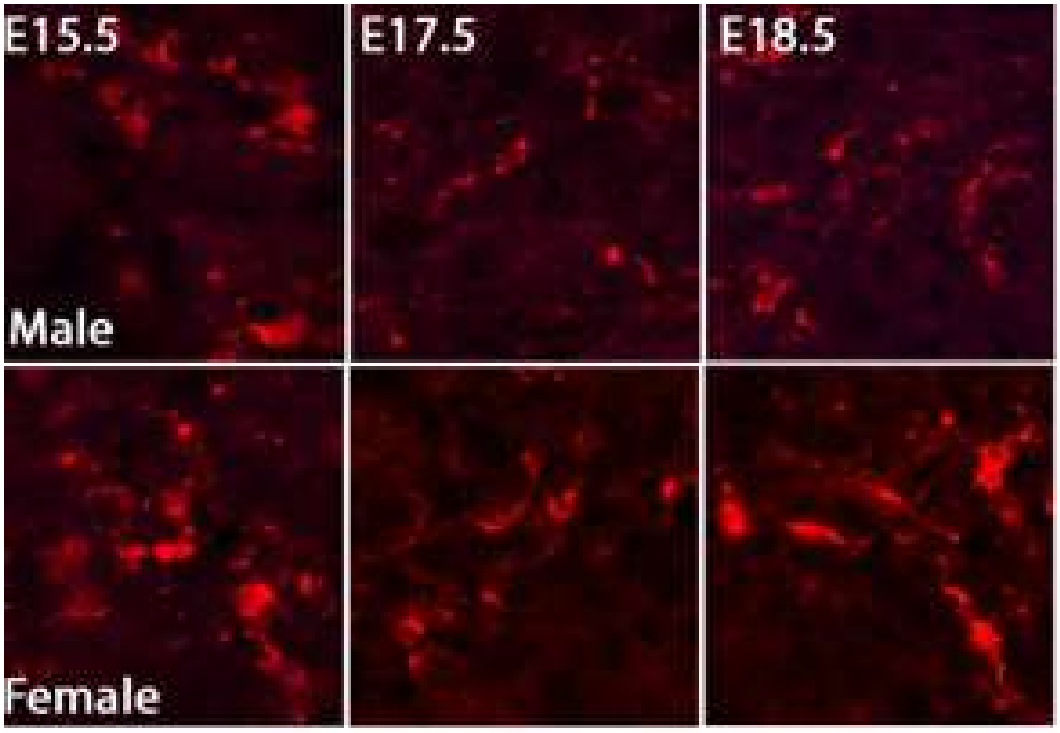


Figure A3. Representative photomicrographs showing GnIH-ir cells in DMH of male (top) and female (bottom) mice across prenatal development. Number of GnIH-ir cells were found to be significantly higher in females than in males at all *in utero* developmental stages.

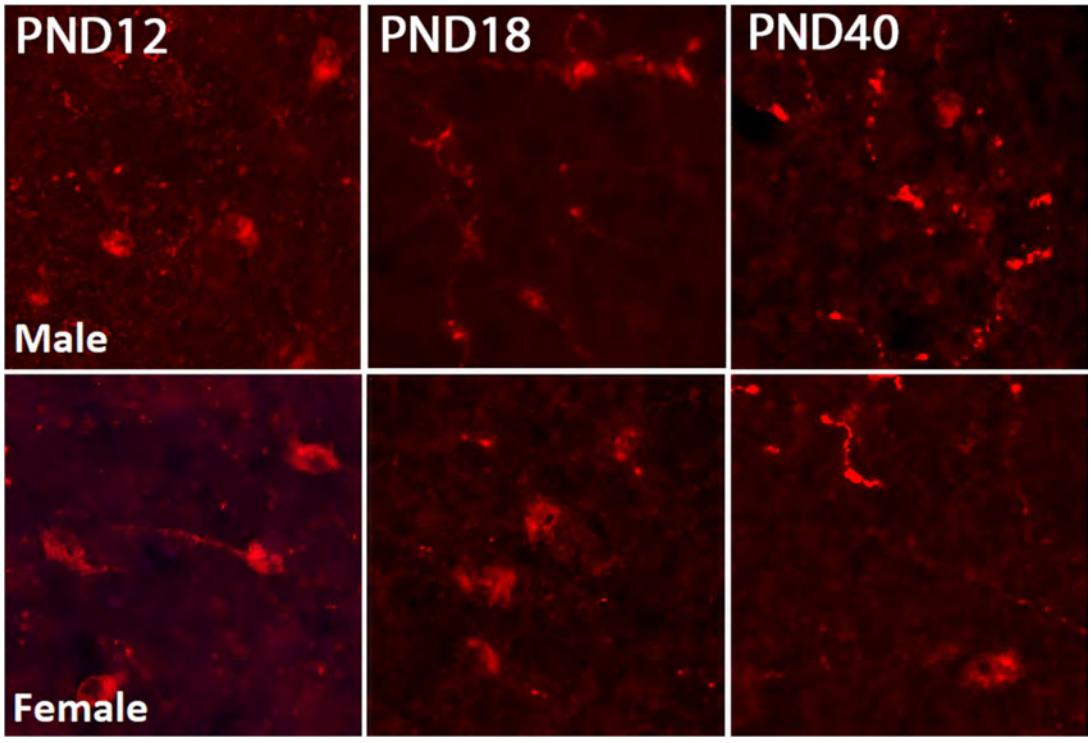


Figure A4. Representative photomicrographs highlighting GnIH-ir cells in DMH of male (top) and female (bottom) mice through postnatal development. Females had significantly higher number of cells throughout postnatal development.

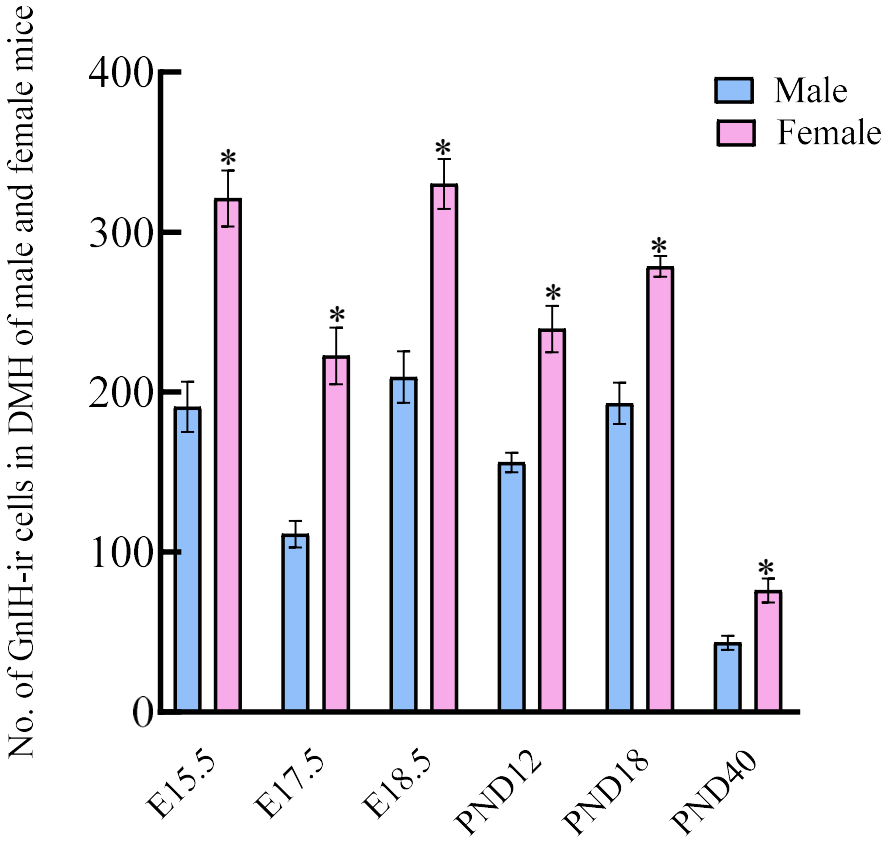


Figure A5. mean±SEM number of GnIH-ir cells in DMH of male and female mice across prenatal and postnatal development. Number of GnIH-ir cells were found to be significantly higher in females than in males at all developmental stages through multiple t-tests. *=P<0.05; (E, embryonic day; PND, Postnatal day)

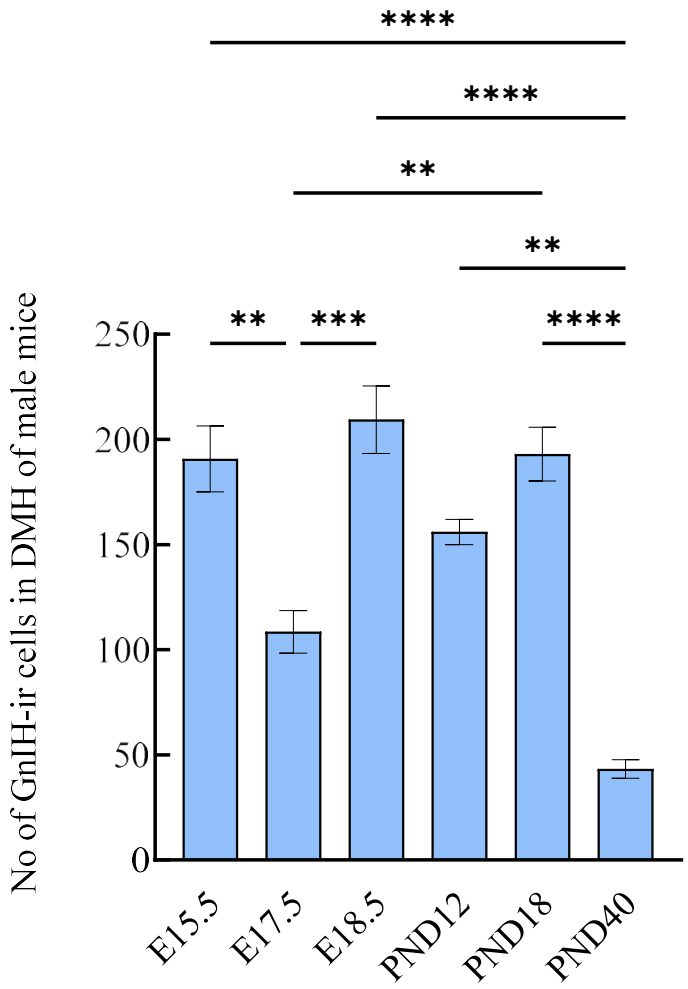


Figure A6. mean±SEM number of GnIH-ir cells in DMH of male mice of different developmental stages. The number of cells were significantly higher at E18.5 than E17.5, which then declined significantly at PND40. **=P<0.01, ***=P<0.001, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

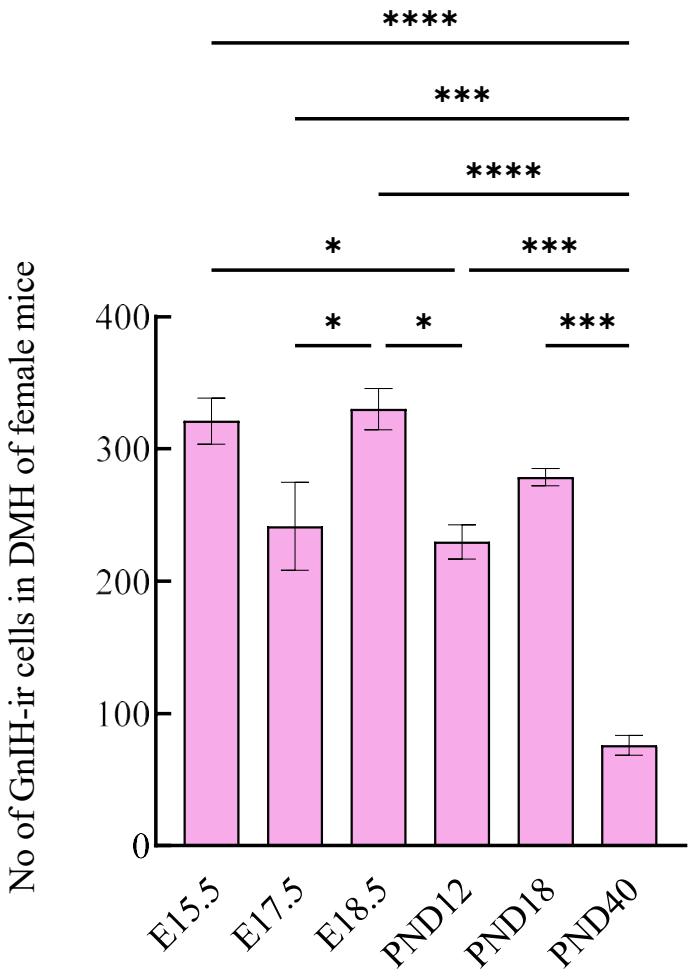


Figure A7. mean±SEM number of GnIH-ir cells in DMH of female mice through development. The number of GnIH-ir cells was significantly higher at E18.5 and PND18, which then declined significantly at PND40. *=P<0.05, ***=P<0.001, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

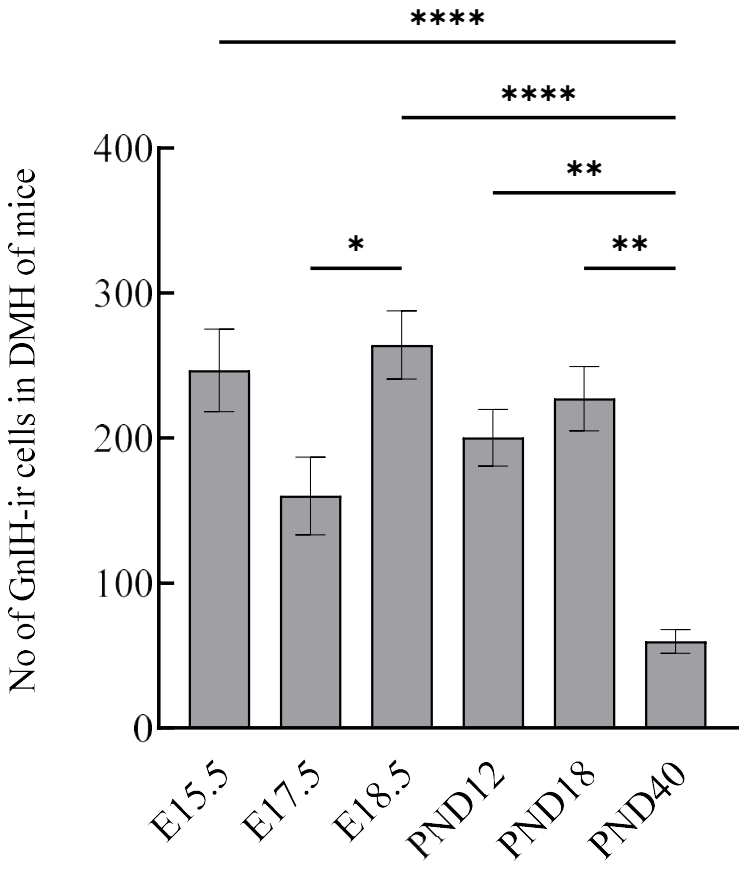


Figure A8. mean±SEM number of GnIH-ir cells in DMH of male and female mice (data collapsed by sex) across development. The number of GnIH-ir cells was significantly higher at E18.5 and PND18 which then declined significantly at PND40. *=P<0.05, **=P<0.01, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

Table A2. Individual and mean number of GnIH-ir terminals manually counted in arcuate region of male and female mice through development

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	12	13	27	67	157	106
	10	3	3	110	652	97
	3	7	2	87	348	54
	0	2	3			
		1	3			
mean±SEM	**6.25±2.84**	**5.20±2.20**	**7.60±4.85**	**88.0±12.40**	**386.0±144**	**85.70±16**
Female	18	5	18	121	696	85
	3	2	2	63	649	56
	0	22	0			68
			3			
mean±SEM	**7.00±5.57**	**9.67±6.23**	**5.75±4.13**	**92.0±29.00**	**673.0±23.50**	**69.70±8.41**
mean±SEM (Collapsed by Sex)	**6.57±2.60**	**6.88±2.56**	**6.78±3.08**	**89.6±11.50**	**500±106.00**	**77.70±8.86**

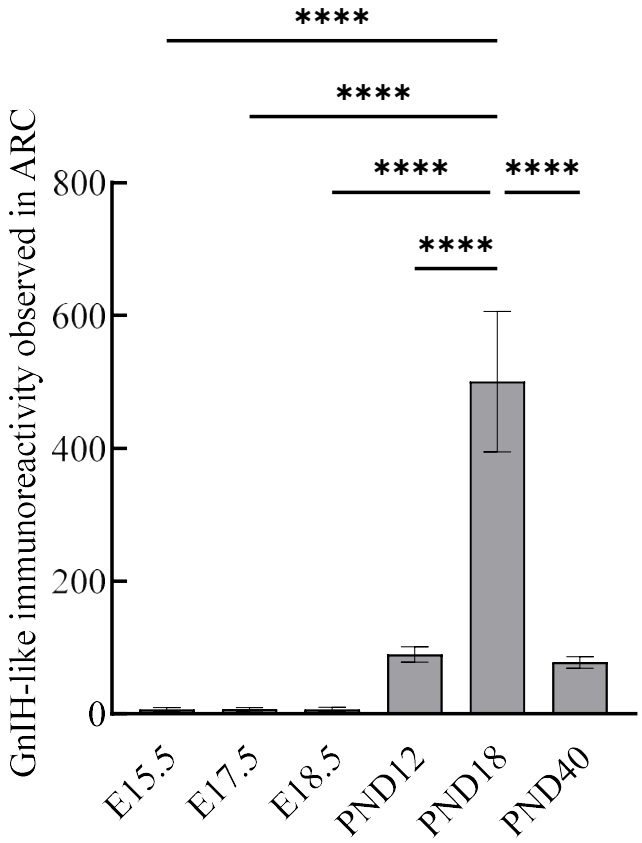


Figure A9. mean±SEM GnIH-like immunoreactivity observed in arcuate region of male and female mice (data collapsed by sex) across development. During prenatal development minimal immunoreactivity was noticed in the ARC, while a significant rise was seen at PND18, declining again at PND40. ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

Table A3. Individual and mean fiber density measured in ARC nucleus of mice across prenatal and postnatal development

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	0.02	0.187	0.173	0.674	1.1785	0.181
	0.03	0.095	0.358	0.405	1.851	0.379
	0.03	0.128	0.367	1.12	0.895	0.505
	0.2	0.092	0.407			
		0.125	0.718			
mean±SEM	**0.07±0.04**	**0.12±0.01**	**0.40±0.08**	**0.73±0.20**	**1.30±0.28**	**0.35±0.09**
Female	0.05	0.085	0.213	0.6	1.39	0.505
	0.03	0.171	0.134	0.52	1.933	1.405
	0.02	0.134	0.332			0.653
			0.12			
mean±SEM	**0.03±0.00**	**0.13±0.02**	**0.19±0.04**	**0.56±0.04**	**1.66±0.27**	**0.85±0.27**
mean±SEM (Collapsed by Sex)	**0.05±0.02**	**0.12±0.01**	**0.31±0.06**	**0.66±0.12**	**1.45±0.19**	**0.60±0.17**

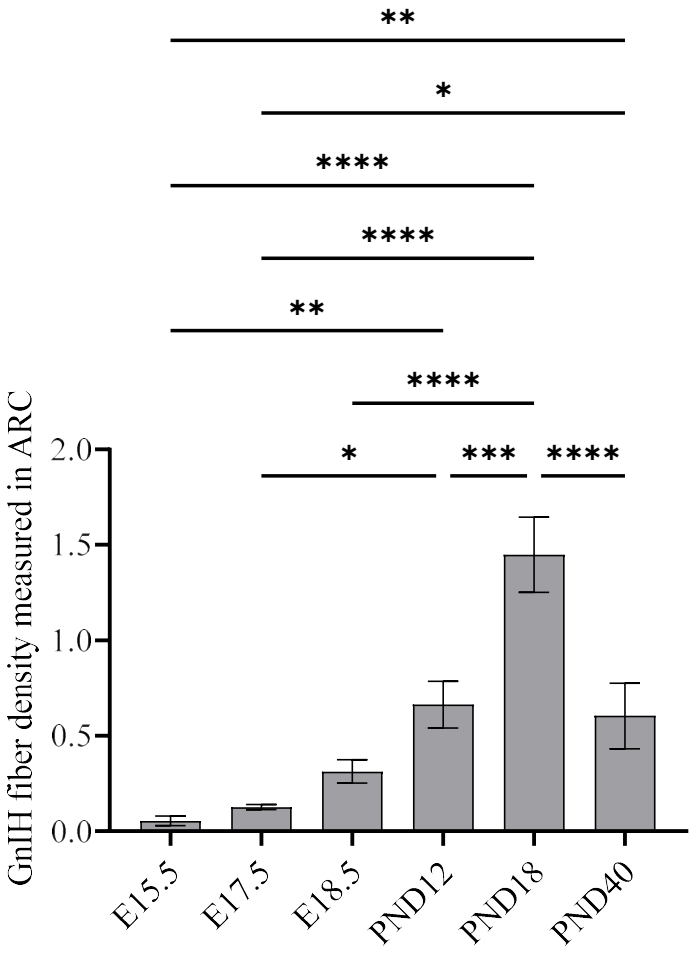


Figure A10. mean±SEM GnIH fiber density measured in arcuate region of male and female mice (data collapsed by sex) across development. During prenatal development minimal fiber density was noticed in the ARC, while a significant rise was seen at PND18, declining again at PND40. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

Table A4. Individual and mean number of GnIH-ir nerve terminals observed in paraventricular nucleus of male and female mice across prenatal and postnatal development

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	778	980	1908	429	2542	719
	816	789	1228	542	6139	605
	514	848	1520	582	5380	673
	869	785	1454			
		884	1914			
mean±SEM	**744±79.00**	**857±35.90**	**1605±134.00**	**518±45.80**	**4687±1095**	**666±33.10**
Female	1009	1475	1920	836	4731	914
	789	768	2038	728	5210	858
	916	715	1854			878
			1598			
mean±SEM	**905±63.80**	**986±245.00**	**1853±93.00**	**782±54.00**	**4971±240**	**883±16.40**
mean±SEM (Collapsed by Sex)	**813±58.40**	**906±86.30**	**1715±91.30**	**623±71.50**	**4800±608**	**775±51.40**

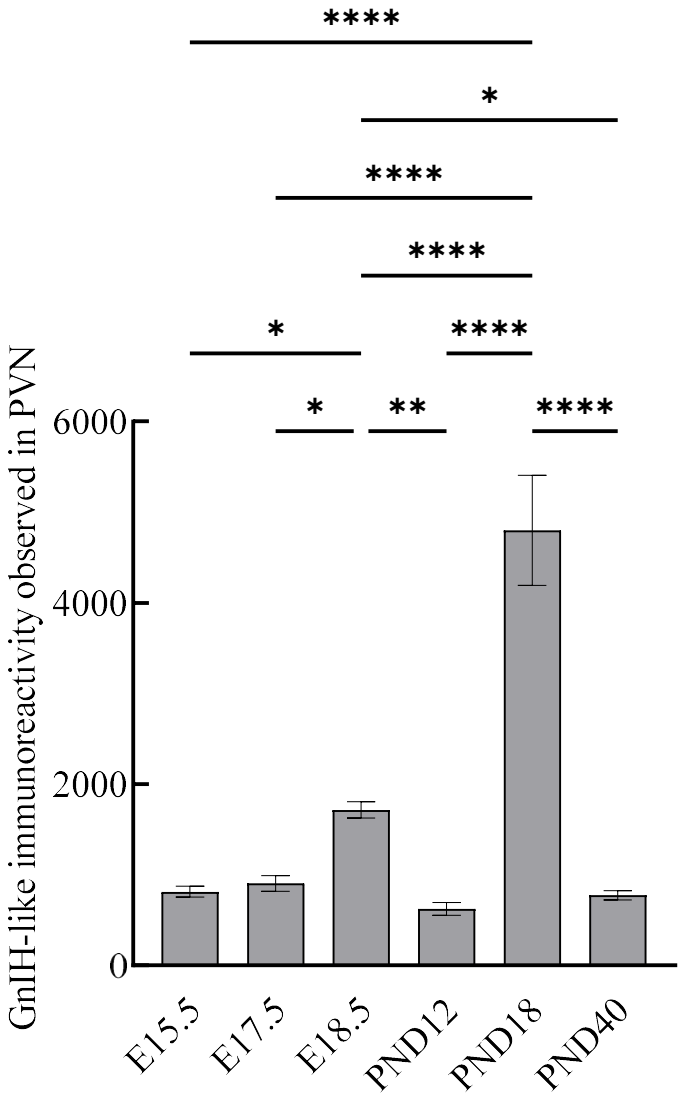


Figure A11. mean±SEM GnIH-like immunoreactivity observed in paraventricular region of male and female mice (data collapsed by sex) across development. A significant rise in GnIH-like-ir was evident at E18.5, declining significantly after birth. Then, a sharp rise was seen at PND18, followed by a significant decline at PND40. *=P<0.05, **=P<0.01, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

Table A5. Individual and mean GnIH fiber density measured in paraventricular nucleus of male and female mice across development

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	0.01	0.101	0.331	0.073	1.747	0.041
	0.02	0.08	0.328	0.08	0.97	0.082
	0.04	0.045	0.421	0.4	1.453	0.293
	0.2	0.021	0.195			
		0.033	1.45			
mean±SEM	**0.06±0.04**	**0.05±0.01**	**0.54±0.22**	**0.18±0.10**	**1.39±0.22**	**0.13±0.07**
Female	0.25	0.158	1.5	0.352	1.643	0.783
	0.132	0.047	1.052	0.02	1.55	0.149
	0.025	0.232	0.732			0.093
			0.953			
mean±SEM	**0.13±0.06**	**0.14±0.05**	**1.05±0.16**	**0.18±0.16**	**1.59±0.04**	**0.34±0.22**
mean±SEM (Collapsed by Sex)	**0.09±0.03**	**0.12±0.03**	**0.77±0.16**	**0.18±0.07**	**1.47±0.13**	**0.24±0.11**

