Study of the role of NPY in the control of prolactin and growth hormone secretion through Y1 receptor subtype in juvenile male rhesus monkey (*Macaca mulatta*)

BY

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Study of the role of NPY in the control of prolactin and growth hormone secretion through Y1 receptor subtype in juvenile male rhesus monkey (*Macaca mulatta*)

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of the requirements for the degree
of
MASTER OF PHILOSOPHY
IN
ENDOCRINOLOGY

BY
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Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad
2023

“In the name of ALLAH, the Most Gracious, the Most Merciful”

بسم الله الرحمن الرحيم

CERTIFICATE

This dissertation "Study of the role of NPY in the control of prolactin and growth hormone secretion through Y1 receptor subtype in juvenile male rhesus monkey" submitted by **Ms. Sonia Kanwal,** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Endocrinology.

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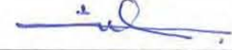

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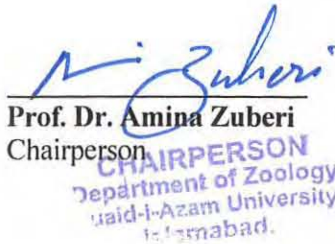

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