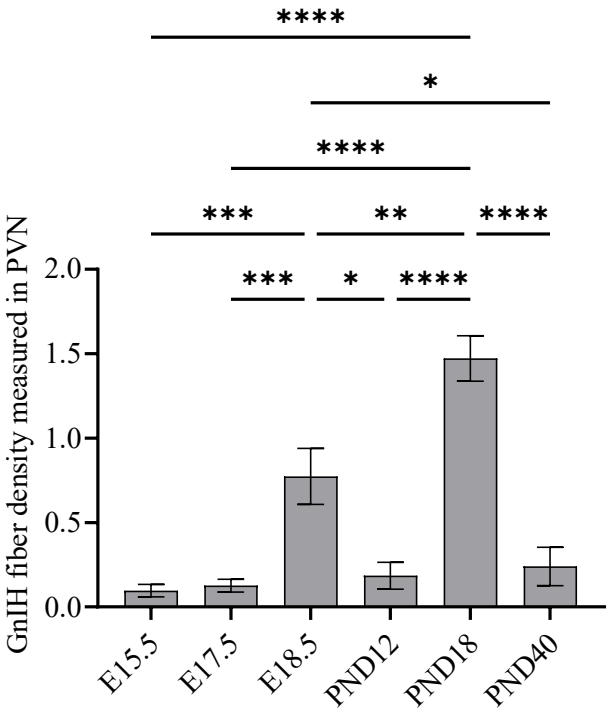


Figure A12. mean±SEM GnIH fiber density measured in paraventricular region of male and female mice (data collapsed by sex) across development. A significant rise was seen in the fiber density at E18.5 and at PND18. *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

Table A6. Individual and mean GnIH fiber density measured in preoptic area of male and female mice

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	0.11	0.256	0.189	0.17	2.448	0.163
	0.12	0.830	0.618	0.166	2.440	0.483
	0.14	0.069	0.018	1.049	1.780	0.205
	0.16	0.079	0.014			
		0.046	0.385			
mean±SEM	**0.13±0.01**	**0.25±0.14**	**0.24±0.11**	**0.46±0.29**	**2.22±0.22**	**0.28±0.10**
Female	0.14	0.134	0.227	0.609	2	0.871
	0.15	0.742	0.765	0.553	1.52	1.358
	0.18	0.470	0.066			0.370
			0.502			
mean±SEM	**0.15±0.01**	**0.44±0.17**	**0.39±0.15**	**0.58±0.02**	**1.76±0.24**	**0.86±0.28**
mean±SEM (Collapsed by Sex)	**0.14±0.00**	**0.32±0.11**	**0.30±0.09**	**0.50±0.16**	**2.03±0.18**	**0.57±0.18**

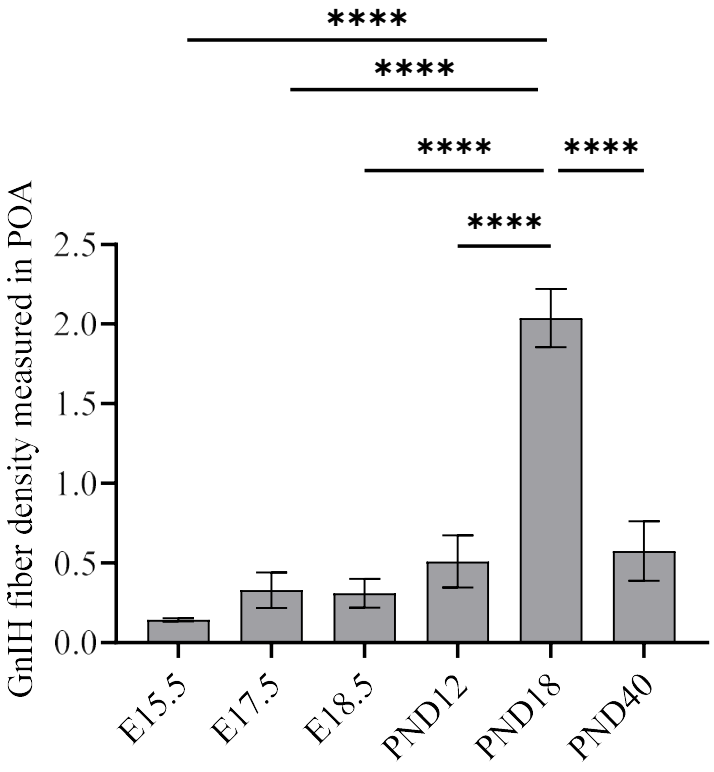


Figure A13. mean±SEM GnIH fiber density measured in preoptic area of male and female mice (data collapsed by sex) across development. Minimal fiber density was seen through prenatal period while a sharp rise was seen at PND18 followed by a significant decline at PND40. ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

Table A7. Individual and mean fiber density measured in lateral septum of male and female mice across development

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	0.10	0.578	0.141	0.245	0.700	0.457
	0.11	0.205	1.290	0.200	1.545	0.541
	0.11	0.066	0.482	0.400	2.314	0.244
	0.13	0.229	0.068			
		0.360	1.048			
mean±SEM	**0.11±0.00**	**0.28±0.08**	**0.60±0.24**	**0.28±0.06**	**1.52±0.46**	**0.41±0.08**
Female	0.12	0.470	0.171	1.200	2.460	2.078
	0.14	0.078	0.359	0.200	2.282	1.371
	0.10	0.661	0.741			0.126
			0.440			
mean±SEM	**0.12±0.01**	**0.40±0.17**	**0.42±0.11**	**0.70±0.50**	**2.37±0.08**	**1.19±0.57**
mean±SEM (Collapsed by Sex)	**0.11±0.00**	**0.33±0.07**	**0.52±0.14**	**0.44±0.19**	**1.86±0.33**	**0.80±0.31**

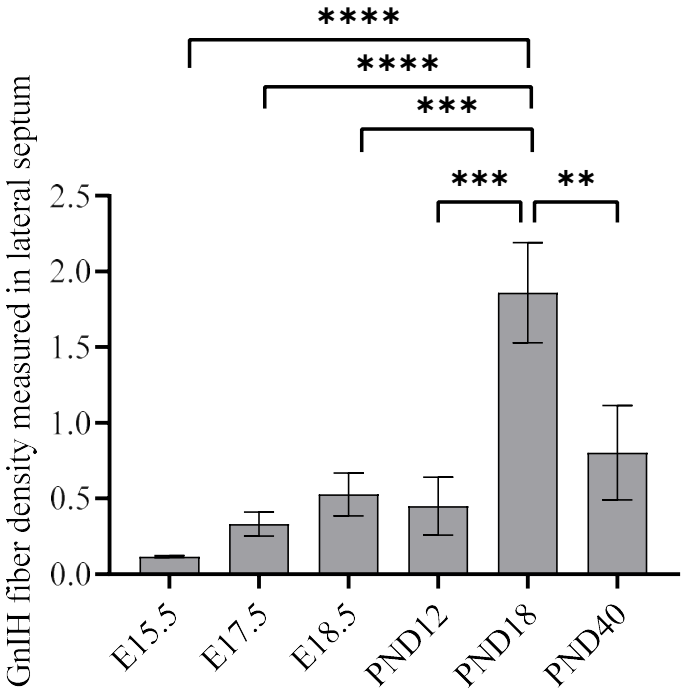


Figure A14. mean±SEM GnIH fiber density measured in lateral septum of male and female mice (data collapsed by sex) across development. A significantly higher fiber density was seen at PND18, with a significant decline at PND40. **=P<0.01, ***=P<0.001, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

Table A8. Individual and mean number of GnIH-ir nerve terminals observed in brain of male and female mice of different developmental ages

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	1309	2188	3468	1142	3868	1362
	1475	1900	3153	1223	9009	1242
	822	3111	3660	1144	7577	1079
	821	1902	2548			
		1897	3057			
mean±SEM	**1202±196**	**2200±235**	**3207±243**	**1183±40.50**	**6818±1532**	**1228±82**
Female	2226	2056	4330	1782	7000	1397
	1343	2011	2881	1326	7791	1387
	1748	2050	2741			1273
			2200			
mean±SEM	**1772±255**	**2039±14.10**	**3038±455**	**1554±228**	**7396±396**	**1352±39.80**
mean±SEM (Collapsed by Sex)	**1594±128**	**2291±182**	**3115±213**	**1339±115**	**7049±860**	**1285±50.10**

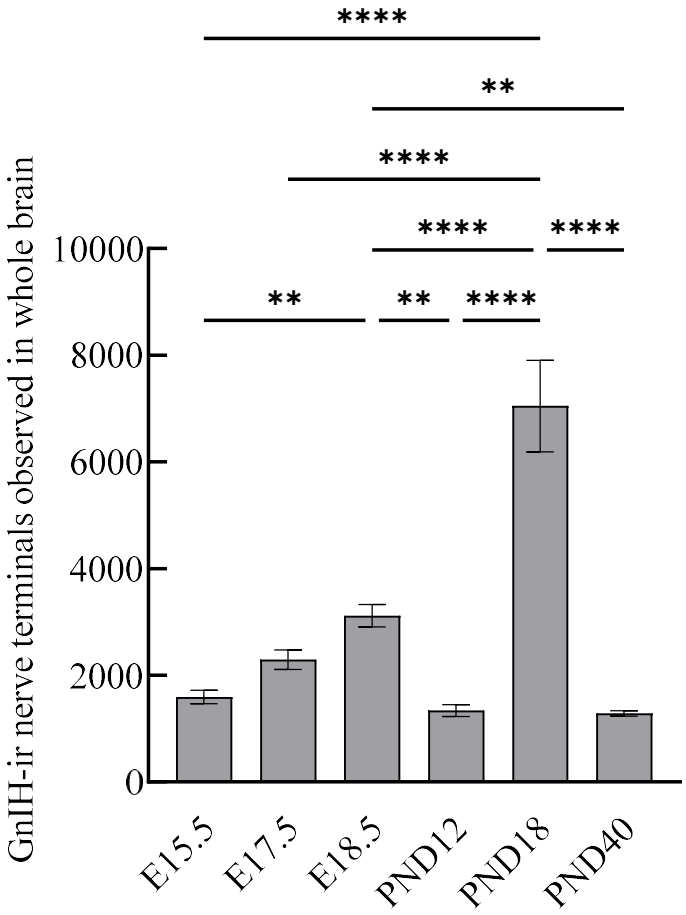


Figure A15. mean±SEM GnIH-like-ir observed in brains of male and female mice (data collapsed by sex) across development. A significant rise in GnIH-like-ir was evident at E18.5, declining significantly after birth. GnIH-like-ir increased at PND18 after birth and declined again at PND40. **=P<0.01, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

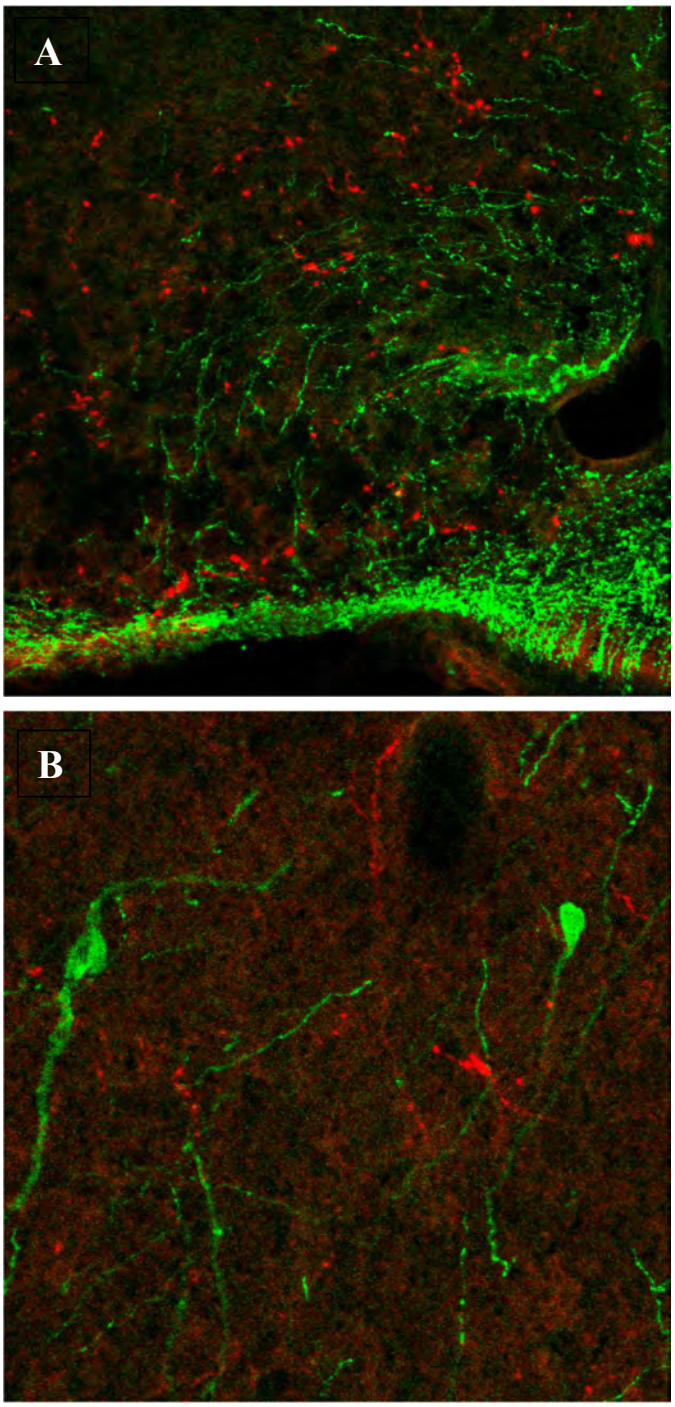


Figure A16. Representative confocal images showing GnIH-like-ir (red) and GnRH-like-ir (green) in arcuate nucleus (A) and POA (B). Dense GnRH-ir fibers are evident in the ARC region.

Table A9. Individual and mean number of GnIH-GnRH axosomatic contacts observed in brain of male and female mice across development

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	0	2	1	0	7	6
	0	2	7	1	9	0
	0	0	0	1	8	0
	2	1	0			
		4	0			
mean±SEM	**0.50±0.50**	**1.80±0.66**	**2.00±1.68**	**0.66±0.33**	**8.00±0.57**	**2.00±2.00**
Female	0	4	0	0	12	4
	0	6	2	1	9	0
	2	1	2			4
			1			
mean±SEM	**0.66±0.66**	**3.66±1.45**	**1.00±0.44**	**0.50±0.50**	**10.50±1.50**	**2.66±1.33**
mean±SEM (Collapsed by Sex)	**0.57±0.36**	**2.50±0.70**	**2.00±0.66**	**0.80±0.58**	**9.00±0.83**	**3.83±0.47**

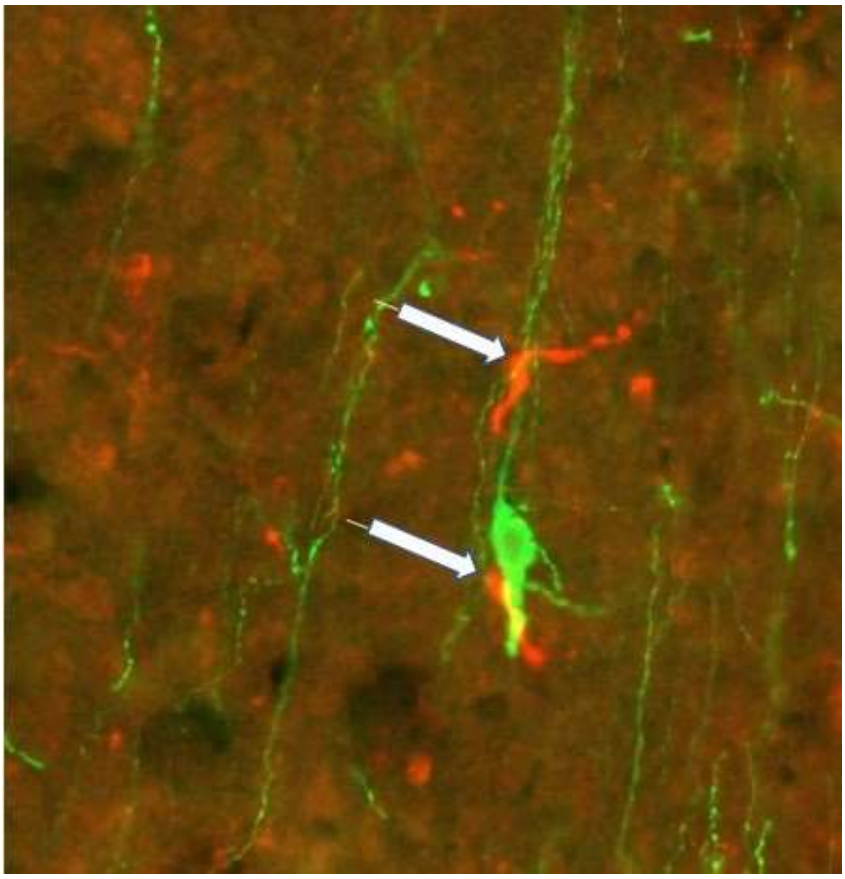


Figure A17. A representative photomicrograph showing GnIH (red) to GnRH (green) axosomatic contact in a PND18 animal.

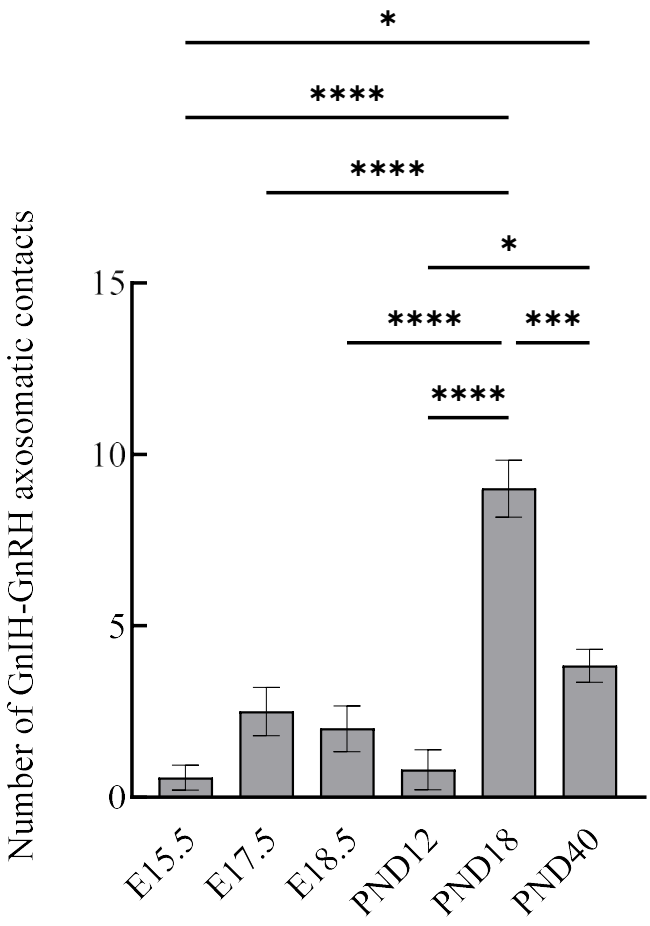


Figure A18. mean±SEM number of GnIH-GnRH axosomatic contacts observed in brains of male and female mice (data collapsed by sex) across development. Only a few contacts were seen during prenatal development while a significant rise in number of contacts was evident at PND18, followed by a significant decline at PND40. *=P<0.05, ***=P<0.001, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

Table A10. Individual and mean number of GnIH-GnRH fiber to fiber contacts observed in brain of male and female mice through prenatal and postnatal development

Sex	Developmental Stage					
	E15.5	**E17.5**	**E18.5**	**PND12**	**PND18**	**PND40**
Male	0	7	12	5	188	29
	0	5	13	4	160	24
	0	1	11	1	147	41
	4	6	9			
		11	4			
mean±SEM	**1.33±1.33**	**6.00±1.61**	**11.25±0.85**	**3.33±1.20**	**165.00±12.09**	**31.33±5.04**
Female	0	16	06	2	146	25
	3	9	5	4	189	47
	2	11	14			27
			11			
mean±SEM	**1.25±0.75**	**12.00±2.08**	**8.00±1.92**	**3.00±1.00**	**167.50±21.50**	**33.00±7.02**
mean±SEM (Collapsed by Sex)	**1.29±0.64**	**8.25±1.61**	**9.44±1.21**	**3.20±0.73**	**166.00±9.51**	**32.20±3.89**

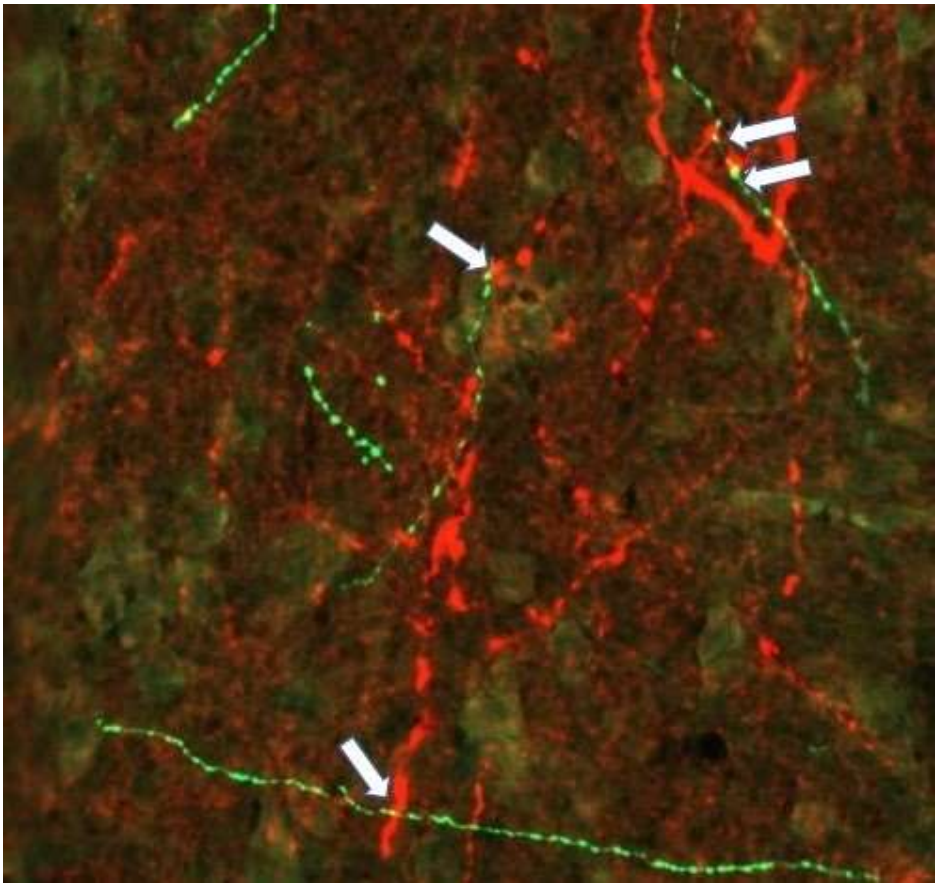


Figure A19. A representative photomicrograph showing GnIH (Red)-GnRH (Green) fiber to fiber contact in a PND18 animal.

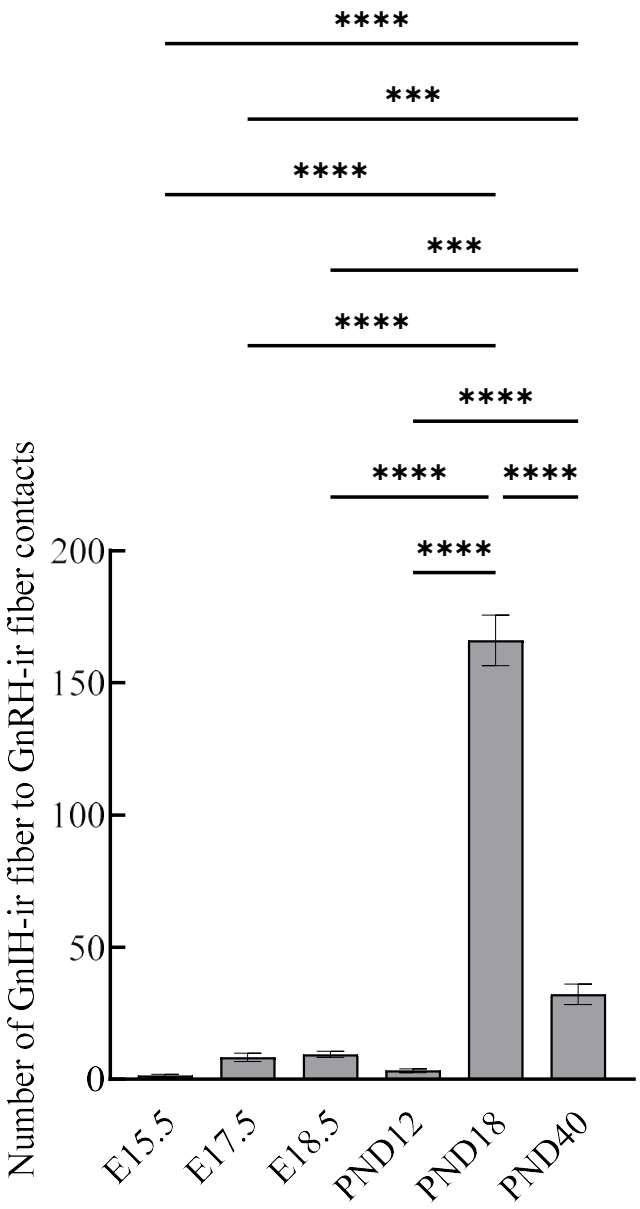


Figure A20. mean±SEM number of GnIH-GnRH fiber to fiber contacts observed in brains of male and female mice (data collapsed by sex) across development. Only a few contacts were seen during prenatal development while a significant rise in number of contacts was evident at PND18, followed by a significant decline at PND40. ***=P<0.001, ****=P<0.0001 (E, embryonic day; PND, Postnatal day)

4.5. Discussion

Although the role of GnIH in reproduction is well established across species (Ukena and Tsutsui, 2001; Bentley *et al*., 2008; Ubuka *et al*., 2009a; 2009b; Peng *et al*., 2016; Muñoz-Cueto *et al*., 2017; Kumar *et al*., 2020), the pattern of expression and interactions with the GnRH system across development required further investigation. Current study examines developmental changes in GnIH protein expression and its crosstalk with GnRH neuronal elements in mice. The results herein suggest that GnIH regulates the activity of the reproductive axis during pre- and postnatal development and hints at a role for this inhibitory pathway in sexual differentiation *in utero* and early postnatally. Likewise, reductions in GnIH just prior to puberty suggest release from inhibitory influences on the HPG axis currently.

As in previous work in rodents, GnIH cells were found to be clustered in the DMH at all developmental stages. Importantly, GnIH cells number was statistically higher in females than in males consistent with the fact that the ovaries are quiescent prior to puberty and do not contribute to sexual differentiation in this sex (Picut *et al*., 2015). This finding contrasts with one previous report where no significant differences were noted between the sexes in adult mice (Poling *et al*., 2012). In addition to this sex difference, a significant rise was noted in the GnIH-ir cells at E18.5, a time when reproductive axis suppression is observed in males (Kuiri-Hänninen *et al*., 2014). In males, following birth, the HPG axis is transiently active for a few days after birth (Bell, 2018). At this time, we observed a reduction in the number of GnIH-ir cells in the DMH that might contribute to this increased activation. At PND18, we observed a significant rise in GnIH positive cells, consistent with the timing of reduced HPG axis activity that remains low until puberty (Bell, 2018). At PND40, there was a precipitous decline in GnIH-ir cell numbers, consistent with the timing of pubertal HPG axis

activation. These postnatal findings are in agreement with previous reports in male and female mice (Poling *et al*., 2012; Semaan and Kauffman, 2015).

To comprehensively examine changes in the GnIH system across development, GnIH projections were examined across the brain. The distribution of GnIH-ir fibers was consistent with previous studies in rodents, with fibers principally targeting midline hypothalamic loci and GnRH cells receiving close appositions (Kriegsfeld *et al*., 2006; Johnson *et al*., 2007), sheep (Smith *et al*., 2008) and humans (Ubuka *et al*., 2009b). Considerable variation in GnIH innervation was seen during the pre- and postnatal development in males and females, hinting at the likelihood of GnIH cells acting through intermediary neuronal systems to control the activity of the HPG-axis during the developmental process. During embryonic development, the ARC region exhibited almost no signs of GnIH-ir, while many GnIH terminals were seen on PND18. This observation suggests that, during the juvenile period, GnIH might suppress the HPG axis activity through actions on ARC kisspeptin cells or GnRH fiber terminals (Yeo and Herbison, 2011). Interestingly, GnIH-GnRH crosstalk was found to be bidirectional in the ventral DMH region. Alongside GnIH cells and fibers interacting with the GnRH somas, some GnRH fibers impinging upon the GnIH somas were also seen. These observations suggest that GnIH neuronal activity may be regulated by the GnRH system.

It is also possible that GnIH nerve terminals in the ARC communicate with orexigenic (i.e., NPY/Agrp) and anorexigenic (CART/POMC) peptide-expressing cells during the postnatal development to modify the activity of the reproductive system and time the onset of puberty based on energy state (Elmquist *et al*., 1999; Qu *et al*., 2020). Interestingly, a marked rise in GnIH positive nerve terminals' number was observed in the paraventricular nucleus on PND18. The PVN plays essential roles in

neuroendocrine/autonomic regulation and stress axis activity (Swanson, 1980; Ferguson, 2008). The hypothalamo-pituitary adrenal axis controlled by secretion of corticotropin-release hormone from the PVN, suppresses reproductive activity during stressful conditions (Einarsson *et al*., 2008). Thus, the GnIH system might indirectly regulate HPG axis activity via this intermediary neuronal system.

In summary, the reproductive circuitry is functional in the fetus of midgestational age and becomes inactive during the latter part of pregnancy. This inhibition is removed at birth, reactivating the reproductive axis followed by quiescence until reactivation at puberty. In the present study, variation observed in the expression profile of the hypothalamic neuropeptide, GnIH, over the gestational and pubertal development in male and female mice mirrors reproductive axis activity. These findings suggest that changes in GnIH during juvenile/prepubertal period may participate in early-life sexual differentiation and subsequent, sex-specific motivated behavior. However, whether changes in GnIH are causative or consequent due to other regulators of reproductive axis activity, remains to be determined. Genomic and pharmacological manipulations in the future will aid in clarifying the particular role of this neurohormone in development and sexual differentiation and help guide the development of novel therapeutic approaches to treat fertility related disorders.

CHAPTER B

Study of hypothalamic pathways governing pubertal progression in rhesus monkey (*Macaca mulatta*)

5.1. Abstract

5.1.1. Background

Initiation of puberty and adult reproduction depends on a dynamic interaction between hormones of the hypothalamus, pituitary, and gonads. Increased gonadotropin releasing hormone (GnRH) release in a pulsatile fashion is crucial for pubertal onset and for the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from pituitary, required for gonadal maturation. A few months after birth in primates, the reproductive axis enters a quiescent stage which persists until the time of puberty. Among many others, gonadotropin inhibitory hormone (GnIH) is thought to be the most important negative regulator of the reproductive axis by central inhibition of the GnRH pulse generation, occasioning a prepubertal brake on the reproductive axis activity. Recent studies have suggested that GnIH signaling might have a role in governing the activity of GnRH pulse generator in many species.

5.1.2. Objective

Current study was architectured to evaluate the regulation of HPG-axis activity by modulation of GnIH signaling in the male rhesus monkey hypothalamus, across pubertal development through protein- and mRNA expression analyses.

5.1.3. Materials and Methods

Hypothalami of fifteen male rhesus monkeys (*Macaca mulatta*) were collected and processed for immunofluorescence and qPCR. These animals were categorized into four different developmental stages based on somatometric and hormonal data: Infant (n=3): body weight 1.033±0.169kg, testicular volume 0.12±0.695ml and testosterone level 0.24±0.0ng/ml; Juvenile (n=4): body weight 2.0±0.129kg, testicular volume 0.326±0.029ml and testosterone level 0.17±0.0ng/ml; Prepubertal (n=4): body weight

4.02±0.131kg, testicular volume 0.34±0.133ml and testosterone level 0.22±0.0ng/ml; Adult (n=4): body weight 11.6±1.24kg, testicular volume 42.4±4.93ml and testosterone level 2.03±0.4ng/ml. Single label immunocytochemistry was performed on three randomly selected 20µm thick hemi-hypothalamic sections to examine the variation in GnIH expression through pubertal development. mRNA was extracted from the other hemi-hypothalamic block of all monkeys and correlative changes in *GnIH*, *GnRH* and *Kiss1* expression were quantified by SYBR green RT-qPCR.

5.1.4. Results

Body weight ($P<0.0001$), plasma testosterone level ($P<0.001$) and total testicular volume ($P<0.0001$) were statistically higher in adult animals compared to all other ages while they were comparable in other groups. Mean GnIH-ir was found to be significantly higher in prepubertal animals ($P<0.01$) compared to infants, while it was significantly reduced in adults ($P<0.001$). Number of GnIH-ir fibers also increased analogously in prepubertal animals ($P<0.01$) decreasing significantly in adults ($P<0.05$). mRNA expression of *GnRH* and *Kiss1* was found to be statistically higher ($P<0.001$) in adult monkeys while *GnIH* mRNA expression was found to be the highest at prepubertal stage ($P<0.001$). Significant negative correlation was seen between *GnIH-GnRH* ($P<0.01$) and *GnIH-Kiss1* ($P<0.001$) mRNA expression.

5.1.5. Conclusion

Our findings suggest that GnIH plays a role in the prepubertal suppression of the reproductive axis, with GnIH decrease causing disinhibition of the adult reproductive axis. A significant inverse age-related relationship between *GnIH*, *GnRH* and *Kiss1* suggests GnIH's potential role in downregulation of the HPG-axis prior to puberty in occasioning the neurobiological brake on the reproductive plexus activation, while

fading away of this brake might trigger puberty onset in higher primates by stimulating the release of GnRH and ultimately pituitary gonadotrophins.

5.2. Introduction

Puberty is defined as the attainment of sexual and somatic maturity (Parent *et al*., 2003; Ojeda *et al*., 2010). Puberty enables an animal to reproduce efficiently and to achieve adult phenotype (Tena-Sempere, 2012). Reproductive axis activity varies during postnatal development in non-human primates (Terasawa and Fernandez, 2001; Plant, 2015). The reproductive axis is reported to remain active during infancy followed by a transient period of inactivity during juvenile and prepubertal stages (Terasawa and Fernandez, 2001; Plant, 2015). Then, the actual increase in reproductive system activity occurs at the start of puberty and lasts throughout adulthood (Kuiri-Hänninen *et al*., 2014; Uenoyama *et al*., 2019).

Various endocrine and neuronal systems act as a coordinated unit, called the hypothalamic-pituitary-gonadal (HPG)-axis to govern reproductive physiology (Lake *et al*., 2008). This axis is comprised of three functional entities: hypothalamus, pituitary, and gonads. The arbitrator between the endocrine and the nervous systems is hypothalamus which secretes gonadotropin releasing hormone (GnRH) responsible for the synthesis and release of anterior pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH induces steroidogenesis while gametogenesis is regulated by FSH, coordinating the reproductive acts through coaction of signals from center and periphery (Thackray *et al*., 2010; Kanda *et al*., 2011).

GnRH release from the hypothalamus follows a peculiar pulsatile fashion and the LH release from the pituitary gland is correlated with this pulsatility in many species (Thiery and Pelletier, 1981; Kawakami *et al*., 1982). The pulsatility in the GnRH release is a prerequisite for the attainment of reproductive success (Knobil *et al*., 1980;

Belchetz *et al*., 1987) and is brought about by the inherent rhythms and multi-unit functioning of neuronal cells.

According to Ojeda, puberty is marked by reactivation of the hypothalamic GnRH system (Ojeda and Skinner, 2006; Ojeda *et al*., 2006; 2010). A multitude of excitatory and inhibitory inputs are reported to modulate GnRH activity (Tena-Sampere, 2012; Terasawa, 2019; Uenoyama *et al*., 2019; Rodríguez-Vázquez *et al*., 2020) but the endocrine regulation of puberty onset is still poorly understood. Other than physiological and environmental factors, many neuronal and endocrine pathways also participate in GnRH's feedback regulation. Gamma amino butyric acid (GABA), glutamate, gonadal steroids and RFamides are some essential factors governing the GnRH secretion (Clarke, 1996; Herbison, 1997; Kuehl-Kovarik *et al*., 2002; Constantin *et al*., 2010).

Rfamides are the peptides released from the brain, having a characteristic C-terminal Arg-Phe-NH_2 sequence (Fukusumi *et al*., 2006). Structurally, RFamides can be subdivided into five different groups including PQRFamide peptides (Yang *et al*., 1985), prolactin-releasing peptides (Hinuma *et al*., 1998), pyroglutamate RFamides (QRFP/26RFamide) (Fukusumi *et al*., 2003), kisspeptins (Ohtaki *et al*., 2001) and gonadotropin inhibitory hormone (GnIH) group (Tsutsui *et al*., 2000). Among these, kisspeptin encoded by the *Kiss1* gene, is thought to be the most important positive regulator of the GnRH secretion (Jayasena *et al*., 2009). It is thought to be the main endocrine player, triggering GnRH release during infancy and at true pubertal onset (Shahab *et al*., 2005; 2018; Garcia *et al*., 2018). Kisspeptin neuronal cells are primarily localized in the arcuate nucleus (ARC) in primates with fiber projections to median eminence (ME), preoptic area (POA) and mediobasal hypothalamus (MBH) (Ramaswami *et al*., 2008). Kisspeptin cells express receptors for steroid hormones

(Adachi *et al*., 2007) while GnRH cells do not (Huang and Harlen, 1993) but they contain the receptor for kisspeptins, GPR54 (Han *et al*., 2005). Based on these findings it is proclaimed that kisspeptin cells transfer the feedback effect of sex steroids on to the GnRH cells.

GnIH, on the contrary, is thought to be the most important negative regulator of the reproductive axis and GnRH secretion. GnIH, a dodecapeptide (SIKPSAYLPLRFamide), was first time discovered in the quail hypothalamus by Tsutsui and colleagues. (2000) and afterwards its existence was confirmed in other vertebrate species (Kriegsfeld *et al*., 2006; Clarke *et al*., 2008; Ubuka *et al*., 2009a; 2009b; Tsutsui *et al*., 2010a; Tsutsui *et al*., 2012). Avian GnIH has several mammalian orthologs including RFRP-1, RFRP-2, and RFRP-3 (Tsutsui *et al*., 2012) however, RFRP-3 is reported to be the only functional ortholog in mammals with the capability of inhibiting the LH/FSH synthesis and release (Johnson *et al*., 2007; Ubuka *et al*., 2012).

GnIH immunoreactive cell bodies are found to be localized in the DMH of mammals while its axon terminals are found in arcuate region of rats and Syrian hamsters (Kriegsfeld *et al*., 2006). In higher primates, GnIH positive cells were seen in anteroventral paraventricular (AVPV) region of hypothalamus and DMH with projections leading to ARC and ME in humans (Ubuka *et al*., 2009a; 2009b). GnIH fibers have been localized to various brain areas in rodents (Ukena and Tsutsui, 2001; Kriegsfeld *et al*., 2006) and POA, PVN, IPe, arcuate and ME in primates including rhesus monkey (Ubuka *et al*., 2009a).

GnIH mediates a wide variety of functions like stress, depression, aggressiveness, sleep (Soga *et al*., 2014; Son *et al*., 2014; Teo *et al*., 2021) and reproduction (Tsutsui *et al*., 2010b; Tsutsui and Ubuka, 2016). Multiple studies have described the suppressive

effect of GnIH on production and secretion of GnRH and pituitary gonadotrophs as well as apoptosis of testicular tissues with seminiferous tubular regression (Ubuka *et al*., 2006; Johnson *et al*., 2007; Clarke *et al*., 2008; Wu *et al*., 2009; Son *et al*., 2012; Ubuka *et al*., 2012). Inhibitory function of GnIH on gonadotropins is conserved across vertebrates including primates (Ciccone *et al*., 2004; Kriegsfeld *et al*., 2006; Clarke *et al*., 2008; Murakami *et al*., 2008; Tsutsui *et al*., 2012) with some exceptions in fish where it works in the opposite fashion (Amano *et al*., 2006; Zhang *et al*., 2010).

Developmental variation in the hypothalamic GnIH expression has been reported in various species, like Zebrafish (Zhang *et al*., 2010), Indian major carp (Biswas *et al*., 2015), European bass (Paullada-Salmerón *et al*., 2017), cichlid (Di Yorio *et al*., 2018), catla (Kumar *et al*., 2020) and European green frog (Pinelli *et al*., 2020). Furthermore, GnIH has been shown to suppress GnRH release through kisspeptin/GPR54 signaling system (Pineda *et al*., 2010b; Kelestimur *et al*., 2013). Moreover, GnIH contacts onto GnRH soma in rodents, sheep, rhesus monkey and humans have been reported (Kriegsfeld *et al*., 2006; Smith *et al*., 2008; Ubuka *et al*., 2009a; 2009b).

As mentioned earlier, developmental variation in GnIH expression is studied in different species of fish and rodents only. The developmental pattern of GnIH expression in higher primates has not been reported till date. Further, age-related relationship in the mRNA expression of *GnIH*, *GnRH* and *Kiss1* in higher primates has not been explored till now. Therefore, this study was aimed at investigating the GnIH immunoreactivity distribution in hypothalamus of male rhesus monkey from infancy to adulthood to decipher the involvement of GnIH neuronal signaling in the prepubertal brake on the HPG-axis in primates using immunofluorescence and RT-qPCR techniques.

As already mentioned, one would anticipate that GnIH immunoreactivity and mRNA expression would be higher during juvenile and prepubertal phase of postnatal development. Likewise, it can also be predicted that reactivation of the HPG-axis in adulthood might be due to reduction in GnIH expression. Furthermore, the goal was to determine the correlative changes between expression of *GnIH*, *GnRH* and *Kiss1* mRNA through pubertal progression in male rhesus macaque. Based on above mentioned data, we hypothesized that suppressed pituitary gonadotroph secretion during juvenile and prepubertal phase is due to the increased GnIH signaling that ultimately suppresses the GnRH and kisspeptin release from the hypothalamus.

5.3. Materials and Methods

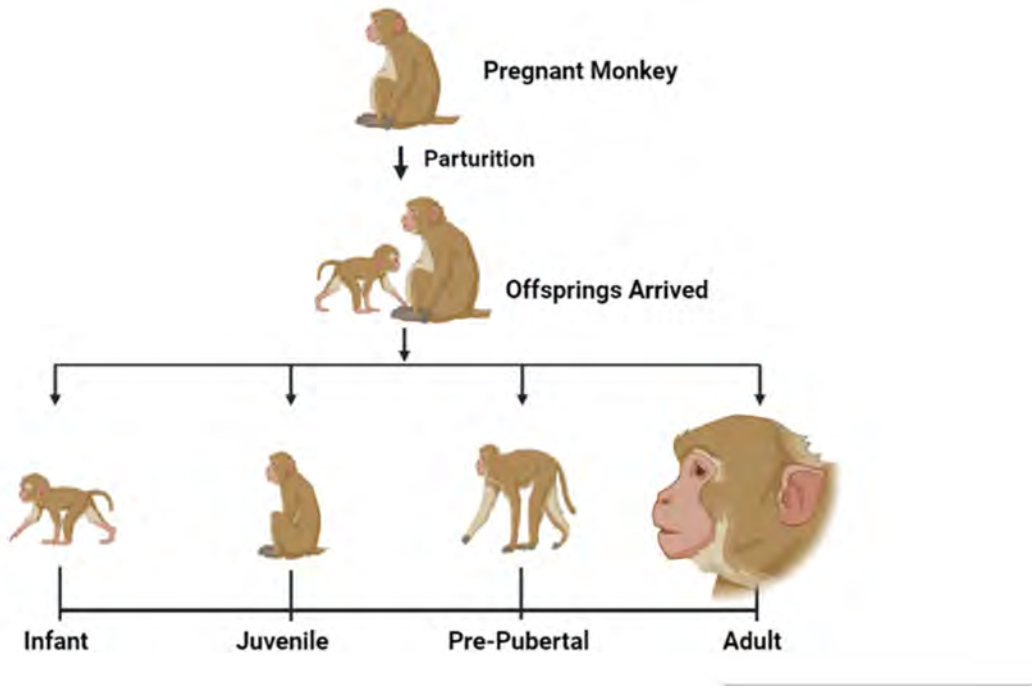
5.3.1. Animals

Fifteen male rhesus monkeys were employed for the present study. These animals were divided into four different age groups i.e., infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4). The animals in each stage were characterized according to their somatometric parameters. Mean±SEM value of these parameters in each group was as follows: Infant (n=3): body weight 1.05±0.17kg, testicular volume 0.13±0.06ml and testosterone level 0.24±0.08ng/ml; Juvenile (n=4): body weight 2.0±0.12kg, testicular volume 0.32±0.02ml and testosterone level 0.17±0.05ng/ml; Prepubertal (n=4): body weight 3.70±0.201kg, testicular volume 0.42±0.07ml and testosterone level 0.32±0.13ng/ml; Adult (n=4): body weight 11.7±1.25kg, testicular volume 42.4±4.93ml and testosterone level 2.04±0.46ng/ml. After being captured from wild, all these monkeys were kept in individual cages under semi-ambient conditions before euthanization.

5.3.2. Tissue Collection

Hypothalamic blocks and testicular tissues were collected from all fifteen animals of specific ages (Figure B1.). Ketamine hydrochloride was used to deeply sedate the animals. Hairs were shaved off the head region with the help of a razor, then skin on the skull was completely cleaned with 70% ethanol and muscle tissue was removed by using a scalpel. Then the brain was exposed after cutting open the cranium in a circle using a bone cutter. The brain was removed immediately from the cranial cavity and was put on a chilled plate of glass. Hypothalamic blocks consisting of MBH and POA mainly were dissected out from the brain, as described previously (Shibata *et al*., 2007).

Study Design



Ages investigated

Figure B1. Brain sampling timeline of male rhesus monkeys through pubertal development

Segregated blocks were washed with normal saline. Further, two hemi-hypothalamic blocks were made by cutting along the mid-line. One hemi-hypothalamic block from all animals was transferred to a fixative and was cryopreserved by sequential passage through sucrose solutions to be used in immunocytochemistry and the second hemi-hypothalamus was quickly dipped in liquid nitrogen and was stored at -80˚C until RNA extraction to be used in RT-qPCR. A single sample of blood was drawn from all monkeys and their testicular dimensions were noted before dissection. For histological purposes, testicular tissue from one testicle of each animal was also collected.

5.3.3. Tissue Processing

After washing, hemi-hypothalamic blocks were fixed for 16 hours at 4°C, after which they were cryopreserved in sucrose solution. Later, these blocks were cut serially into 20µm thick sections on a cryostat in horizontal plane and preserved in an antifreeze solution at -20°C until immunofluorescence. Testicular tissues were fixed in sera and were passed through ascending grades of alcohol for dehydration. After which they were embedded in molten wax and were fixed onto wooden blocks.

5.3.4. Testicular Volume

At the time of dissection, the testicular volume of all the monkeys was calculated as described before (Steiner and Bremner, 1981).

5.3.5. Plasma Testosterone Measurement

Total plasma testosterone concentration was determined using a commercially available enzyme immunoassay (EIA) kit (Astra Biotech GmBH, Luckenwalde, Germany) and the manufacturer's instructions. The assay sensitivity was 0.05 ng/ml, and the variation coefficients were less than 9% and 10%, respectively.

5.3.6. Testicular Morphology

To study the morphological parameters of the testis of monkeys across different ages, paraffin embedded tissues were stained with eosin and hematoxylin for morphological analysis. The height of the epithelial cells and diameter of the seminiferous tubules were quantified and compared across postnatal development.

5.3.7. Fluorescence Immunocytochemistry

Four sections from the hypothalamus of each animal were selected for standard single label immunocytochemistry protocol. Out of these four, primary antibody solution was applied to three sections while one section was employed as a primary antibody omitted control. All sections were rinsed with 0.1M PBS. Then, non-specific binding sites were blocked by incubating the sections with 10% normal goat serum for one hour. Then, after washing, sections were incubated with primary antibody (rabbit anti-, white-crowned sparrow GnIH antibody (PAC123,124, antigen sequence SIKPFSNLPLRF), used at dilution 1:5000) in a buffer solution for 2 days at 4°C, followed by washing. Control sections were incubated without primary antibody. Later, sections were placed in a solution of secondary antibody (Cy3-goat anti rabbit, Cat# 111-165-003; Jackson Immonoresearch Laboratories Inc, used at dilution 1:200) for two hours at room temperature, in dark. Control sections were also incubated with the secondary antibody solution, followed by rinsing.

Then, sections were mounted on super frosted glass slides (CrystalCruz^{R}, Cat # Sc-363562; Santa Cruz Biotechnology Inc) and left for drying overnight at 4°C in dark. Next day, the slides were coverslipped (Microscope Cover Glass, MAS GmbH, Leonberg, Germany, 24x50mm) using Immu-Mount^{TM} (Thermo Shandon Limited, Cheshire, UK, Cat# 238402) as a medium for mounting of sections and the slides were stored at 4°C after drying.

5.3.8. Microscopy

GnIH immunoreactivity was studied utilizing an Olympus fluorescent microscope (Olympus BX51, Tokyo, Japan) and photomicrographs were captured, simultaneously. The total number of GnIH immunoreactive nerve terminals and fibers in midline hypothalamic regions specially ARC and PVN in all sections was manually counted and mean was calculated for each animal. Olympus CX51 light microscope was used to study testicular morphological parameters in H&E-stained tissues of each animal.

5.3.9. Real Time-quantitative PCR

5.3.9.1. RNA Extraction and Synthesis of cDNA

Wizol^{TM} Reagent (Wizbiosolutions, Seongnam, South Korea) was used to isolate total RNA from the hemi-hypothalamus of all animals, according to the manufacturer's instructions. The RNA quality and quantity were measured using a Thermo Scientific Nanodrop 1000 spectrophotometer (Wilmington, DE, USA). A cDNA synthesis kit (WizScript^{TM}, Wizbiosolutions) was employed to prepare cDNA from this RNA, using the supplier's protocol with the random hexamer primers.

5.3.9.2. Real Time PCR

The expression of the *GnIH*, *Kiss1*, and *GnRH* genes was determined using real-time polymerase chain reaction with the Wizpure^{TM} qPCR Master (SYBR) kit (Wizbiosolutions). Each 10µl reaction contained 5µl of SYBR Green, 0.45µl of each primer set, and 1.6µl of RNAse-free water. The sequences of all primers used are as follows *GnIH* F 5- CCTCGTGAGACGGGTTCTTA-3, R 5- ATTGGCACATGGTGAATGC -3 (NM-001033115.2; product length 118); *Kiss1* F 5- GGACCTGCCGAACTACAACT-3, R 5-TGACTCCTCTGGGGTCTGAA -3 (XM-028852143.1; product length 141); *GnRH* F 5- AGATGCCGAAAATTTGATGG -3, R

5-TTTCCAGAGCTCCTTTCAGG-3 (S- 75918; product length 134); *GAPDH* F 5-TGTTGCCATCAATGACCCCT -3, R 5- TTGATGACGAGCTTCCCGTT -3 (NM-001195426.1; product length 119). Pre-denaturation temperature was 95°C for 180s, denaturation temperature was 95°C for 10s, annealing temperature was 60°C for 15s, and elongation temperature was 72°C for 20s. All amplifications were repeated, and cycle threshold values (Ct) were calculated using CFX Maestro software version 2.3. (Biorad, Hercules, CA, USA). The relative Ct method was used to calculate gene expression levels. The $2^{-\Delta\Delta CT}$ method was used to normalize each sample to endogenous housekeeping gene GAPDH expression (Livak and Schmittgen., 2001; Thayil et al., 2020), with the infant group serving as the calibrator.

5.3.10. Statistical Analyses

All data are presented here as mean±SEM. One-way analyses of variance were employed to compare body weight, plasma testosterone measurement, GnIH-like ir and *GnIH* expression relative to *GnRH* and *Kiss1* across developmental stages. Pearson's correlation was used to establish the correlation among *GnIH-GnRH* and *GnIH-Kiss1* mRNA expression through pubertal development. $P \leq 0.05$ was statistically significant.

5.4.Results

5.4.1. Somatometric Data

Fifteen male rhesus monkeys were categorized into four developmental stages (infant, juvenile, prepubertal and adult) based on somatometric parameters i.e., body weight, plasma testosterone level, testicular volume, and histology. These animals were used to assess immune- and gene expression of *GnIH*, *GnRH* and *kisspeptin* in hypothalamus. Data on body weight, testicular volume and plasma testosterone concentration of individual animals are given in Table B1. and B2., respectively. Body weight and testicular volume showed a prominent increase with the progressing age of the monkeys. Statistically, there was no significant difference ($P>0.05$) in body weight, testicular volume and testosterone level of infant, juvenile and prepubertal group, while adults showed significantly higher body weight ($F_{3,11}=50.71$; $P<0.0001$), testicular volume ($F_{3,11}=65.39$; $P<0.0001$) and testosterone level ($F_{3,11}=11.89$; $P<0.01$) as compared to rest of the developmental groups (Figures B2.B4.).

5.4.2. Testicular Histology

Prominent differences were evident in histological examination of H&E-stained testicular sections of monkeys in different groups. With advancing age, clear variation in maturation and differentiation of spermatogonia was noticeable. Tubular lumen was closed in infants (Figure B5. A, B) whereas juveniles showed a very small luminal space (Figure B5. C, D). Monkeys in the prepubertal stage showed a relatively larger lumen (Figure B5. E, F) whereas adult monkeys had the maximum luminal space with active spermatogenesis (Figure B5. G, H) that represents the reproductive state of the animals. Epithelial height and tubular diameter showed a prominent increase with age, data presented in Table 2.3. Adults showed significantly higher epithelial height

($F_{3,11}$=37.82; P<0.0001) and seminiferous tubule diameter ($F_{3,11}$=49.30; P<0.0001) compared to all other developmental groups (Figure B6. and B7., respectively).

5.4.3. Developmental Variation in GnIH Immunoreactivity

Individual and mean number of GnIH-like-ir nerve terminals in mediobasal hypothalamus and arcuate area during developmental process is shown in Table B4. Representative photomicrographs showing GnIH like-ir in infant, juvenile, prepubertal and adult male rhesus monkeys are included in Figure B8. A significant variation in GnIH-ir in mediobasal hypothalamus across pubertal development was noticed ($F_{3,11}$=15.39; P<0.001). More specifically, mean GnIH-ir was found to be significantly higher in prepubertal animals as compared to infants (P<0.01) while it was significantly reduced (P<0.001) in adult animals (Figure B9.). A similar trend was noticed in GnIH-ir in the arcuate area, where GnIH immunoreactivity increased significantly during pubertal development followed by a precipitous decline ($F_{3,11}$=23.50; P<0.0001). A significantly higher GnIH expression was noticed in the prepubertal animals as compared to infants (P<0.01) and juveniles (P<0.001) while a significantly reduced expression was seen in adults as compared to prepubertal (P<0.0001) and infant groups (P<0.05) (Figure B10.).

5.4.4. Pubertal Expression of GnIH-ir Fibers

Individual and mean number of GnIH-ir fibers in mediobasal hypothalamus and arcuate area are presented in Table B5. Expression of GnIH-ir nerve fibers in the mediobasal hypothalamus varied significantly across development ($F_{3,11}$=9.12; P<0.01). Precisely, mean number of GnIH-ir fibers was significantly higher in prepubertal animals as compared to infants (P<0.01) and juvenile animals (P<0.01) while it was found to be significantly reduced in adult animals (P<0.05) as compared

to prepubertal animals (Figure B11.). GnIH-ir fiber expression varied analogously in the arcuate region of male monkeys across development ($F_{3,11}$=19.09; P<0.001). Specifically, mean number of GnIH-ir fibers expressed in the arcuate area of prepubertal animals was significantly higher than infants (P<0.01) and juveniles (P<0.001) while a significantly reduced number of GnIH-ir fibers was seen in adults as compared to prepubertal (P<0.001) animals (Figure B12.).

5.4.5. RT-qPCR

Efficiency curves of primers for *GnRH, Kiss1, GnIH* and *GAPDH* genes are presented in Figure B13. Figures B14.-B16. show comparative changes in the expression of *GnRH*, *Kiss1*, and *GnIH* mRNA in the macaque hypothalamus during pubertal development, respectively. A significant variation was noted in the expression of *GnRH* ($F_{3,11}$=11.58; P<0.001), *Kiss1* ($F_{3,11}$=12.07; P<0.001) and *GnIH* ($F_{3,11}$=14.80; P<0.001) across pubertal development. Significantly higher expression of reproductive genes *GnRH* (P<0.01) and *Kiss1* (P<0.01) in the adult group agree with active breeding state of the adult animals. *GnIH* expression was found to be significantly higher in the prepubertal animals compared to juvenile monkeys (P<0.05) while a sharp decline (P<0.001) in its expression was seen in adult animals as compared to prepubertal animals. A significant, inverse correlation was seen between *GnIH-GnRH* (F=13.34; P<0.01) and *GnIH-Kiss1* (F=17.52; P<0.001) expression (Figure B17. A, B).

Table B1. Individual and mean body weight and testicular volume of male rhesus monkeys of different ages

Parameter	Developmental Stage			
	Infant	**Juvenile**	**Prepubertal**	**Adult**
Body Weight (kg)	0.70	2.30	3.90	9.00
	1.25	2.10	3.80	11.00
	1.15	1.90	4.00	11.50
		1.70	3.10	15.00
mean±SEM	**1.05±0.17**	**2.00±0.12**	**3.70±0.20**	**11.75±1.25**
Testicular Volume (ml)	0.10	0.38	0.42	53.48
	0.16	0.31	0.27	30.63
	0.23	0.34	0.64	39.00
		0.24	0.35	46.83
mean±SEM	**0.13±0.06**	**0.32±0.02**	**0.42±0.07**	**42.49±4.93**

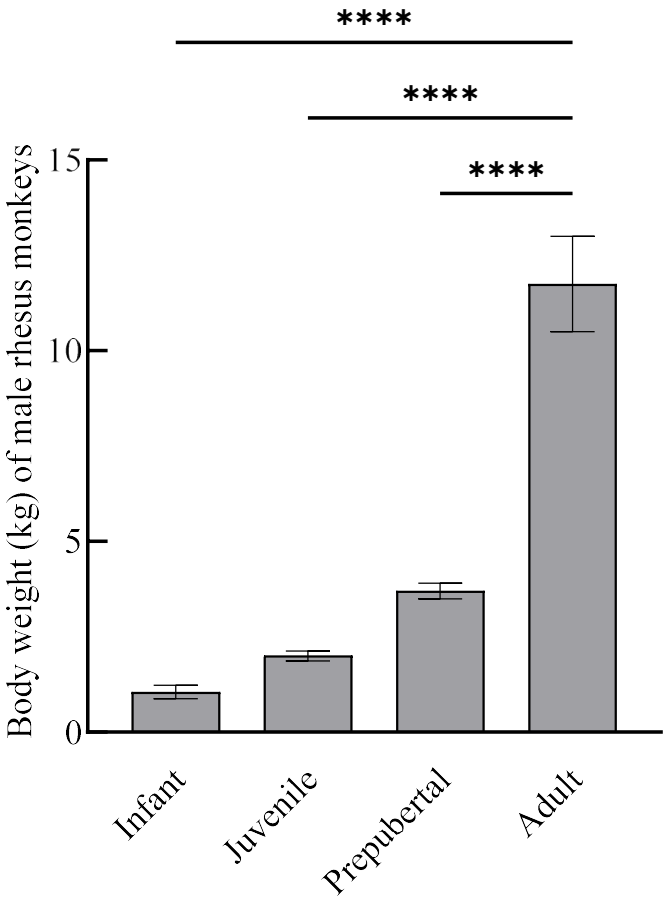


Figure B2. Comparison of mean±SEM body weight of infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4) male rhesus monkeys. One-way ANOVA shows that adults have significantly higher body weight compared to all other groups. ****P<0.0001

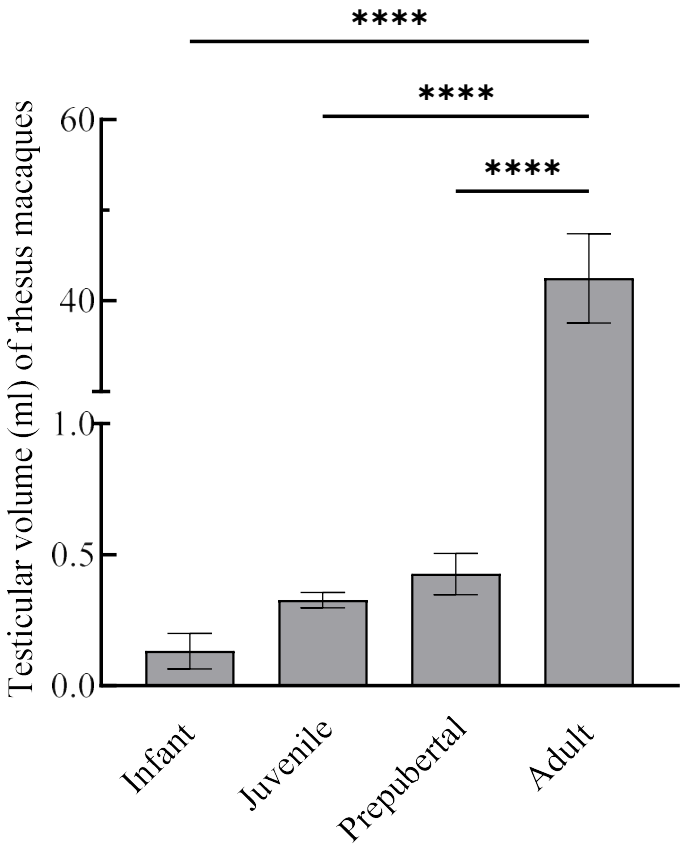


Figure B3. Comparison of mean±SEM testicular volume of infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4) male rhesus monkeys. Adults have higher testicular volume compared to all other groups. ****P<0.0001

Table B2. Plasma testosterone levels observed in individual male rhesus monkeys at different developmental stages

Parameter	Developmental Stage			
	Infant	**Juvenile**	**Prepubertal**	**Adult**
Plasma Testosterone (ng/ml)	0.10	0.05	0.27	0.85
	0.40	0.17	0.34	1.73
	0.23	0.14	0.22	2.82
		0.31	0.05	2.75
mean±SEM	**0.24±0.08**	**0.17±0.05**	**0.32±0.13**	**2.04±0.46**

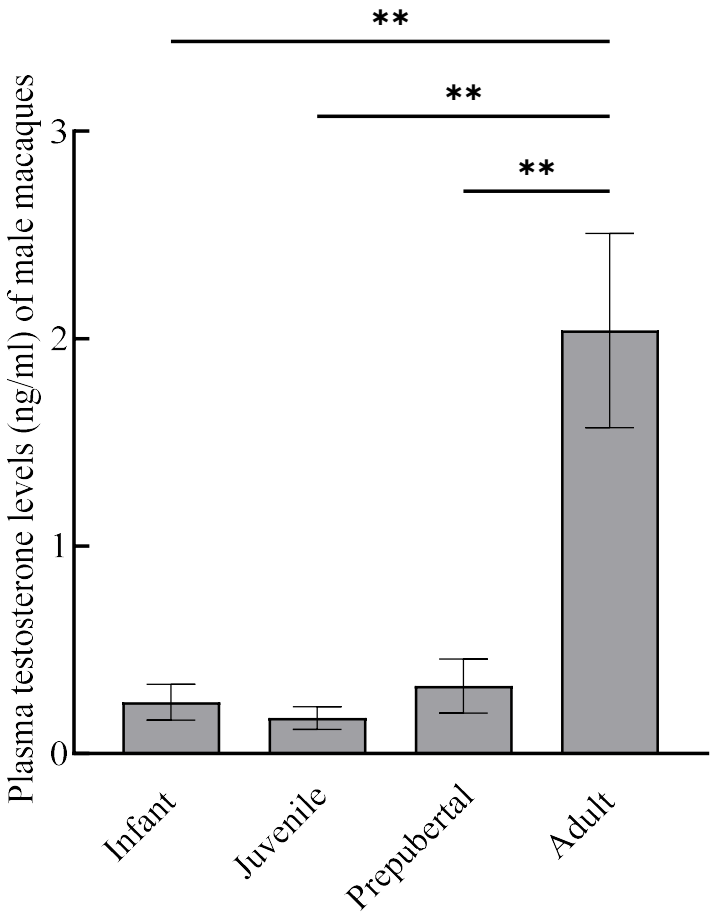


Figure B4. Comparison of mean±SEM plasma testosterone level of infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4) male rhesus monkeys. Mean plasma testosterone concentration of adults was significantly higher compared to all other groups. **P<0.01

Table B3. Individual and mean epithelial height and seminiferous tubule diameter in the testis of male rhesus monkeys through pubertal development

Parameter	Developmental Stage			
	Infant	**Juvenile**	**Prepubertal**	**Adult**
Epithelial height (µm)	17.08	0.00	15.50	63.83
	12.37	16.00	17.33	49.26
	13.08	14.87	17.50	47.88
		17.50	12.63	67.77
mean±SEM	**14.18±1.46**	**12.09±4.06**	**15.74±1.13**	**57.15±5.04**
Seminiferous tubule diameter (µm)	43.50	0.00	44.83	195.00
	33.16	43.60	45.80	178.80
	34.33	46.33	41.20	172.13
		86.10	37.10	212.33
mean±SEM	**37.00±3.26**	**44.01±17.59**	**42.23±1.97**	**189.60±8.98**

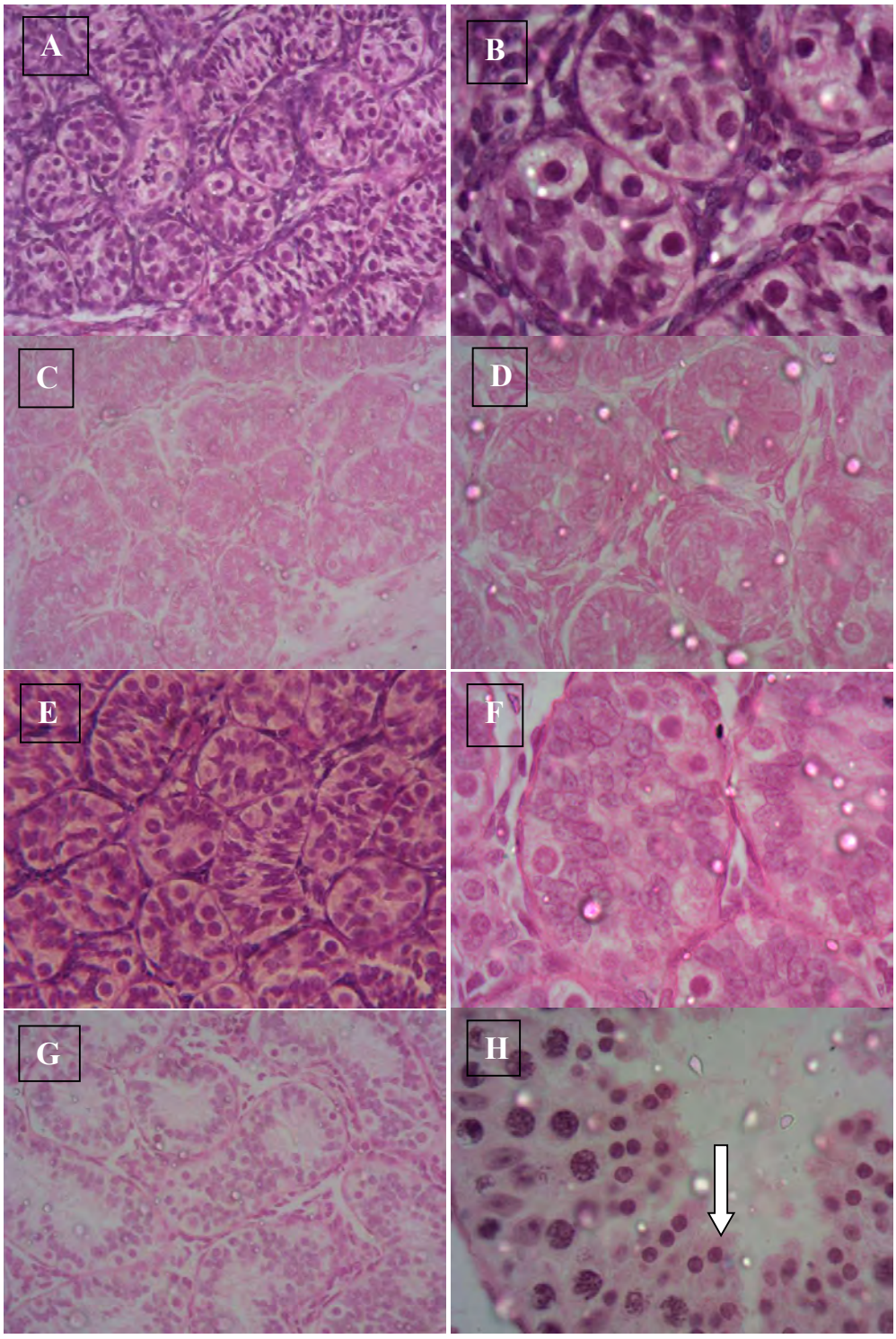


Figure B5. Representative photomicrographs showing testicular histology of infants (A=40x, B=100x), juvenile (C=40x, D=100x), prepubertal (E=40, F=100x) and adult (G=40x, H=100x) male rhesus monkeys. Adult monkeys have active spermatogenesis (shown by an arrow) and a large lumen.

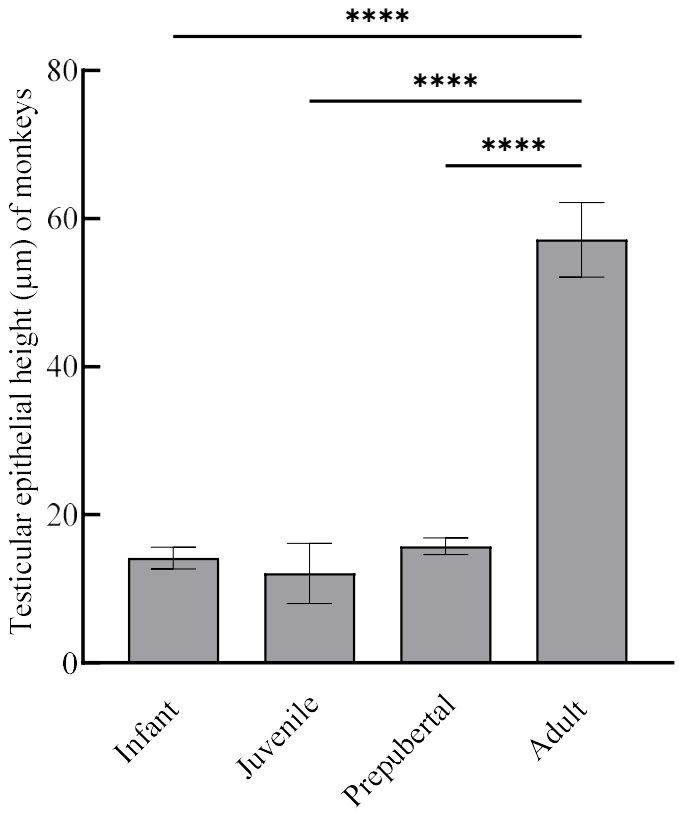


Figure B6. Comparison of mean±SEM epithelial height per tubule of infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4) male rhesus monkeys. Mean epithelial height in adults was significantly higher as compared to all other groups. ****P<0.0001

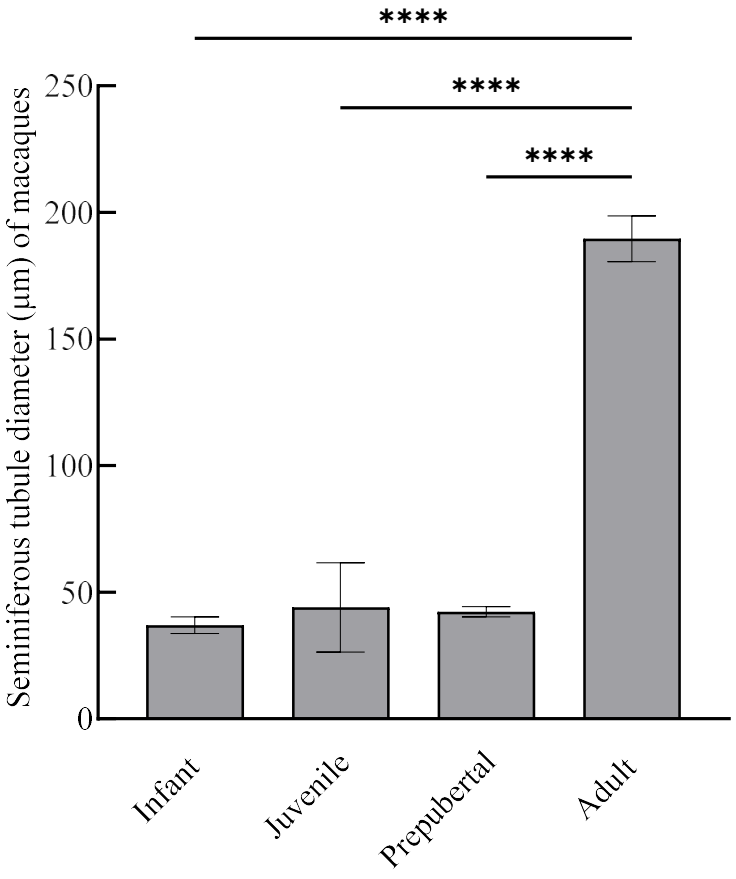


Figure B7. Comparison of mean±SEM seminiferous tubule diameter per tubule of infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4) male rhesus monkeys. Mean tubular diameter in adults was significantly higher than all other ages. ****P<0.0001

Table B4. Individual and mean GnIH-ir observed in mediobasal hypothalamus and arcuate area of male rhesus monkeys at different developmental stages

Region	Developmental Stage			
	Infant	**Juvenile**	**Prepubertal**	**Adult**
Mediobasal Hypothalamus	469	851	731	388
	516	536	1122	342
	496	750	830	301
		517	953	277
mean±SEM	**493.70±13.62**	**663.50±81.83**	**909.00±84.28**	**327.00±24.36**
Arcuate Area	174	248	330	67
	181	192	296	62
	191	119	381	83
		88	296	65
mean±SEM	**184.70±7.44**	**161.80±36.08**	**325.80±20.08**	**69.25±4.69**

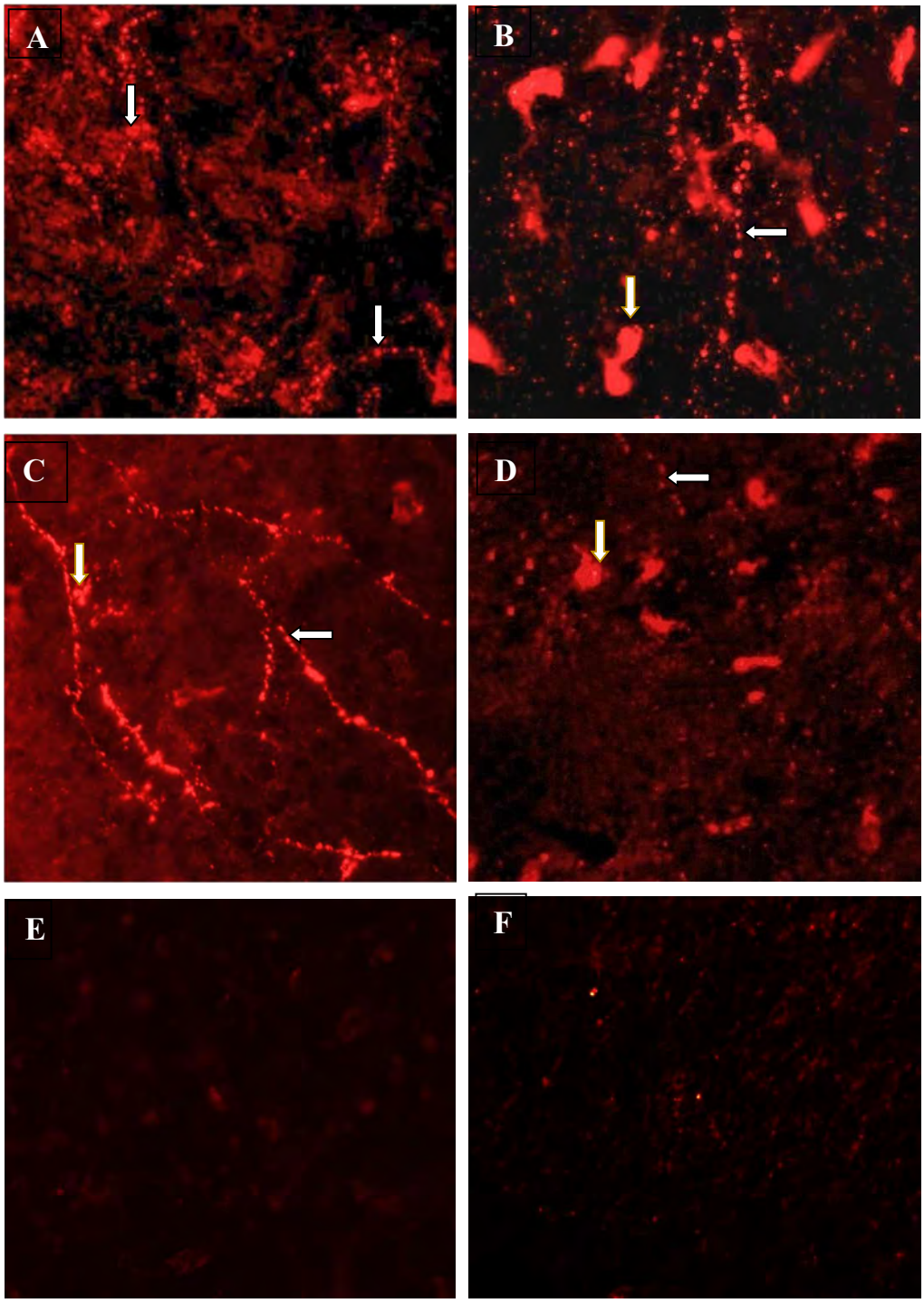


Figure B8. Representative photomicrographs showing GnIH like-ir nerve terminals (gold arrows) and fibers (white arrows) in the MBH of infant (A), juvenile (B), prepubertal (C) and adult (D) male rhesus monkeys. All images were taken at 40X magnification. Primary antibody omitted control sections lacked discrete GnIH like-ir (E, F).

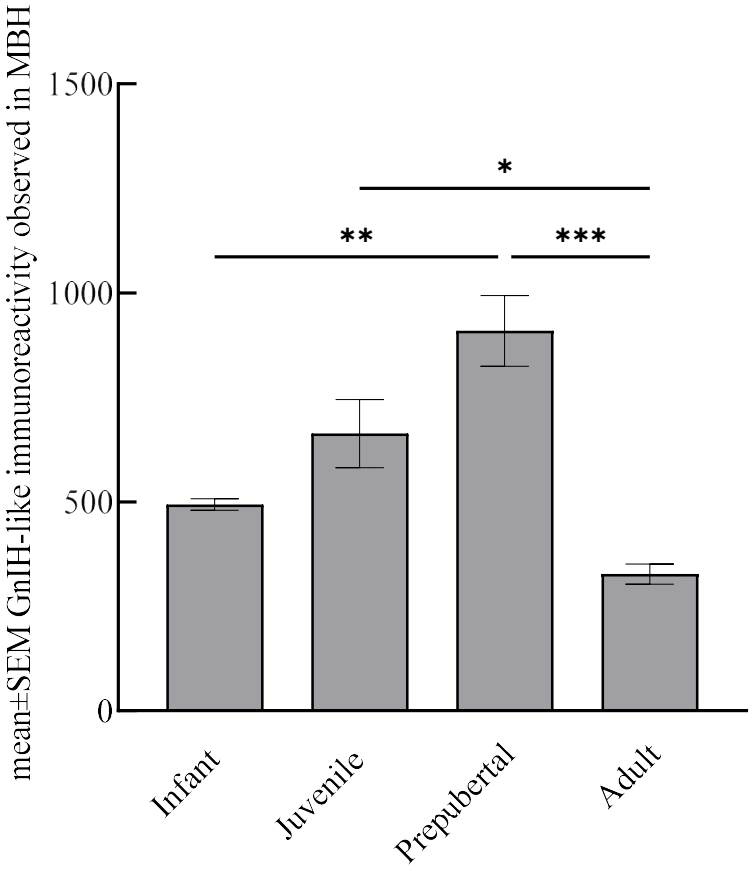


Figure B9. Comparison of mean±SEM GnIH-like immunoreactive nerve terminals observed in mediobasal hypothalamus of infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4) male rhesus monkeys. Mean GnIH-ir in prepubertal monkeys was significantly higher than all other age groups, followed by a significant decline in adult animals. *P<0.05, ** P<0.01, ***P<0.001

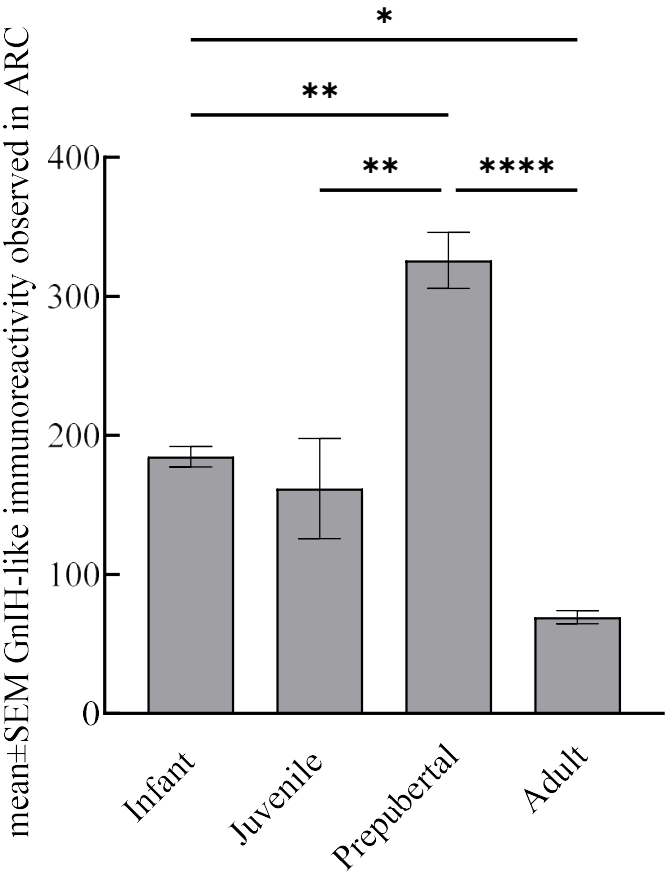


Figure B10. Comparison of mean±SEM GnIH-like immunoreactive nerve terminals observed in arcuate area of infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4) male rhesus monkeys. Mean GnIH-ir in prepubertal monkeys was significantly higher than all other ages while in adults it was significantly lower compared to all other groups. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Table B5. Individual and mean number of GnIH-ir fibers observed in mediobasal hypothalamus and arcuate area of male rhesus monkeys through pubertal development

Region	Developmental Stage			
	Infant	**Juvenile**	**Prepubertal**	**Adult**
Mediobasal Hypothalamus	278	326	723	280
	240	240	465	321
	205	292	392	307
		274		303
mean±SEM	**241.00±21.08**	**283.00±17.94**	**526.70±100.40**	**302.80±8.50**
Arcuate Area	113	108	338	74
	136	97	230	73
	126	98	205	132
		95		117
mean±SEM	**125.00±6.65**	**99.50±2.90**	**257.70±40.81**	**99.00±15.04**

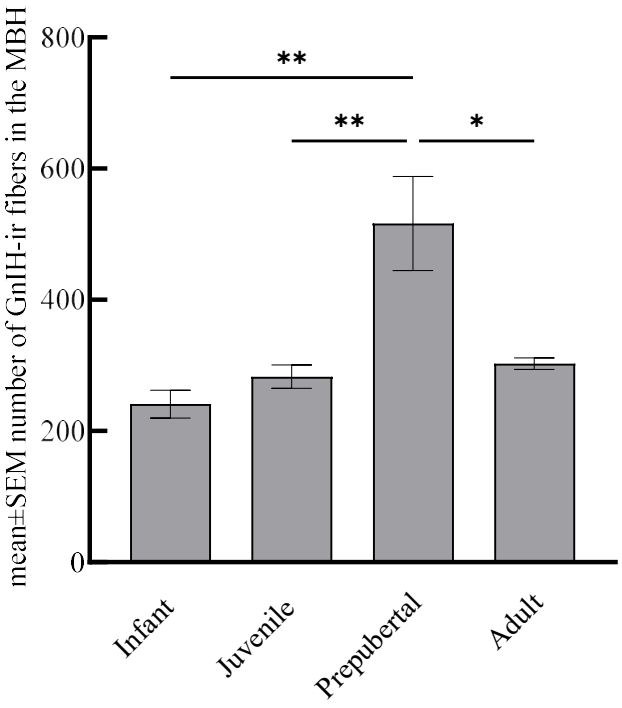


Figure B11. Comparison of mean±SEM number of GnIH-ir nerve fibers observed in mediobasal hypothalamus of infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4) male rhesus monkeys. The number of GnIH-ir fibers increased significantly till prepubertal stage of development followed by a significant decline in adult age. *P<0.05, **P<0.01

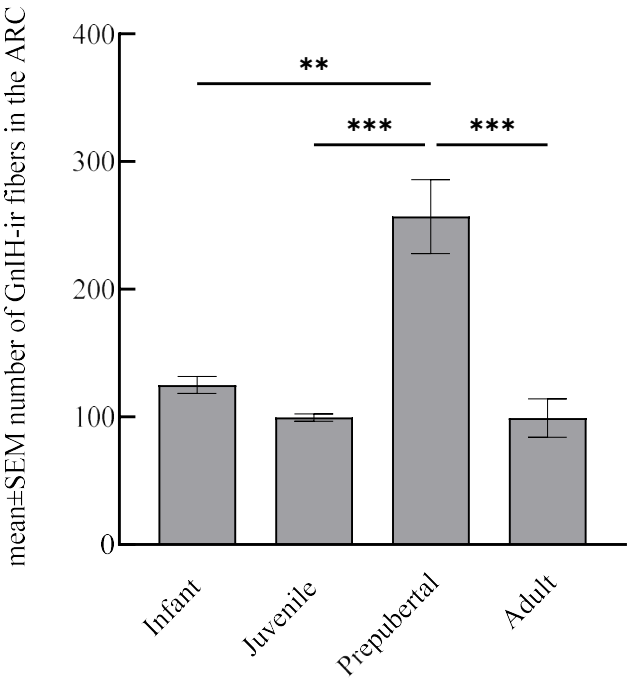


Figure B12. Comparison of mean±SEM number of GnIH-ir fibers observed in arcuate area of infant (n=3), juvenile (n=4), prepubertal (n=4) and adult (n=4) male rhesus monkeys. Number of GnIH-ir fibers in arcuate region increased significantly through postnatal development, declining significantly in adult age. **P<0.01, ***P<0.001

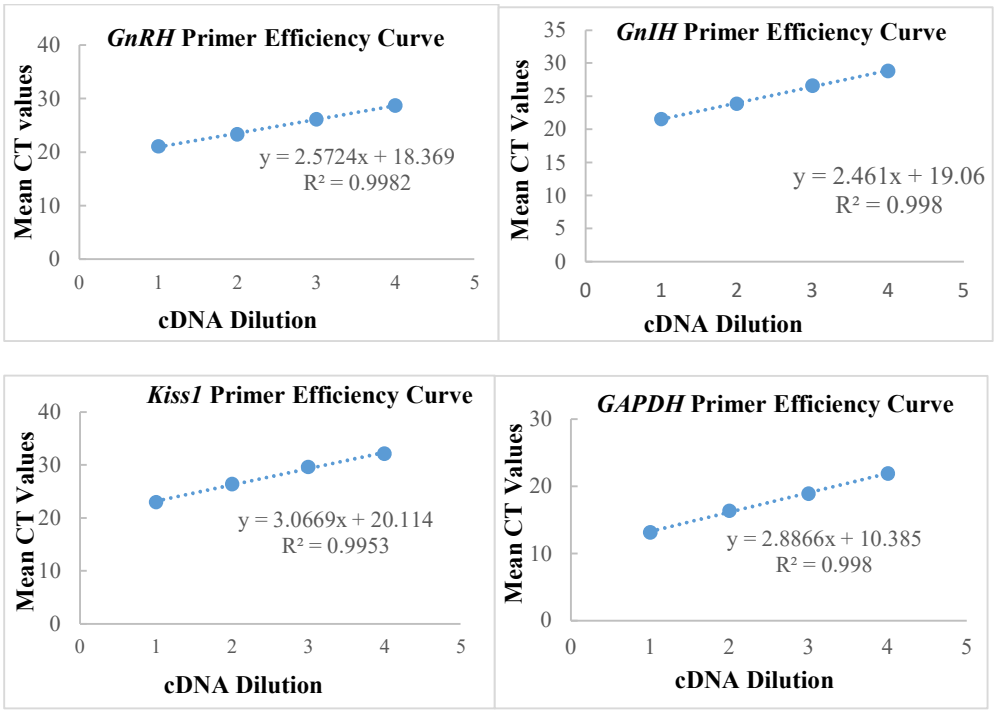


Figure B13. Determination of efficiency of primers to amplify the *GnRH, Kiss1, GnIH* and *GAPDH* genes. The mean threshold cycles of 4 dilutions 1:1, 1:5, 1:50 and 1:500 is represented as 1, 2, 3 and 4 data points on x-axis.

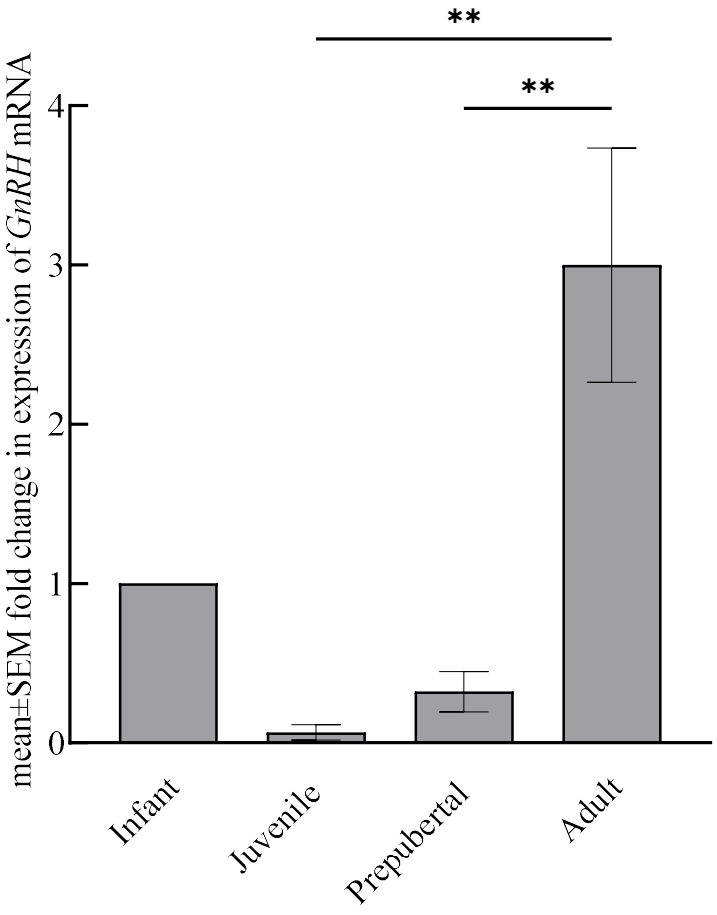


Figure B14. mean±SEM fold change in expression of *GnRH* mRNA during pubertal development in male rhesus monkeys. *GnRH* mRNA expression was significantly higher at adult stage compared to all other age groups. **P<0.01

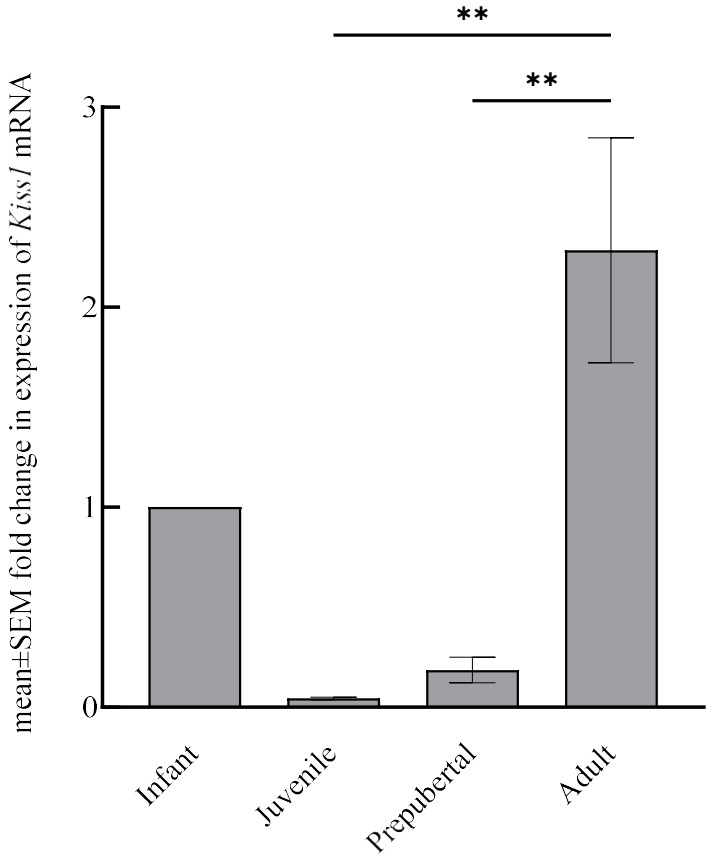


Figure B15. mean±SEM fold change in expression of *Kiss1* mRNA during pubertal development in male rhesus monkeys. *Kiss1* expression was significantly higher in adult animals compared to all other ages. **P<0.01

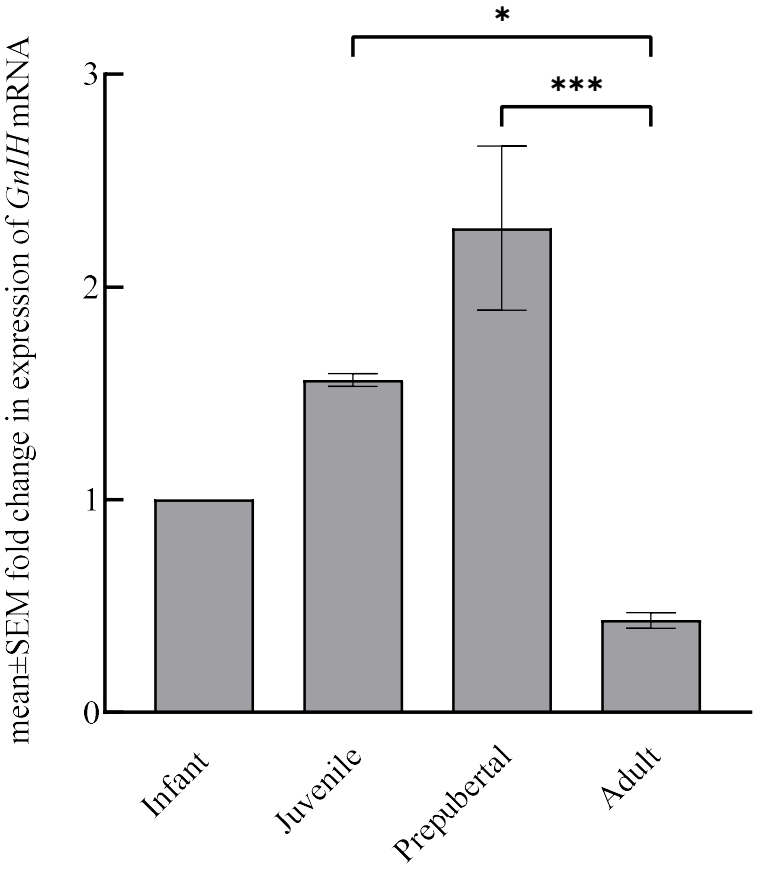


Figure B16. mean±SEM fold change in expression of *GnIH* mRNA during pubertal development in male rhesus monkeys. *GnIH* mRNA expression increased till prepubertal stage, followed by a significant decline at adult age. *P<0.05, ***P<0.001

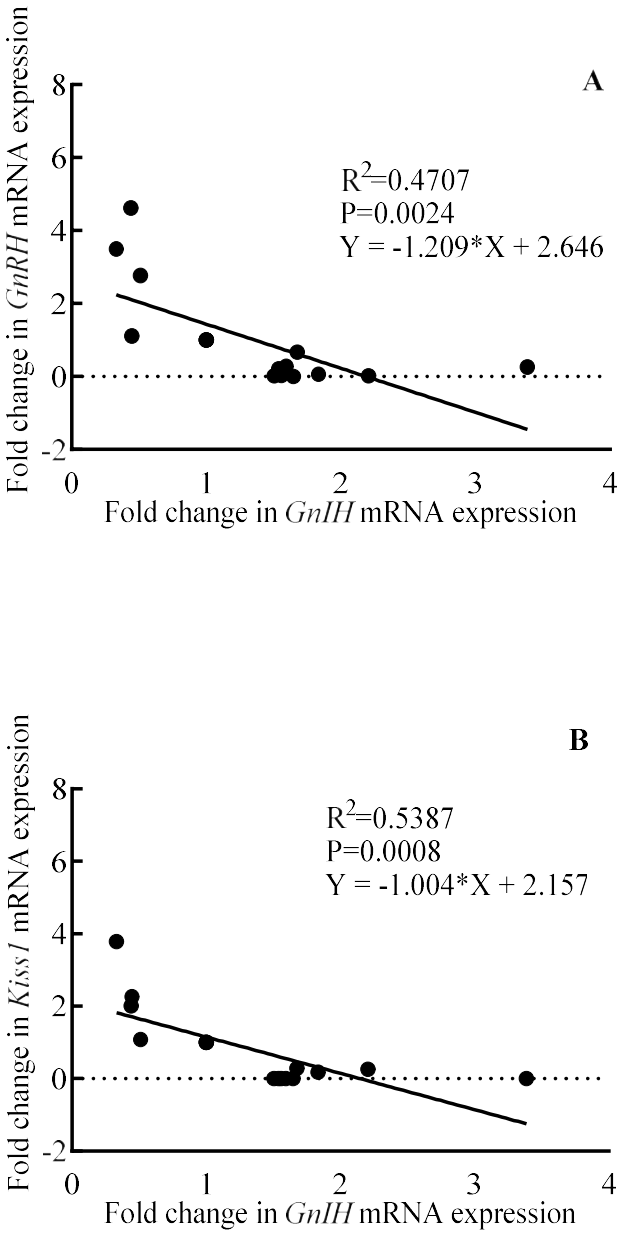


Figure B17. Correlation between hypothalamic fold change in *GnRH* and *Kiss1* expression with *GnIH* expression during pubertal development in male rhesus monkeys. Significant correlation was seen between *GnRH* and *GnIH* (A; **P<0.01) and *Kiss1* and *GnIH* expression (B; ***P<0.001)

5.5. Discussion

Gonadotropin releasing hormone's release from birth till onset of puberty follows two basic patterns in higher primates. GnRH release is robust during infancy causing steroidogenesis but not gametogenesis (Plant and Witchel, 2006; Terasawa and Kurian, 2012). Then, during the juvenile phase of development GnRH release gets dampened resulting in relative quiescence of the reproductive axis due to hypogonadotropism (Plant and Witchel, 2006; Terasawa and Kurian, 2012). At the end of prepubertal phase of development in primates, pulsatile GnRH secretion in the portal blood resumes causing the release of pituitary gonadotropins, that ultimately act on the gonads. In response to pituitary gonadotropins, gonads start producing the sex steroid hormones and the gametes, thus activating the reproductive axis, also known as onset of true puberty (Terasawa *et al*., 2013).

This break in the release of GnRH before onset of puberty can be seen as a neural brake, halting GnRH production till puberty onset (Plant, 1988). Worthy to mention here is that this conceptual brake can be brought about by either the enforcement of an inhibitory signal and/or dampening of a stimulatory drive to the GnRH neurons (Plant and Witchel, 2006). Terasawa and Fernandez. (2001) proposed the idea that this biological brake can be an inhibitory neurocircuitry lying upstream to GnRH neurons. Since the discovery of GnRH neuronal system in 1970s (Matsuo *et al*., 1971), a lot of research has been done to decipher the switch that turns the pulsatile GnRH release on at the end of juvenile phase of development triggering the onset of puberty (de Roux *et al*., 2003; Seminara *et al*., 2003). Kisspeptin neurons have emerged as an important player in the pubertal onset and regulation of the gonadal function by maintaining the activity of the neuroendocrine axis during adulthood, in primate and non-primate species (Semple *et al*., 2005). But the switch that turns the GnRH release off in the

infantile primates has received little attention. In the year 2000, Tsutsui and colleagues identified a neurohormone that inhibited pituitary gonadotropin release in quail brain and named it gonadotropin inhibitory hormone (GnIH) (Tsutsui *et al*., 2000). Since its discovery, a plethora of research in various species suggests that GnIH may act as part of this conceptual neurobiological brake that keeps the GnRH release in check during pubertal development. But its role in regulation of reproductive axis activity during pubertal development in higher primates has never been studied before. Therefore, the present study was designed with the aim to elucidate the regulation of HPG-axis activity by GnIH neuronal signaling during pubertal development in non-human primates. Another aim of this study was to decipher the variation in interaction of GnIH neurons with elements of the GnRH pulse generator (a network of neurons stimulating the release of GnRH) in the hypothalamus of rhesus monkey during postnatal development.

Our immunofluorescence and qPCR data shows a significant increase in the expression of GnIH-ir nerve terminals and fibers during the prepubertal phase of postnatal development as compared to the infantile male rhesus monkey, a representative higher primate. Plasma testosterone level, testicular volume and various histological features correspond to an inactive reproductive axis in these animals. Then, a precipitous decline in the GnIH-ir neuronal elements was seen in adult animals. These animals had significantly higher plasma testosterone level and testicular volume compared to the prepubertal animals. Their testicular histology showed a fully active reproductive axis with sufficient spermatogenesis. Our results are in line with previous findings in mice where GnIH immunoexpression was found to be significantly decreased in pubertal animals compared to prepubertal animals (Poling *et al*., 2012; Semaan and Kauffman, 2015). Thus, it can be inferred that higher GnIH signaling before the onset of puberty

causes a hiatus in the reproductive axis activity keeping steroidogenesis and gametogenesis in check while reduced GnIH signaling in adult animals causes the onset of puberty by reactivation of the reproductive axis activity in these animals.

We also quantified the *GnRH* and *Kiss1* mRNA expression in male rhesus monkeys of various developmental ages. Our results show a higher *GnRH* and *Kiss1* mRNA expression in the adult animals compared to juvenile and prepubertal animals. These findings are consonant with previous findings in rodents and primates (Navarro *et al*., 2004a; Shahab *et al*., 2005; Keen *et al*., 2008; Takumi *et al*., 2011; Ramaswami *et al*., 2013). Significant negative correlation between *GnIH*-*GnRH* and *GnIH*-*Kiss1* mRNA expression was another interesting finding of this study. This finding further strengthens our postulation that higher GnIH signaling during juvenile and prepubertal phase of development, keeps GnRH pulse generator activity in check by downregulating the *Kiss1* expression in the hypothalamus while a decrease in the GnIH signaling at the end of prepubertal phase brings about the resumption of the GnRH pulse generator activity, thus activating the reproductive axis.

GnIH-ir nerve terminals and fibers were seen in midline hypothalamic nuclei (ARC and PVN) in this study. This complies with previous findings in other species (Clarke *et al*., 2008, Kriegsfeld *et al*., 2006) and in higher primates (Ubuka *et al*., 2009a; 2009b). A subtle variation in the expression of GnIH neuronal elements was noticed with advancing age in rhesus monkeys. This may imply that GnIH may directly inhibit GnRH neuronal signaling or might do so via intermediary neuronal systems present in these brain areas, for example kisspeptin neuronal population in the ARC region. It is also highly plausible that GnIH regulates GnRH pulse generation activity of an individual based on energy state of the body, via interaction with the energy sensitive POMC neurons also present in the ARC nucleus (Elmquist *et al*., 1999; Qu *et al*.,

2020). It also suggests that GnIH might regulate other physiological functions by modulating the activity of other neuronal systems present in MBH nuclei like regulation of prolactin release via dopamine neurons present in ARC (Ben-Jonathan and Hnasko, 2001). Similarly, GnIH can downregulate the reproductive axis activity under stressful conditions by modulating the activity of hypothalamic pituitary adrenal axis by directly affecting the corticotropin releasing hormone (CRH) secretion from the PVN (Einarsson *et al*., 2008).

In summary, the hiatus in the reproductive axis activity during juvenile and prepubertal phase of development in higher primates appears to coincide with a rise in the GnIH tone, as evidenced by increased GnIH immune- and gene expression at these stages. Furthermore, negative correlation of *GnIH* expression with *GnRH* and *Kiss1* expression implies that GnIH might serve as an important player in the neurobiological brake on the reproductive axis activity by decreasing the kisspeptin and GnRH release directly, or via intermediary neuronal systems. We also suggest that other than reproductive axis activity, GnIH might also regulate other neuronal activities such as motivated behaviors including reproductive behavior.

GENERAL DISCUSSION

6. General Discussion

HPG-axis governs the reproductive process and is mandatory for the completion of reproductive activity. HPG-axis regulation is mediated by various neuropeptides that are synthesized and released by hypothalamus and pituitary gland that in turn regulate the gonadal function. Gonadotropin inhibitory hormone is found to be a major regulator of HPG-axis along with GnRH, kisspeptin and other various neurohormones in vertebrate species.

GnRH was discovered in the 1970s as a hypothalamic decapeptide that triggers the production of LH/FSH from the adenohypophysis in mammals (Matsuo *et al*., 1971; Burgus *et al*., 1972). Although more than 20 different sequences of GnRH have been found in various vertebrate species, its amino acid sequence is found to be highly conserved (King and Millar, 1982; Miyamoto *et al*., 1982; 1984; Sherwood *et al*., 1986). Decades long comprehensive experimentation on this neuropeptide led to the development of the notion that it is the only neurohormone governing pituitary gonadotropin release in vertebrate species. However, three decades after, Tsutsui and group identified a neuropeptide of the hypothalamic origin that inhibited the LH/FSH release in the quail and named it GnIH (Tsutsui *et al*., 2000).

Subsequent research of Tsutsui and colleagues has proven that sequence of GnIH is conserved in all vertebrates (Tsutsui *et al*., 2006; Tsutsui, 2009; Tsutsui *et al*., 2012; Kriegsfeld *et al*., 2015; Tsutsui, 2016), and that GnIH can act as an inhibitory neuro-signal for the reproductive system. In addition, GnIH is shown to have a multitude of actions other than reproductive system regulation (Tobari *et al*., 2014; Ubuka *et al*., 2014).

Recent reports on the GnIH system have proposed that irregular expression of GnIH may lead to pubertal disorders and reproductive system malfunction in mammals. But developmental and pubertal variation in GnIH expression with sufficient spatiotemporal detail has never been studied in rodents and higher primates. Therefore, this study was designed with the aim to delineate the expression profile of GnIH during prenatal and postnatal development in mice (*Mus musculus*) and during the pubertal development in male rhesus monkeys (*Macaca mulatta*). Another aim was to decipher the GnIH regulation of the HPG-axis activity via its correlation with stimulatory neuropeptides; GnRH and kisspeptin.

These objectives were achieved by performing different immunostainings and gene expression analyses. Firstly, we performed dual labelled immunostaining for GnIH and GnRH neuronal elements in whole brain of mice at various prenatal and postnatal stages. Additionally, GnIH expression level of male and female mice at various developmental time points was compared to decipher the role of GnIH in sexual differentiation. Secondly, expression pattern of GnIH during pubertal development in male rhesus monkeys was studied at various postnatal stages via single label immunostaining and RT-qPCR. *GnRH* and *Kiss1* mRNA were also quantified and correlated to *GnIH* expression at various postnatal developmental stages. Body weight, testicular volume, plasma testosterone levels and testicular histology of rhesus monkeys was also evaluated to confirm the age and reproductive state of the animal.

Overall, our results demonstrate that reproductive axis activity through development and puberty onset is governed by excitatory and inhibitory inputs in mice and monkeys. In first part of this dissertation, we quantified the number of GnIH-ir cells in DMH of male and female mice during prenatal and postnatal development. This is a novel approach to study the ontogenetic trends in GnIH expression from embryonic till

postnatal stages and which describes the meaningfulness of the present study to delineate the role of GnIH signaling in development of the reproductive-axis elements. To further add to the meaningfulness of this study, GnIH-ir nerve terminals and fibers were also counted at the whole brain level, specifically the ARC and PVN regions as these brain regions play very important role in reproductive axis activity and age-related variation in the GnIH peptide content of these regions has never been studied before. To confirm the inhibitory effect of GnIH neurosecretion on the reproductive axis activity, axosomatic and fiber to fiber contacts between GnIH and GnRH were also quantified. This approach further adds to the novelty of our study since this has never been studied before and has never been correlated with age-specific maturational events of the reproductive axis in any species. Results of this study demonstrate that the GnIH expression modulates at various time points, being the highest near term and at PND 18, a developmental stage with minimal reproductive activity. Results of this study reveal that decreased reproductive axis activity might be caused by the reduced GnRH pulse generation, as number of axosomatic and fiber to fiber contacts between GnIH and GnRH were found to be increased at this time point, increasing a possibility of the direct inhibition of the GnRH neuronal activity. Results of this study also hint at the possible role of GnIH in sexual differentiation as GnIH expression was found to be higher in females than in males across development.

GnIH-ir cells were found to be exclusively localized in the DMH of male and female mice across development. Our results accord with the earlier reports in rodents (Kriegsfeld *et al*., 2006), macaques (Ubuka *et al*., 2009a) and humans (Ubuka *et al*., 2009b). Another novel finding of this study is that the number of GnIH-ir cells in the DMH were found to be higher in females than in males at all developmental stages which suggests that different mechanisms might be operative in regulating the GnIH

expression in males and females. This finding is consonant with the fact that ovaries are quiescent before puberty and do not contribute to sexual differentiation (Picut *et al*., 2015). This finding is in contrast with one previous finding which reported no significant difference among GnIH cell number in adult male and female mice (Poling *et al*., 2012). This difference might be due to the experimental technique applied, as we measured the peptide, and they measured the mRNA content and there is a possibility of sex differences in post-transcriptional events that change protein content even when mRNA is the same and we believe that peptide expression is more likely to be of more functional relevance than mRNA expression.

Other than this sex difference, a significant rise was noted in the number of GnIH-ir cells at E18.5. Reproductive axis is reported to be suppressed near term in mice (Kuiri-Hänninen *et al*., 2014) and this rise in the number of GnIH-ir cells might contribute to this suppression. Then, the reproductive axis reactivates and is reported to remain active for a few days after birth, as evidenced by the detectable circulatory testosterone levels (Bell, 2018). We saw a transient reduction in the number of GnIH-ir cells in DMH of both sexes at this stage which might contribute to the activation of gonadal steroidogenesis. Plasma testosterone levels are then reported to decline a few days after birth and remain low until puberty (Bell, 2018). At this age (PND18), we observed a significant rise in the GnIH-ir cells that might contribute to the prepubertal hiatus of the reproductive axis either directly via inhibiting GnRH pulse generation or indirectly by intermediary neuronal systems. Then again, a precipitous drop in GnIH-ir in the DMH of males and females was noted at PND40. Reproductive axis is reported to be active at this stage as mice attain puberty (Poling *et al*., 2012). This finding is in agreement with previous reports where GnIH protein expression was seen to be significantly higher during juvenile period (PND15 to PND20) in male and female

mice followed by a decrease in the GnIH-ir as mice reach adulthood (Poling *et al*., 2012, Semaan and Kauffman, 2015). This decline in number of GnIH-ir cells might contribute to removing the brake on the GnRH pulse generation, fully activating the HPG-axis.

For a comprehensive ascertainment of the GnIH paradigm during the pubertal development, GnIH innervation of the midline hypothalamic nuclei (medial septum/diagonal band of Broca (dBB), pre-optic area (POA), anterior hypothalamus, and PVN) was also quantified in the present study. Distribution of GnIH-ir nerve terminals and fibers in the midline hypothalamic regions is consonant with previous studies in mammals (Kriegsfeld *et al*., 2006). GnIH immunoreactive fibers are seen to be present in proximity with GnRH neurons in the POA in both sexes. This finding is in agreement with previous studies in rodents (Kriegsfeld *et al*., 2006, Johnson *et al*., 2007), sheep (Smith *et al*., 2008) and humans (Ubuka *et al*., 2009b). A considerable variation in the GnIH innervation of various hypothalamic regions was noted during the pre- and post-natal development in mice which hints at the likelihood that GnIH system may function *via* various intermediary neural systems to control the activity of the HPG-axis during the developmental process.

An important finding of this study is the delineation of GnIH-ir in the arcuate nucleus of mice. During embryonic development, the ARC region showed almost no signs of GnIH-ir while a few cells and a large number of GnIH nerve terminals and fibers with many presumptive boutons were seen in this region on PND18. This observation suggests that during the juvenile period, GnIH might directly suppress the GnRH neuronal activity or might indirectly do so via kisspeptin neuronal subpopulation that also resides in the ARC region (Yeo and Herbison, 2011). Variation in expression of GnIH nerve terminals in the ARC area during the postnatal development also suggests

that GnIH neuronal system can modify the activity of the reproductive system according to nutritional state of the individual, turning it on when an individual attains the required body mass at puberty, as the ARC neurons are sensitive to nutritional state of the individual (Elmquist *et al*., 1999; Qu *et al*., 2020). Functional studies in the mice have also reported changes in fasting- and -obesity associated hypogonadotropism in NPFF1R null mice (Leon *et al*., 2014; Leon and Tena-Sempere, 2016).

Interestingly, a robust rise was seen in GnIH nerve terminals and fibers' expression in the paraventricular nucleus on PND18. PVN is a bilateral structure that regulates neuroendocrine and autonomic functions (Swanson and Sawchenko, 1980; Williams *et al*., 2000). It is reported to contain cell bodies of neurons regulating hypothalamic-pituitary-adrenal axis (HPA), thyroid axis and growth and development along with reproductive axis (Ferguson *et al*., 2008). HPA-axis is reported to suppress the reproductive activity under stressful conditions (Einarsson *et al*., 2008) while a normal thyroid function is mandatory to maintain normal reproduction (Krassas *et al*., 2010). It suggests that other than direct effect, GnIH system might indirectly regulate the GnRH neuronal activation via these intermediary neuronal systems and other hormonal processes mediated by these neuronal elements present in the PVN.

In summary, this study's findings demonstrate that the hypothalamic-pituitary axis is functional in the midgestational fetus and becomes inactive during the latter part of pregnancy in male and female mice. Then at birth, this axis gets reactivated and again becomes quiescent until the onset of true puberty. Presently, variation in the expression profile of hypothalamic neuropeptide GnIH over the gestational and postnatal growth in mice correlates with the reproductive axis activity. It suggests that the increase in GnIH-ir in midline hypothalamic regions near term and during juvenile/prepubertal

period may help to transfer the effect of the neural inhibition to the reproductive axis and regulate multiple motivated behaviors.

Two major hypothalamic events governing the GnRH release regulate the puberty onset in higher primates (Plant and Witchel, 2006; Terasawa and Kurian, 2012). At first, a decline in the pulsatile GnRH secretion at late infancy causes reduced pituitary gonadotropins release that continues through the remainder of the pubertal development, ensuring a hiatus in the reproductive activity in juveniles (Plant and Witchel, 2006; Terasawa and Kurian, 2012). Then, reactivation of reproductive axis activity marked by initiation of steroidogenesis due to robust increase in GnRH pulsatility marks the second phase of pubertal onset, also known as true puberty (Plant and Witchel, 2006; Terasawa and Kurian, 2012).

Since reproductive axis activity undergoes striking changes during postnatal development in higher primates, in the second part of this dissertation we determined the expression profile of GnIH neuronal elements in male rhesus monkey, a representative higher primate. For this purpose, we quantified the number of GnIH-like-ir terminals and fibers in the mediobasal hypothalamus, specifically in the ARC and PVN regions at various stages of pubertal development.

Secondly, mRNA expression of *GnIH*, *GnRH* and *Kiss1* were also quantified, compared, and correlated at various ages of pubertal development. GnIH expression and signaling was found to vary across development, being the highest at the prepubertal stage and the lowest in adult animals. These results show that prepubertal hiatus of the reproductive axis activity might be caused by the higher expression and signaling of GnIH neuronal system. It can be postulated that increased GnIH signaling at prepubertal stage causes reduced GnRH pulse generation, ultimately leading to decreased steroidogenesis and gametogenesis. And decrease in GnIH signaling releases

this break on GnRH pulse generation causing the onset of true puberty. Significant age-related negative correlation of *GnIH* with *GnRH* and *Kiss1* mRNA expression provides evidence for this postulation. Lower plasma testosterone level and testicular volume with reduced spermatogenesis in prepubertal animals compared to adult animals further strengthens this hypothesis.

Our immunofluorescence data is in line with the previous findings where GnIH-like-ir nerve terminals are reported in MBH of rodents (Kriegsfeld *et al*., 2006), birds (Ubuka *et al*., 2008), monkeys (Ubuka *et al*., 2009a) and humans (Ubuka *et al*., 2009b). Abundant GnIH-ir fibers were seen in ARC, PVN and POA. This observation complies with previous findings in quail (Ukena *et al*., 2003), rats (Yano *et al*., 2004) and rhesus macaque (Ubuka *et al*., 2009a). Based on this finding, it can be postulated that GnIH might downregulate the reproductive axis activity by direct inhibition of GnRH neuronal activity or might indirectly do so by acting on kisspeptin neurons, which are also present in the arcuate region (Terasawa *et al*., 2013). The presence of GnIH-ir fibers in these brain areas also suggests that GnIH might have role in regulating different physiological functions like homeostasis and instinctive behaviors (Nestler, 2001; Leknes and Tracey, 2008). For example, dopamine neurons are reported to be present in the ARC and periventricular regions of the hypothalamus and project to median eminence, working as major regulatory system for prolactin release (Ben-Jonathan and Hnasko, 2001). Therefore, it can be presumed that GnIH increases prolactin release by hindering the activation of dopaminergic neuronal cells, as reported in rodents (Hinuma *et al*., 2000).

These brain regions are equally important for reproductive behavior in vertebrates (Schober and Pfaff, 2007) indicating that GnIH signaling may function to regulate sexual behavior. Presence of GnIH-ir fibers in these regions in macaque brain makes it

highly plausible that GnIH may regulate sexual behavior in this species as well as GnIH's central administration to white crowned sparrows (Bentley *et al*., 2006) and rats (Johnson et al., 2007) reduces sexual behavior in these species. Similarly, presence of GnIH-ir fibers in the ARC area makes it plausible that they may interact with β-endorphin neurons and POMC neuronal elements also expressed in these regions, (Bancroft, 2005) to regulate the sexual activity in different physiological conditions.

Present study revealed a significant increase in the expression of GnIH-ir terminals at the prepubertal stage, followed by a precipitous decline in adult animals. This finding is in line with previous reports in rodents where significant reduction in GnIH expression was seen in adult mice as compared to prepubertal mice (Poling *et al*., 2012; Semaan and Kauffman, 2015) and with findings of chapter A of this study, where significantly higher expression of GnIH cells is seen in PND18 mice followed by a significant decline at PND40 stage. In primates, reproductive axis is reported to be active during infancy (also termed as minipuberty) (Terasawa *et al*., 2013) with robust GnRH pulse generator activity caused by kisspeptin release. Then, kisspeptin and GnRH release is reported to pause during the juvenile and prepubertal phase of development (Terasawa *et al*., 2013). At this age, we observed a significant rise in the expression of GnIH-ir neuronal elements which suggests that prepubertal hiatus in the reproductive axis activity might be caused by higher GnIH signaling at this age. Then at the end of the prepubertal phase, GnRH pulse generation is reported to resume its proper functionality indicating the onset of true puberty, marked by initiation of steroidogenesis and gametogenesis (Terasawa *et al*., 2013). At this point, we observed a drop in GnIH neuronal elements' expression which correlates with the reproductive axis activity in adult monkeys. Plasma testosterone level and testicular volume with evident spermatogenesis, were found to be higher in adult animals which corresponds

to the reproductive axis activation at this phase of development (Plant *et al*., 2005; Garcia *et al*., 2018).

Adult animals had significantly higher *GnRH* mRNA expression than prepubertal and juvenile monkeys. This finding is consonant with previous reports where GnRH pulse generation activity was found to be lower in juvenile animals (Ramaswami *et al*., 2013). Another finding of this study is the gradual increase in *Kiss1* expression from juvenile to adult stage of development. This finding agrees with previous reports in rodents (Navarro *et al*., 2004a; Takumi *et al*., 2011) and primates (Shahab *et al*., 2005; Keen *et al*., 2008) where higher *Kiss1* expression is noted in adult animals. This finding also agrees with a study in human females where higher *Kiss1* levels were noted in girls with precocious puberty (de Vries *et al*., 2009). Our finding contrasts with one previous report where *Kiss1* expression did not vary significantly in prepubertal female monkeys and ovariectomized adult monkeys (True *et al*., 2017). This discrepancy in results might be either due to gender or gonadal status difference. Interestingly, GnRH response induced by administration of kisspeptin agonists to female monkeys is reported to be more robust than in males, which suggests that males might be subjected to stronger central neural inhibition (Gurriero *et al*., 2012b; Garcia *et al*., 2018).

Inverse age-related correlation of *GnIH* mRNA expression with *GnRH* and *Kiss1* mRNA expression reported in this study advances the assumption that GnIH might regulate GnRH pulse generation through sexual development. This notion is further strengthened by the observation that GnIH administration reduces the firing activity of GnRH neurons (Ducret *et al*., 2009; Wu *et al*., 2009) and immediate early gene expression in them (Anderson *et al*., 2009). GnIH application is also reported to reduce gonadotropin release in isolated pituitary cells in sheep (Clarke *et al*., 2008; Sari *et al*.,

2009). Centrally administered GnIH reduces gonadotropin release in white crowned sparrows (Bentley *et al*., 2006), Syrian hamsters (Kriegsfeld *et al*., 2006) and rats (Johnson *et al*., 2007). Similarly, peripheral application of GnIH also reduces pituitary gonadotropin release in birds (Osugi *et al*., 2004; Ubuka *et al*., 2006), rodents (Kriegsfeld *et al*., 2006; Rizwan *et al*., 2009), sheep (Clarke *et al*., 2008) and cattle (Kadokawa *et al*., 2009). Based on present findings, it can be inferred that decline in GnIH led inhibition towards the end of prepubertal age, might have a role in chartering kisspeptin-dependent excitatory signal to GnRH cells triggering the puberty onset.

Taken together, results of both experiments conducted for this dissertation complement each other and suggest a plausible mechanism of neuroendocrine regulation of the onset of puberty in higher primates. Based on the observations of this study, it can be suggested that GnIH acts as an important player of the neuronal brake on the GnRH pulsatility during juvenile and prepubertal phase of development, corresponding to higher expression of GnIH neuronal elements during these ages. Then, at the end of prepubertal phase of development when an individual attains desired body weight and under normal physiological conditions, this neurobiological brake is released causing the onset of true puberty. Lower GnIH signaling at this point produces robust GnRH pulse generation, causing steroidogenesis and gametogenesis, leading to successful reproductive activity.

6.1. Optimization of the Experimental Techniques

Our results of immune- and gene expression of this study were validated at various steps. First, characterization of animals into different developmental groups was based on their somatometric and hormonal parameters. Body weight, plasma testosterone level, testicular volume, testicular epithelial height, and seminiferous tubular diameter were found to be significantly higher in adult animals as compared to all other

developmental groups. Hematoxylin and eosin staining showed an active spermatogenesis in adult age animals while none was seen in all other groups. The same criterion has been used previously for characterization of male rhesus monkeys (Garcia *et al*., 2018, Bano *et al*., 2022). Antibodies used for immunofluorescence analysis have been used previously in rodents and primates and have produced reproducible results (Kriegsfeld *et al*., 2006; Ubuka *et al*., 2009a) and the antibody against GnIH did not show cross reactivity with other RFamides (Smith *et al*., 2008). Secondly, the sections for immunostainings were selected after comparison with rhesus monkey brain atlas and GnIH-like ir pear-shaped terminals and fibers were carefully manually counted to verify our experimental validity and special caution was exercised to avoid counting the same fibers/terminals twice. In mice study, we used every fourth 30µm thick section, so there were very low chances of double counting the same boutons or fibers because of the distance (~90µm) between the selected sections. In addition to this, primary antibody omitted control sections were used in rhesus monkey immunostainings. Lack of fluorescence in the antibody omitted control sections verified the absence of nonspecific binding of the secondary antibody, employing that our results of immunofluorescence are likely to be specific and valid. Similarly, to evaluate *GnIH* mRNA-expression, primers efficiency was confirmed by standard curve before performing original reaction and samples were also run in duplicate manner to avoid any ambiguity in our results. *GAPDH* was chosen as a normalization control for RT-qPCR analysis. Overall, these quality control checks at various stages have increased the specificity and validity of our results.

6.2. Limitations of the Study

Though modulation in GnIH expression is clearly visible during pre- and postnatal development in mice, we could not perform hormonal analysis (testosterone/estradiol)

to confirm the reproductive axis activity at particular time points. And the findings tend to be correlational, and we can only speculate about the changes in GnIH expression driving puberty and *in-utero* differentiation in mice. In addition, all animals were euthanized during a small-time window during the day and gene expression levels and neuronal activity may follow other circadian rhythms outside of this window. Though there is a clear tendency of age-related changes in GnIH expression, we have a small sample size. Smaller sample size is usual in studies involving higher primate because of ethical considerations, especially the studies that involve euthanizing the animals for collection of tissue and where the animals cannot be used again (Wahab *et al*., 2013; Shamas *et al*., 2015a; 2015b; Aliberti *et al*., 2019; Wahab *et al*., 2019; Zubair *et al*., 2022). Also, although hypothalamic sections for the primate study were carefully selected, only a limited number of sections were scanned. Examination of the whole hypothalamus may have provided a more detailed view of the modulation in GnIH signaling with advancing age. Moreover, the ages of monkeys cannot be mentioned with certainty as they were captured from the wild. Although, body weight, plasma testosterone and testicular morphology together allow a relatively accurate assessment of age by comparison to previous work (Plant *et al*., 2005; Simorangkir *et al*., 2012; Garcia *et al*., 2018). It is possible that we did not observe differences from infant to prepubertal groups due to age variability in these animals. We could not perform dual labelling with GnIH and GnRH and confocal imaging of the monkey tissues, which could have provided a clearer picture of the maturational events taking place during pubertal progression in higher primates. Also, lack of more direct assessment of functionality precludes to be assertive on the functional consequences of the expression data obtained in mice, especially since

immunohistochemical data might not directly correlate with produced or released peptide.

6.3. Prospects of the Study

Findings presented in this document provide a cogent framework for the regulation of reproduction in mammals. However, as the potential role of GnIH signaling in prepubertal hiatus of the reproductive axis is suggested here, it is to be discerned whether the presumptive GnIH signal is integral or itself is regulated by other upstream/downstream neural signals. This would also be interesting to know whether GnIH directly inhibits the GnRH pulse generation by inhibiting the GnRH neuronal activity or does so by intermediary neural pathways present in the DMH, ARC, POA and PVN. The study of sexual differentiation in GnIH knockout mice could shed light on the potential role of this neuropeptide in sex determination during in utero development. Manipulation of reproductive hormones and observation of their effects on the functionality of the GnIH system will also be an intriguing future prospect.

7. Conclusion

In conclusion, the findings of this study strengthen our knowledge about the neuroendocrine regulation of pubertal progression in rodents and non-human higher primates. In addition, our current understanding about the trajectory of GnIH expression during various stages of pubertal development has also been enhanced. Furthermore, clinical trials based on advanced genomic and pharmacological interventions in various animal models may aid in understanding the role of this neuropeptide in pubertal development and sexual differentiation, potentially leading to the development of novel therapeutic approaches and in the treatment of hormone-dependent diseases like precocious puberty, endometriosis, uterine fibroids, benign

prostatic hyperplasia, and prostatic and breast cancers in humans. Further, human GnIH has the potential to be used as a novel contraceptive and to treat fertility-related disorders. Based on the findings of this dissertation, representative models about the GnIH regulation of the neuropeptides involved in onset of puberty in rodents (Figure 3) and primates (Figure 4) are presented here.

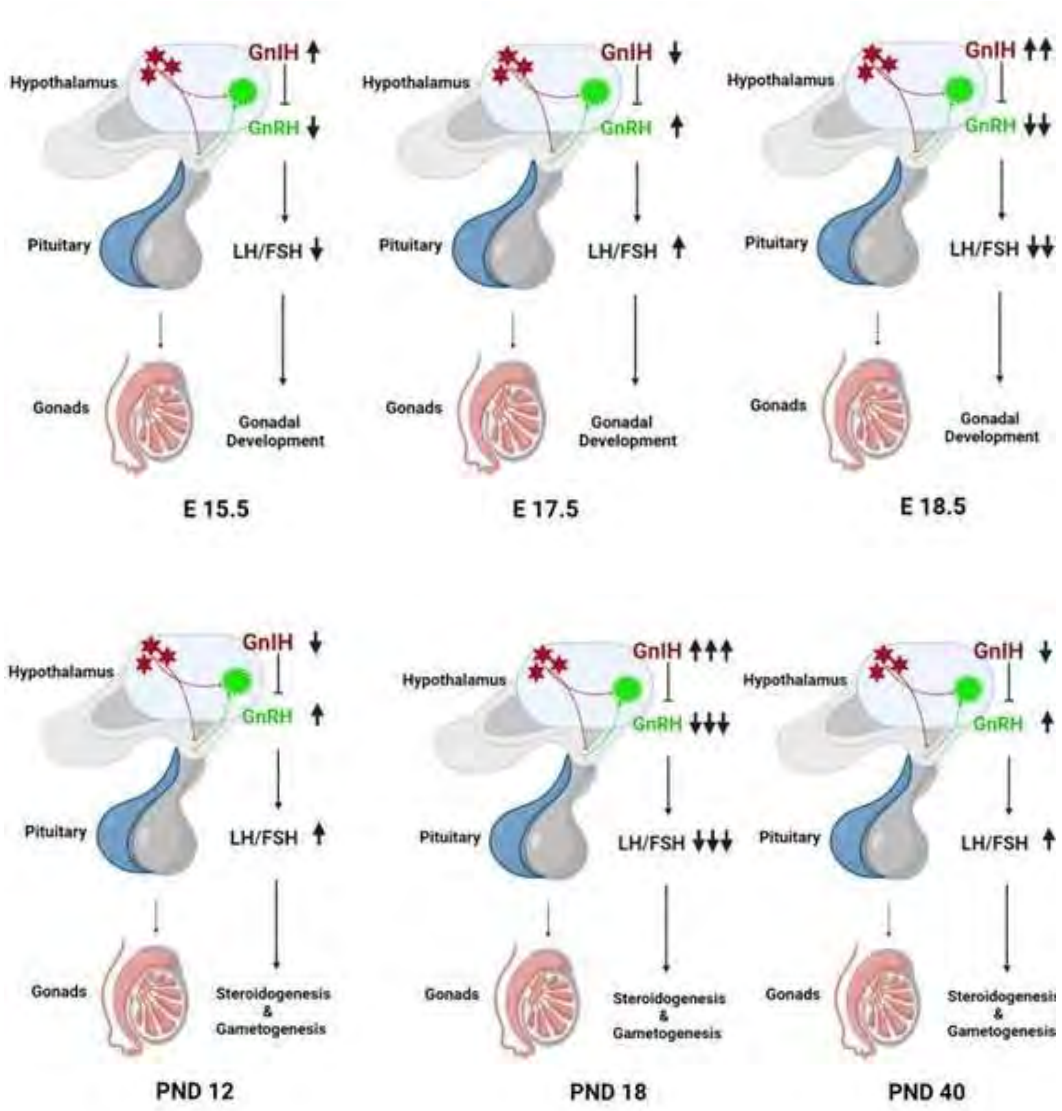


Figure 2: A proposed model for the association of GnIH signaling with pre- and postnatal activity of the reproductive axis in male rodents. (↑: Normal expression, ↑↑: High expression, ↑↑↑: Very high expression, ↓: Low expression, ↓↓: Lower expression, ↓↓↓: Very low expression). We could not measure the hormone levels, this model is based on hormonal levels reported by Bell, 2018 in male mice.

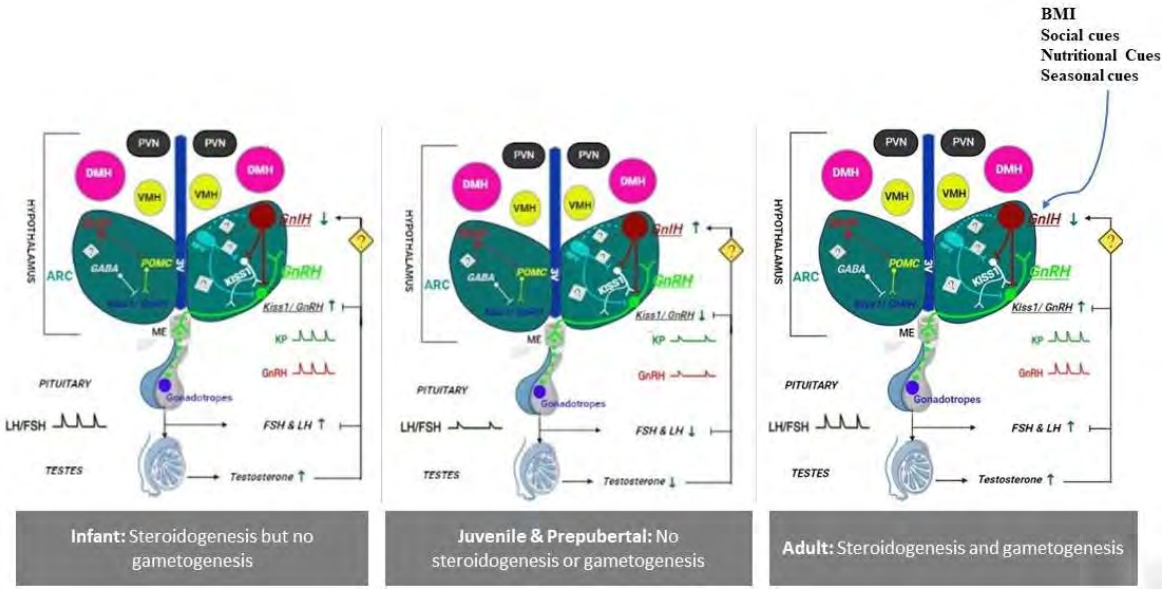


Figure 3: A proposed model showing the hypothalamic neurobiological cues associated with pubertal initiation in higher primates. (ME: Media eminence, ARC: Arcuate nucleus, PVN: Paraventricular nucleus. DMH: Dorsomedial hypothalamus, VMH: ventromedial hypothalamus, 3V: Third ventricle, GABA: Gamma amino butyric acid, POMC: Pro-opiomelanocortin neurons, KISS1/Kiss1/KP: Kisspeptin, ↓: Low expression/release, ↑: High expression/release, BMI: Body mass index

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Variation in Hypothalamic GnIH Expression and Its Association with *GnRH* and *Kiss1* during Pubertal Progression in Male Rhesus Monkeys (*Macaca mulatta*)

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Simple Summary: An increase in the pulsatile release of gonadotropin-releasing hormone (GnRH) is essential for the onset of puberty. However, the mechanisms controlling pubertal increases in GnRH release are still unclear. In primates the GnRH neurosecretory system is active during the neonatal period but subsequently enters a dormant state in the juvenile period. The present study examined developmental changes in the gonadotropin inhibitory hormone (GnIH) neuronal system, an inhibitory neuropeptidergic system upstream of GnRH neurons, and the relationship among changes that were observed in GnRH and kisspeptin (Kiss1) gene expression during puberty. A significant inverse age-related relationship between GnRH, Kiss1, and GnIH was observed, suggesting GnIH's potential role in reproductive suppression prior to puberty, with this theoretical 'brake' during the pubertal transition. These findings underscore the need for further research on the role that is played by GnIH in reproductive transition to aid in the development of potential GnIH-based drugs to treat pubertal disorders and adult fertility.

Abstract: Modulation of pulsatile gonadotropin-releasing hormone (GnRH) secretion across postnatal development in higher primates is not fully understood. While gonadotropin-inhibitory hormone (GnIH) is reported to suppress reproductive axis activity in birds and rodents, little is known about the developmental trajectory of GnIH expression in rhesus monkeys throughout the pubertal transition. This study was aimed at examining the variation in GnIH immunoreactivity (-ir) and associated changes among GnIH, GnRH, and Kiss1 mRNA expression in the hypothalamus of infant, juvenile, prepubertal, and adult male rhesus monkeys. The brains from rhesus macaques were collected from infancy until adulthood and were examined using immunofluorescence and RT-qPCR. The mean GnIH-ir was found to be significantly higher in prepubertal animals ($p < 0.01$) compared to infants, and significantly reduced in adults ($p < 0.001$). Significantly higher ($p < 0.001$) GnRH and Kiss1 mRNA expression was noted in adults while *GnIH* mRNA expression was the highest at the prepubertal stage ($p < 0.001$). Significant negative correlations were seen between *GnIH-GnRH* ($p < 0.01$) and *GnIH-Kiss1* ($p < 0.001$) expression. Our findings suggest a role for GnIH in the prepubertal suppression of the reproductive axis, with disinhibition of the adult reproductive axis occurring through decreases in GnIH. This pattern of expression suggests that GnIH may be a viable target for the development of novel therapeutics and contraceptives for humans.

Keywords: GnIH; GnRH; Kisspeptin puberty; rhesus macaque

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1. Introduction

Puberty is defined as the attainment of sexual and somatic maturity [1–3]. Puberty enables animals to reproduce and to achieve the adult phenotype [4]. Reproductive axis activity varies during postnatal development in non-human primates [5,6]. More specifically, the reproductive axis remains active during infancy, followed by a transient period of inactivity during juvenile and prepubertal stages [5,6]. At the onset of puberty, an increase in the activity of the reproductive system occurs that continues throughout adult life [7,8]. According to Ojeda, puberty is marked by the reactivation of the hypothalamic gonadotropin-releasing hormone (GnRH) system [3,9]. While a multitude of excitatory (e.g., glutamate) and inhibitory (e.g., gamma amino butyric acid (GABA)), inputs modulate GnRH activity [4,8,10,11], the neuroendocrine regulation of puberty onset is still poorly understood.

Beginning early in this millennium, the discovery of RFamide peptides (peptides having a characteristic Arg-Phe-NH_2 motif at their C-terminal) [12] with potent actions on the reproductive axis have helped to clarify the mechanisms that contribute to fertility. Among these, kisspeptin is thought to be the most important positive regulator of the reproductive axis, triggering GnRH release during infancy [13,14] and at pubertal onset [15–17]. Kisspeptin neurons are primarily localized in the arcuate nucleus region (ARC) of primates with fibers projecting to the mediobasal hypothalamus (MBH), preoptic area (POA), and median eminence (ME) [18]. Kisspeptin-releasing neurons have sex steroid receptors [19], while GnRH neurons do not [20], suggesting that gonadal steroid feedback occurs at kisspeptin cells. GnRH neurons express GPR54, the receptor for kisspeptin [21]. In contrast to kisspeptin, gonadotropin-inhibitory hormone (GnIH) is thought to be the most important negative regulator of the reproductive axis and GnRH secretion. GnIH is a dodecapeptide (SIKPSAYLPLRFamide) that was discovered in the quail brain by Tsutsui and colleagues [22] and subsequently found in other vertebrates from agnathans to humans [23–29]. GnIH mediates a wide variety of functions including stress, depression, aggression, sleep [30–32], and reproduction [33,34]. A multitude of studies has confirmed the suppressive effect of GnIH on the synthesis and release of GnRH and pituitary gonadotropins as well as apoptosis of testicular tissues with seminiferous tubular regression [35–37]. Developmental variation in hypothalamic GnIH expression has been reported in various species, including zebrafish [38], Indian major carp [39], European bass [40], cichlids [41], catla [42], European green frogs [43], and mice [44,45].

GnIH has been shown to suppress GnRH release through direct actions on the GnRH system and indirectly through alterations of kisspeptin/GPR54 signaling [46,47]. GnIH terminal fiber contacts onto GnRH soma have been reported in rodents, sheep, rhesus monkeys, and humans [23,26,27,48]. However, the developmental pattern of GnIH expression and its association with *GnRH* and *Kiss1* expression has not been investigated in higher primates. Based on findings to date, one can reasonably hypothesize that GnIH might serve as an important prepubertal neurobiological 'brake' on the reproductive axis in primates and waning of this neural brake might allow for kisspeptin-dependent/independent GnRH release and the onset of puberty. Therefore, this study examined developmental variation in GnIH expression by immunofluorescence and real time PCR and the relationship between *GnIH-GnRH* and *GnIH-Kiss1* mRNA expression during pubertal development in male rhesus monkeys, a representative higher primate.

2. Materials and Methods

Variation in GnIH expression in male rhesus monkeys from infancy through postnatal development was evaluated via protein and gene expression analyses. Initially, the animals at each developmental stage were characterized according to physical and hormonal parameters, as described previously [16,49]. Hypothalamic blocks of infant, juvenile, prepubertal, and adult animals were collected and processed after euthanizing the animals. Standard single-label immunofluorescence histology was used to stain GnIH nerve terminals and fibers while *GnIH*, *GnRH*, and *Kiss1* mRNA expression was quantified and correlated via RT-

qPCR analysis. The association between *GnIH* and *GnRH* and *Kiss1* mRNA was examined to determine whether high levels of GnIH correlated with reproductive axis suppression (i.e., low *GnRH/Kiss1* expression) and low GnIH with reproductive axis competence (i.e., high *GnRH/Kiss1* expression).

2.1. Animals

A total of fifteen intact male rhesus monkeys were employed for the present study. These animals were divided into four different age groups i.e., infant (n = 3), juvenile (n = 4), prepubertal (n = 4), and adult (n = 4), according to their body weight, testicular volume, and plasma testosterone levels. The mean ± SEM value of these parameters was as follows: infant: body weight 1.033 ± 0.169 kg, testicular volume 0.12 ± 0.695 mL and testosterone level 0.24 ± 0.0 ng/mL; juvenile: body weight 2.0 ± 0.129 kg, testicular volume 0.326 ± 0.029 mL and testosterone level 0.17 ± 0.0 ng/mL; prepubertal: body weight 4.02 ± 0.131 kg, testicular volume 0.34 ± 0.133 mL and testosterone level 0.22 ± 0.0 ng/mL; and adult: body weight 11.6 ± 1.24 kg, testicular volume 42.4 ± 4.93 mL and testosterone level 2.03 ± 0.4 ng/mL. All the animals were taken captive from Margalla Hills National Park, Islamabad, Pakistan. Each animal was kept in a separate cage under semi-ambient conditions at the Primate Facility of the Department of Zoology, Quaid-i-Azam University, Islamabad, Pakistan. All the animals were fed fresh fruits, peanuts (0900–0930 h), boiled eggs (1100 h), and bread (1300–1330 h). Water was available *ad libitum*. All the experimental procedures were carried out in accordance with the guidelines of the Departmental Committee for Care and Use of Animals (BEC-412).

2.2. Tissue Collection and Processing

Ketamine hydrochloride (Ketamax, Trittau, Germany; 10–20 mg/kg BW, im) was used to deeply sedate the animals prior to brain and testicular tissue collection. Hair was shaved off the head region with a razor, then skin on the skull was thoroughly scrubbed with 70% ethanol and muscle tissue was removed by using a scalpel. After cutting the skull bone in a circular manner with the aid of a sharp bone cutter, the brain was removed from the cranial cavity and immediately placed on a cold glass plate. Hypothalamic blocks, including POA and MBH, were dissected out from the brain as described previously [50]. Briefly, through the mammillary bodies, coronal cuts were made anterior to the optic chiasm. On either side of the midline, a parasagittal cut was made at approximately 4 mm distance. Then, a final horizontal cut was made dorsal to the anterior commissure. Segregated blocks were washed with normal saline. Further hemi-hypothalamic blocks including MBH and POA were made by a cut along the medial line. One hemi-hypothalamic block from all animals was transferred to a fixative (4% paraformaldehyde (PFA)) and was cryopreserved by sequential passage through sucrose solutions to be used in immunocytochemistry. Later, these hemi-hypothalamic blocks were cut into serial sections of 20µm thickness on a cryostat (Bright OTF 5000, A-M systems, Sequim, WA, USA; temperature −25 °C) in the horizontal plane and sections were stored at −20 °C in an anti-freeze solution. The other hemi-hypothalamic block was flash frozen in liquid nitrogen and stored at −80 °C until RNA extraction for RT-qPCR. Single blood samples were collected from all animals in heparinized syringes and their testicular dimensions were noted using Vernier calipers before dissection. For histological purposes, testicular tissue from one testicle of each animal was also collected and was fixed in Bouin's fixative for 16 h. Testicular tissue was then dehydrated by sequential passage through ascending grades of alcohol, embedded in molten wax, and fixed onto wooden blocks.

2.3. Plasma Testosterone and Testicular Volume Measurement

The concentration of the total testosterone was measured by using a commercially available human enzyme immunoassay (EIA) kit (Astra Biotech GmBH, Luckenwalde, Germany) according to manufacturer's instructions. Assay sensitivity was 0.05 ng/mL and the inter- and intra-assay coefficient of variation was less than 9% and less than

10%, respectively. At the time of dissection, the testicular volume of all the monkeys was calculated by using the formula $V = (\pi w^2 l)/6$, in which 'w' denotes width (mm), 'l' represents the length (mm) of each testis, and 'V' is the volume in ml [51]. The volume of the left and right testis was added to get the total volume of the testes.

2.4. Testicular Morphology

To examine the changes in testicular morphology of monkeys across different ages, the paraffin-embedded tissues were stained with eosin and hematoxylin. For deparaffinization, sections were given two washes in xylol, each for five min. The sections were then rehydrated by passing through descending grades of alcohol (100%, 90%, and 70% each for one minute). Then, the sections were placed in hematoxylin stain for 5 min. Sections were then washed in tap water for 2 min. The sections were then dipped 2–3 times in 1% acid alcohol followed by a 2 min wash with tap water before being placed in eosin for 2 min. After washing with tap water, the sections were dehydrated and given two xylol washes of 1 min each. The sections were then cover-slipped. The epithelial height and tubular diameter were measured and compared among all the monkeys across postnatal development.

2.5. Fluorescence Immunocytochemistry

A total of four sections from each animal were processed using a standard single label immunocytochemistry protocol. Of these 4 sections, 3 were treated with a primary antibody solution while one was used as a primary antibody omitted control section. All the sections were washed in 0.1 M phosphate buffer saline (PBS, pH 7.3; 8 × 15 min each) at room temperature prior to staining. Then, the sections were incubated in a blocking solution containing 10% normal goat serum, 0.05% bovine serum albumin (BSA), and 0.05% Triton-X100 (T-X100) in PBS for one hour at room temperature to block non-specific binding. The sections were then washed with PBS 3 × 15 min. The sections were then incubated with a primary GnIH antibody (rabbit anti-, white-crowned sparrow GnIH antibody (PAC123,124, antigen sequence SIKPFSNLPLRF, generous gift of George Bentley, Berkeley, CA, USA; used at dilution 1:5000) in a buffer solution containing 0.05% BSA and 0.05% TX-100, for 48 h at 4 °C on a shaker, followed by washing for 3 × 15 min. Control sections were incubated without the primary antibody. Later, the sections were incubated in secondary antibody (Cy3-goat anti-rabbit, Cat# 111-165-003; Jackson Immonoresearch Laboratories Inc, West Grove, PA, USA; used at dilution 1:200) solution containing 0.05% BSA and 0.05% TX-100 in PBS for two hours at room temperature, in the dark on a shaker. The control sections were also incubated with the secondary antibody at this stage. Subsequently, sections were washed with PBS 3 × 15 min. After washing, the sections were mounted on super frosted glass slides (CrystalCruz^{R}, Cat # Sc-363562; Santa Cruz Biotechnology Inc, Dallas, TX, USA) and left to dry overnight at 4 °C in dark. The next day, the slides were cover-slipped (Microscope Cover Glass, MAS GmbH, Leonberg, Germany, 24 × 50 mm) using anti-fade medium (Immu-Mount^{TM}, Cat# 238402, Thermo Shandon Limited, Cheshire, UK). The slides were stored at 4 °C in the dark until fluorescent microscopy was conducted.

2.6. Microscopy

GnIH immunoreactivity was examined by using an Olympus fluorescent microscope (Olympus BX51, Tokyo, Japan) and photographs were taken using a digital camera attached to the microscope. GnIH-ir was visualized using the standard wavelength for Cy-3 (568 nm). The whole MBH area was scanned in three random sections from each animal. The total number of GnIH immunoreactive nerve terminal boutons and fibers in midline hypothalamic regions, especially in the ARC area in each section, was manually counted and the mean ± SEM were calculated for each animal. Testicular sections were viewed under a light microscope and morphological parameters were measured using a micrometer.

2.7. Real Time-Quantitative PCR

2.7.1. Isolation of RNA and cDNA Synthesis

The total RNA was isolated from hemi-hypothalamic block using Wizol™ Reagent (Cat # W76100, Wizbiosolutions, Seongnam, Republic of Korea) according to manufacturer's instructions. The RNA quantity was measured by using a Thermo Scientific Nanodrop 1000 spectrophotometer (Wilmington, DE, USA). cDNA was synthesized from this RNA using a first strand cDNA synthesis kit (WizScript™, Cat# W2211, Wizbiosolutions) using the supplier's protocol with the random hexamer primers in a thermocycler (T100 Bio-Rad Thermocycler, Hercules, CA, USA). Briefly, the process involved initial incubation for 5 min at 65 °C, then further incubation at 37 °C for 60 min followed by termination for 10 min at 70 °C. The cDNA samples were placed at −20 °C until further analysis.

2.7.2. Quantitative Real Time PCR

The expression of *GnIH*, *Kiss1*, and *GnRH* genes was evaluated using real-time polymerase chain reaction. The reactions were done by using qPCR Master (SYBR) kit (Wizpure™, Cat # W1401-5, Wizbiosolutions). Each reaction of 10 µL included 5 µL of SYBR Green, 0.45 µL of each primer, 2.5 µL cDNA (1:4 dilution), and 1.6 µL of RNAse-free water. The primers that were used were synthesized by Macrogen company (Seoul, Republic of Korea). The sequences of all the primers that were used with their accession numbers are provided in Table 1. Reaction conditions were pre-denaturation temperature at 95 °C for the 180 s, denaturation temperature 95 °C for 10 s, annealing temperature 60 °C for 15 s, and elongation temperature 72 °C for 20 s. All the reactions were run in duplicate and cycle threshold (Ct) was calculated by using software CFX Maestro software version 2.3 (Biorad, Hercules, CA, USA). Comparative expression was calculated by using the relative Ct method. Every sample was normalized to the endogenous housekeeping gene *GAPDH* expression by using the $2^{-\Delta\Delta CT}$ method [52,53], taking infant group as calibrator.

Table 1. Sequence, accession number, and product length of the primers that were used for RT-qPCR.

Gene	Accession#	Primer Sequence (5′ to 3′)	Product Length
GnRH	S- 75918	Rev: TTTCCAGAGCTCCTTTCAGG For: AGATGCCGAAAATTTGATGG	134
Kiss1	XM-028852143.1	Rev: TGACTCCTCTGGGGTCTGAA For: GGACCTGCCGAACTACAACT	141
GnIH	NM-001033115.2	Rev: ATTGGCACATGGTGAATGC For: CCTCGTGAGACGGGTTCTTA	118
GAPDH	NM-001195426.1	Rev: TTGATGACGAGCTTCCCGTT For: TGTTGCCATCAATGACCCCT	119

2.8. Statistical Analyses

GraphPad Prism Version 8 was used to perform data analysis (GraphPad Software Inc., La Jolla, CA, USA) and the data are expressed as means ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc tests, were employed to compare body weights, plasma testosterone levels, testicular morphological parameters, GnIH-ir in the hypothalamus and *GnIH* expression relative to *GnRH* and *Kiss1* at different developmental stages in rhesus monkeys. Pearson's correlation was used to determine the correlative changes between *GnIH-GnRH* and *GnIH-Kiss1* mRNA expression. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Body Weight, Plasma Testosterone, and Testicular Volume

Body weight and testicular volume showed a prominent increase with the progressing age of the monkeys. Statistically, there was no significant difference ($p > 0.05$) in body weight, testicular volume, and testosterone levels of infant, juvenile, and prepubertal groups, while adults showed significantly higher body weight ($F_{3,11} = 50.71$; $p < 0.0001$),

testicular volume ($F_{3,11}$ = 65.39; $p < 0.0001$), and testosterone levels ($F_{3,11}$ = 11.89; $p < 0.01$) as compared to all the other developmental groups (Figure 1).

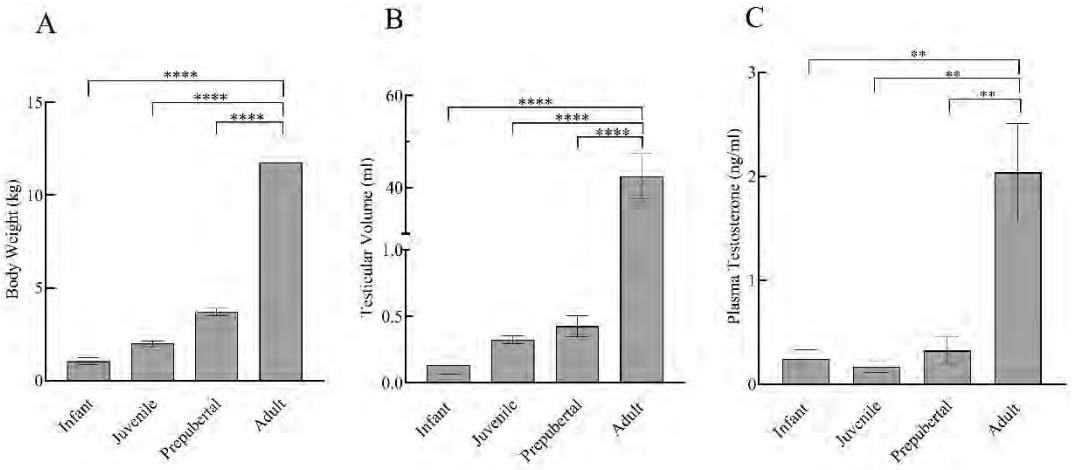

Figure 1. Body weight (**A**), testicular volume (**B**), and plasma testosterone levels (**C**) of infant (n = 3), juvenile (n = 4), prepubertal (n = 4), and adult (n = 4) male rhesus monkeys. All data are presented as the mean ± SEM. ** $p < 0.01$, **** $p < 0.0001$.

3.2. Testicular Morphology

Prominent differences were evident in the histological examinations of hematoxylin- and eosin-stained testicular sections of monkeys in different groups. With advancing age, clear variation in maturation and differentiation of spermatogonia was noticeable. The tubular lumen was closed in infants (Figure 2A,B) whereas juveniles showed a very small luminal space (Figure 2C,D). Monkeys in the prepubertal stage showed a relatively larger lumen (Figure 2E,F) whereas adult monkeys had the maximum luminal space with active spermatogenesis (Figure 2G,H). Epithelial height and tubular diameter showed a prominent increase with age. Adults showed significantly higher ($F_{3,11}$ = 37.82; $p < 0.0001$) epithelial height and seminiferous tubule diameter ($F_{3,11}$ = 49.30; $p < 0.0001$) compared to all the other developmental groups (Figure 3A,B, respectively).

3.3. Developmental Variation in Number of GnIH-ir Terminals Expression Fluorescence

GnIH-ir terminal boutons were quantified in the ARC and MBH (Figure 4). Significant variation in the number of GnIH-ir nerve terminals in monkey hypothalamus across pubertal development was observed. Specifically in the ARC area, GnIH-ir terminals increased significantly during pubertal development followed by a precipitous decline in adulthood ($F_{3,11}$ = 23.50; $p < 0.0001$). Significantly higher GnIH-ir terminals were observed in prepubertal animals as compared to infants ($p < 0.01$) and juveniles ($p < 0.001$) while significantly reduced expression was seen in adults as compared to prepubertal ($p < 0.0001$) and infant groups ($p < 0.05$) (Figure 5A). The mean number of GnIH-ir terminal boutons in the MBH increased analogously across postnatal development, showing a sharp decline at the adult stage ($F_{3,11}$ = 15.39; $p < 0.001$). Specifically, the number of GnIH-ir terminals was found to be significantly higher in prepubertal animals as compared to infants ($p < 0.01$) and significantly reduced ($p < 0.001$) in adult animals (Figure 5B).

3.4. Developmental Variation in Expression of GnIH-ir Fibers

GnIH-ir fiber expression varied significantly in the ARC region of male monkeys across development ($F_{3,11}$ = 19.09; $p < 0.001$). Specifically, the mean number of GnIH-ir fibers that were expressed in the arcuate area of prepubertal animals was significantly higher than infants ($p < 0.01$) and juveniles ($p < 0.001$), while a significantly reduced number of GnIH-ir fibers was seen in adults as compared to prepubertal ($p < 0.001$) animals (Figure 5C). The expression of GnIH-ir nerve fibers in the MBH varied analogously through pubertal

development ($F_{3,11} = 9.12$; $p < 0.01$). More precisely, the mean number of GnIH-ir fibers was significantly higher in prepubertal animals as compared to infants ($p < 0.01$) and juvenile animals ($p < 0.01$) while staining was found to be significantly reduced in adult animals ($p < 0.05$) as compared to prepubertal animals (Figure 5D).

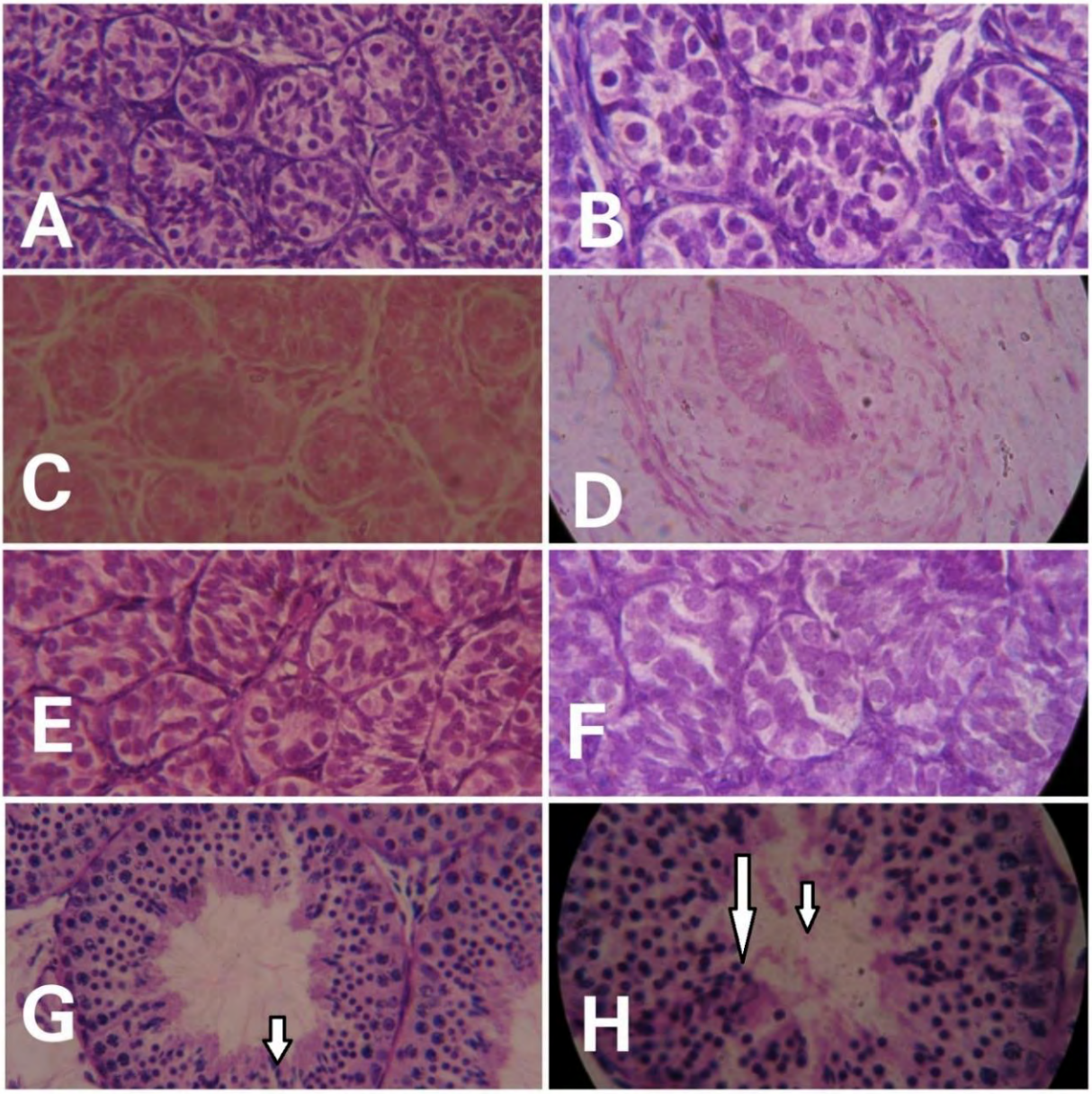

Figure 2. Representative photomicrographs showing the testicular histology of infants ((**A**) = 40×, (**B**) = 100×), juvenile ((**C**) = 40×, (**D**) = 100×), prepubertal ((**E**) = 40, (**F**) = 100×), and adult ((**G**) = 40×, (**H**) = 100×) male rhesus monkeys. Active spermatogenesis (indicated by arrows) and a wide lumen is evident in adult monkeys.

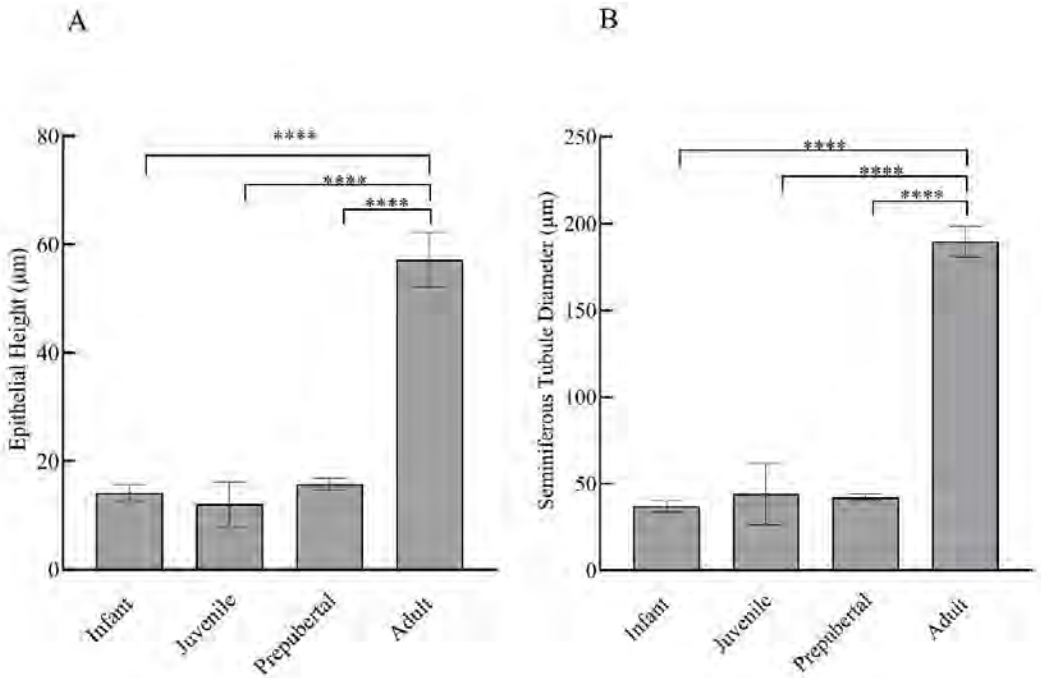

Figure 3. Mean ± SEM epithelial height per tubule (**A**) and seminiferous tubular diameter (**B**) of male rhesus monkeys through postnatal development. **** $p < 0.0001$.

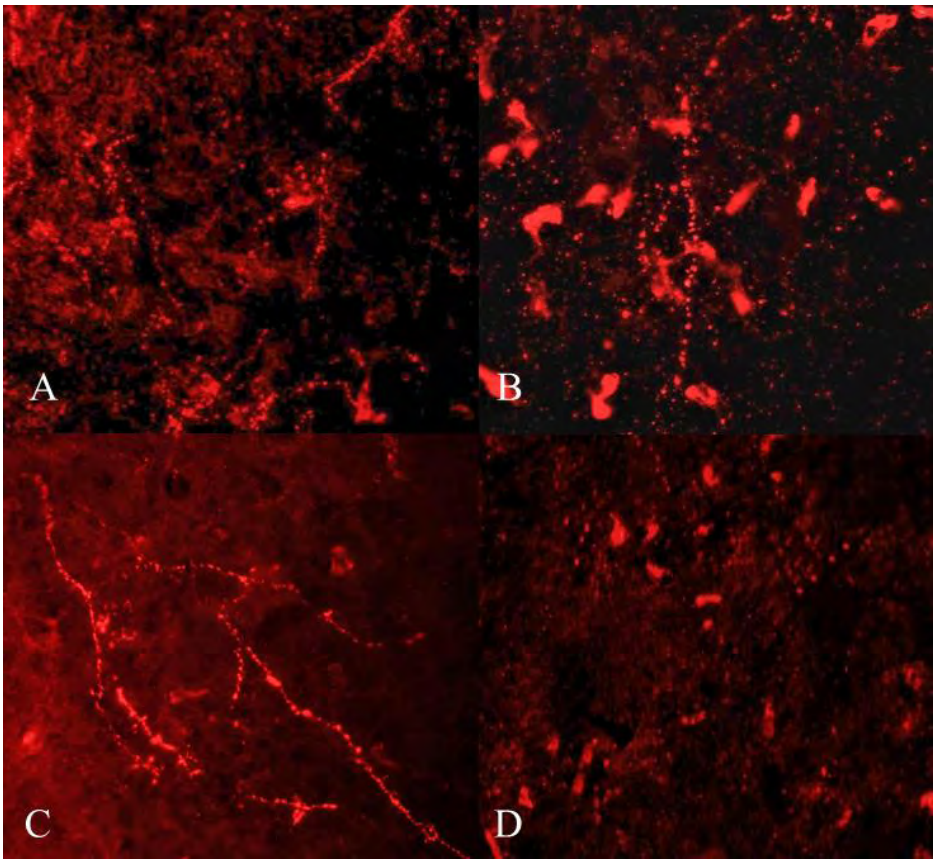

Figure 4. Representative photomicrographs showing GnIH-like immunoreactive terminal boutons and fibers in infant (**A**), juvenile (**B**), prepubertal (**C**), and adult (**D**) male rhesus monkeys.

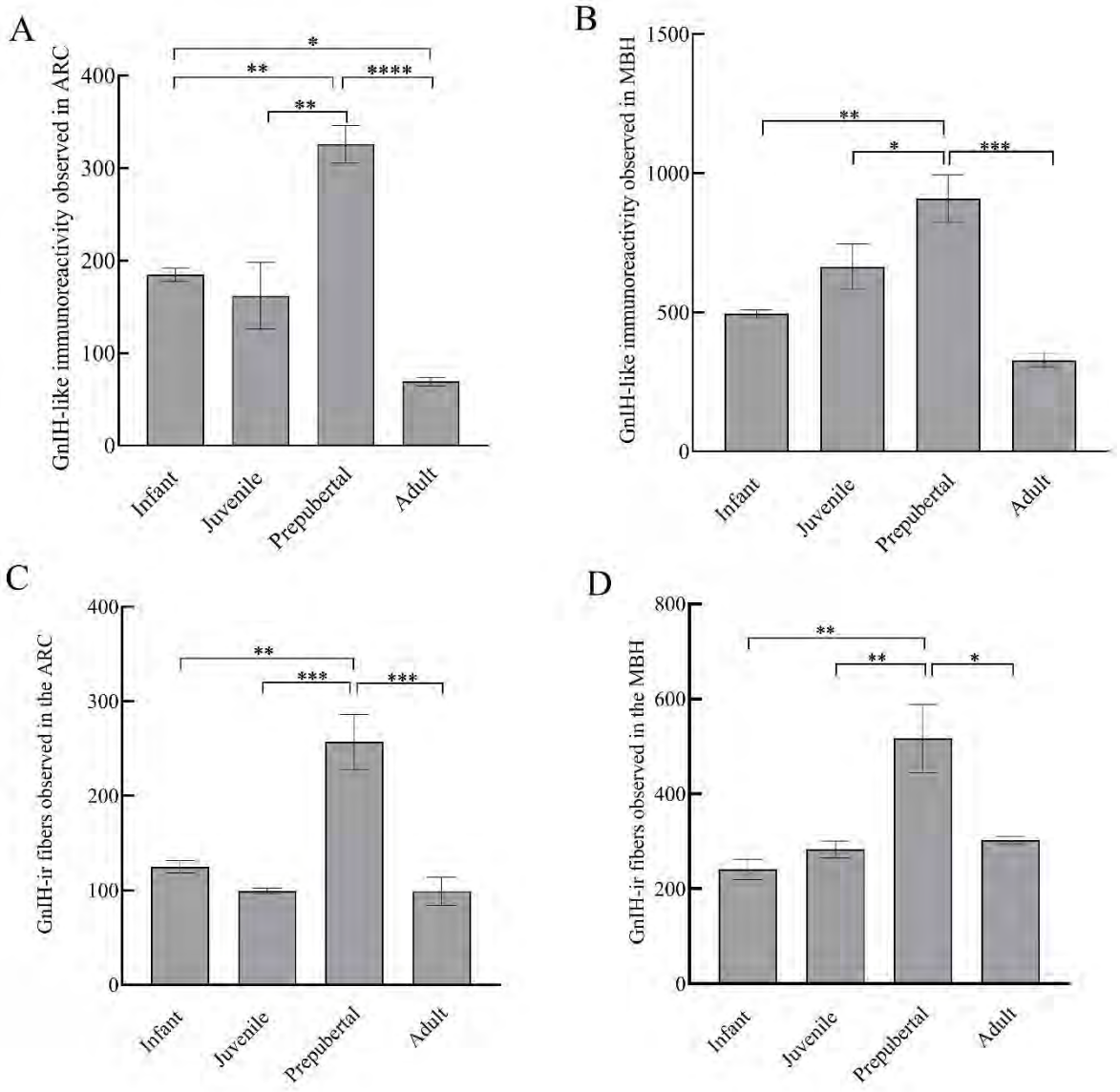

Figure 5. GnIH-like immunoreactive nerve terminals that were observed in arcuate area (**A**), MBH (**B**); and GnIH-like-ir fibers in arcuate area (**C**) and MBH (**D**) of male rhesus monkeys through pubertal development. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5. RT-qPCR

Comparative changes in the expression of *GnRH, Kiss1,* and *GnIH* mRNA in the hypothalamus of male rhesus monkey during pubertal development are shown in Figure 6. A significant variation was noted in the expression of *GnRH* ($F_{3,11} = 11.58$; $p < 0.001$), *Kiss1* ($F_{3,11} = 12.07$; $p < 0.001$), and *GnIH* ($F_{3,11} = 14.80$; $p < 0.001$) across pubertal development. Significantly higher expression of *GnRH* ($p < 0.01$) and *Kiss1* ($p < 0.01$) in the adult group agree with the active breeding state of the adult animals. *GnIH* expression was found to be significantly higher in prepubertal animals compared to juvenile monkeys ($p < 0.05$) while a sharp decline ($p < 0.001$) in *GnIH* expression was seen in adult animals as compared to prepubertal animals. A significant, inverse correlation was seen between *GnRH-GnIH* ($F = 13.34$; $p < 0.01$) and *Kiss1-GnIH* ($F = 17.52$; $p < 0.001$) expression (Figure 7).

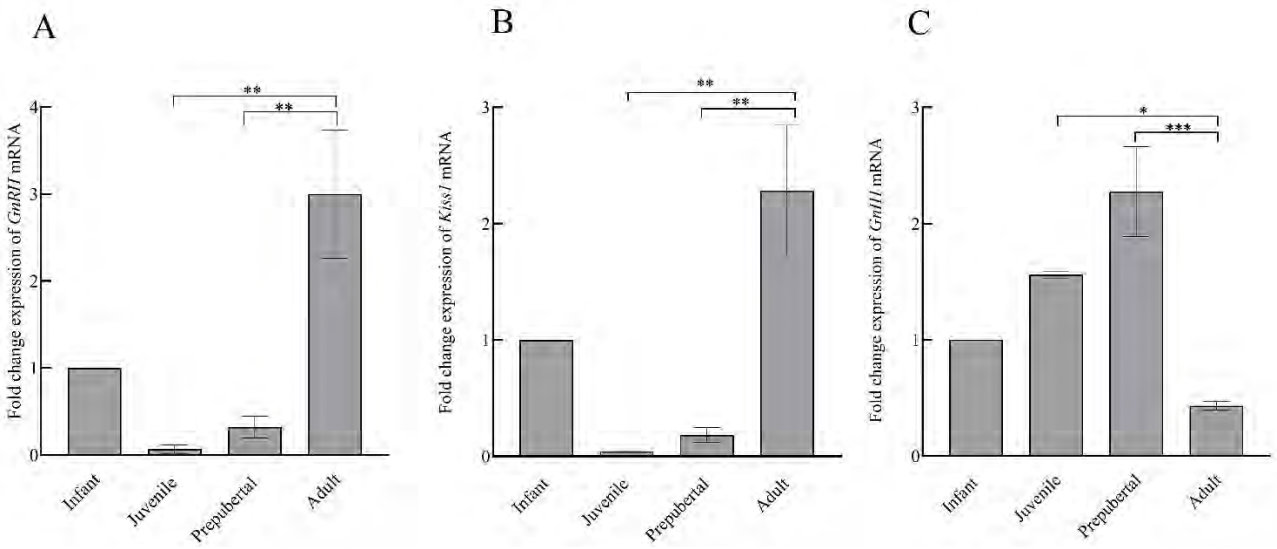

Figure 6. The mean ± SEM hypothalamic fold change expression of *GnRH* (**A**), *Kiss1* (**B**), and *GnIH* (**C**) mRNA during pubertal development in male rhesus monkeys. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

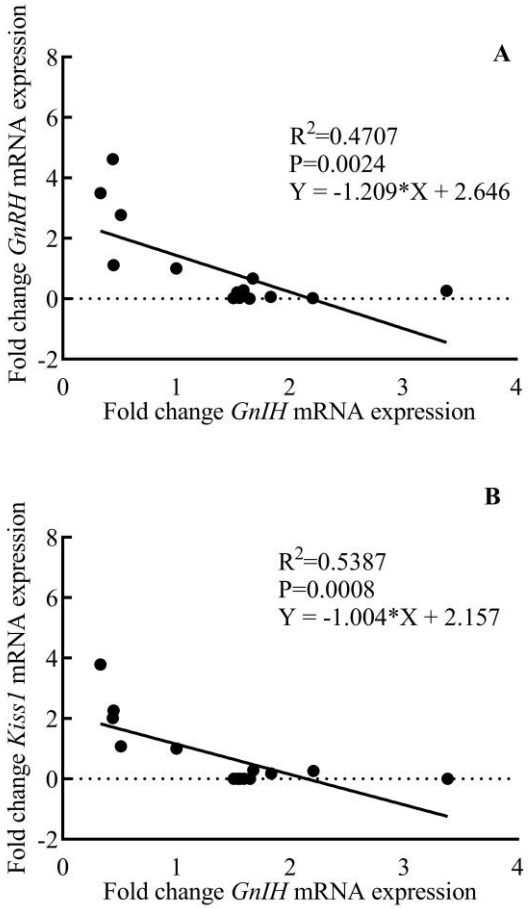

Figure 7. Inverse age-related correlation between hypothalamic fold change *GnRH* (**A**) and *Kiss1* (**B**) expression with *GnIH* expression during pubertal development in male rhesus monkeys.

4. Discussion

In the present study, we examined the pubertal changes in GnIH protein and mRNA expression and its correlation with *GnRH* and *Kiss1* mRNA expression in male rhesus monkeys, a representative higher primate. Our immunofluorescence and qPCR data show

a significant increase in the expression of GnIH-ir nerve terminals, fibers, and mRNA during the prepubertal phase of postnatal development as compared to the infantile male rhesus monkeys. In adult animals, we observed a precipitous decline in the number of GnIH-ir neuronal elements. These animals had significantly higher plasma testosterone levels and testicular volume compared to the prepubertal animals, with their testicular histology indicative of a fully active reproductive axis with sufficient spermatogenesis. Together, the present findings suggest that elevated GnIH signaling prior to the onset of puberty causes a hiatus in reproductive axis activity, keeping steroidogenesis and gametogenesis in check, while reduced GnIH signaling in adult animals allows for the onset of puberty by reactivation of the reproductive axis activity.

In primates, GnRH release is robust during infancy causing steroidogenesis (i.e., sex steroid production) but not gametogenesis (i.e., sperm production) [13,54]. During the juvenile phase of development, GnRH release is dampened, resulting in the relative quiescence of the reproductive axis due to hypogonadotropism [13,54]. At the conclusion of the prepubertal phase of development in primates, pulsatile GnRH secretion in the portal blood resumes, resulting in the release of pituitary gonadotropins, that ultimately act on the gonads. In response to pituitary gonadotropins, the gonads produce sex steroid hormones and gametes, thus activating the reproductive axis, also known as onset of true puberty [14]. Since the discovery of the GnRH neuronal system in the 1970s [55], substantial research has been conducted to decipher the switch that initiates the pulsatile release of GnRH release at puberty [56,57]. Kisspeptin neurons have emerged as an important player in pubertal onset and regulation of gonadal function by maintaining the activity of the neuroendocrine axis during adulthood in primate and non-primate species [58]. As a result, we also quantified *GnRH* and *Kiss1* mRNA expression in male rhesus monkeys of various developmental ages. Our results show higher *GnRH* and *Kiss1* mRNA expression in adult animals compared to juvenile and prepubertal animals. These findings are consistent with previous findings in rodents and primates [15,59–62]. Likewise, girls with precocious puberty have higher kisspeptin levels compared to prepubertal girls [63], possibly releasing the GnRH pulse generator from the neurobiological brake.

Despite significant research aimed at determining the neurobiology underlying the developmental regulation of the reproductive axis, the switch that turns GnRH release off in infantile primates has received little attention. After the discovery of GnIH in the year 2000 [22], substantial research has been performed to establish its role in reproductive axis activity in many species [26,27,42,64–66]. To completely ascertain the role of GnIH in the conceptual brake on GnRH neuronal activity, we studied the pattern of expression of *GnIH* mRNA during pubertal development and its correlation with *GnRH* and *Kiss1* gene expression. We saw a significant negative correlation between *GnIH-GnRH* and *GnIH-Kiss1* mRNA expression. This finding further strengthens our postulation that higher GnIH signaling during juvenile and prepubertal phases of development keeps the GnRH pulse generator activity in check by downregulating *Kiss1* expression in the hypothalamus, while a decrease in GnIH signaling at the end of the prepubertal phase brings about the resumption of the GnRH pulse generation, thus activating the reproductive axis.

GnIH-ir nerve terminals and fibers were seen in midline hypothalamic nuclei (ARC) where GnRH neurons are located, suggesting possible innervation by GnIH projections, a finding that is consistent with previous findings in other species [23,25] and in higher primates [26,27]. A subtle variation in the expression of GnIH neuronal elements was noticed with advancing age in rhesus monkeys. Our results are in line with previous findings in mice where GnIH-ir was found to be significantly decreased in pubertal animals compared to prepubertal animals [44,45]. This finding suggests that GnIH may directly inhibit GnRH neuronal signaling or might do so via intermediary neuronal systems that are present in these brain areas such as the kisspeptin neuronal population in the ARC region. Previously, it was established that centrally administered GnIH interferes with the pituitary gonadotropins release in white-crowned sparrows [67], Syrian hamsters [23], and rats [36] and reduces the firing activity of the GnRH neurons [68,69]. Thus, variation

in GnIH expression during pubertal development might directly modulate the GnRH pulse generator activity controlling the activation of the reproductive axis. However, ascertaining morphological interactions between GnIH with GnRH and kisspeptin neuronal elements will help in understanding the role of GnIH signaling during sexual maturation of higher primates. It is also highly plausible that GnIH regulates GnRH pulse generation activity of an individual based on energetic state via an interaction with energy-sensitive POMC neurons in the ARC [70,71]. GnIH might regulate other physiological functions by modulating the activity of other neuronal systems that are present in MBH nuclei, including the regulation of prolactin release via dopamine neurons that are present in ARC [72]. Finally, GnIH may downregulate the reproductive axis activity under stressful conditions by modulating the activity of the hypothalamic pituitary adrenal axis by directly affecting corticotropin-releasing hormone (CRH) secretion from the PVN [73].

Although there is a clear trend of age related changes in GnIH expression, our sample size is small. Smaller sample sizes are common in studies of higher primates compared to studies in rodents because of ethical considerations, especially the studies that involve euthanizing the animals for tissue collection and where animals cannot be repeatedly examined [74–79]. Also, although hypothalamic sections were carefully selected, only a limited number of sections were scanned. Examination of the whole hypothalamus may have provided a more detailed view of the modulation in GnIH signaling with advancing age in various brain nuclei. The ages of the animals cannot be determined with certainty as all the animals were captured from wild. Although body weight, testicular morphology, and plasma testosterone measures, together allow a relatively accurate assessment of age by comparison to previous work [16,49,80]. It is possible that we did not observe differences from infant to prepubertal animals due to age variability in these groups. Future studies involving confocal analysis of the colocalization and interactions of GnIH with GnRH and kisspeptin and other neuropeptides may provide a more detailed assessment of the mechanisms that are responsible for the neuroendocrine regulation of puberty onset in higher primates.

5. Conclusions

In summary, the findings of the present study suggest that suppression of reproductive axis activity during the juvenile and prepubertal phase of development in higher primates is associated with an increase in GnIH tone as indicated by an increase in the GnIH peptide and gene expression at these stages. Furthermore, the negative correlation of *GnIH* expression with *GnRH* and *Kiss1* expression that was observed in this study implies that GnIH might serve as an important player in the neurobiological brake on reproductive axis activity by decreasing kisspeptin and GnRH activity directly, or via intermediary neuronal systems. Based on the current findings, it can also be suggested that, in addition to reproductive axis activity, GnIH might also regulate other neuronal populations to influence motivated behaviors, including reproductive behavior. The present findings set the stage for future studies examining whether GnIH directly inhibits GnRH pulse generation by inhibiting GnRH neuronal activity or by intermediary neural pathways that are present in the DMH, ARC, POA, and PVN. Further, advanced genomic and pharmacological studies will advance understanding the role of this neuropeptide in pubertal development and sexual differentiation and guide the development of novel therapeutic approaches in the treatment of hormone-dependent diseases such as precocious puberty, endometriosis, uterine fibroids, benign prostatic hyperplasia, and prostatic and breast cancers. Human GnIH may also have potential as a novel contraceptive and in the treatment of fertility related disorders.

Author Contributions: Conceptualization, H.Z.; Data curation, H.Z.; Funding acquisition, M.S. (Muhammad Shahab); Methodology, H.Z., M.S. (Muhammad Saqib), S.S. and S.I.; Resources, M.S. (Muhammad Shahab); Software, H.Z. and M.N.K.; Supervision, M.S. (Muhammad Shahab); Writing—original draft, H.Z.; Writing—review and editing, M.S. (Muhammad Shahab). All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bio Ethics Committee of Quaid-i-Azam University, Islamabad. The ethical approval code is BEC-412.

Data Availability Statement: Not Applicable.

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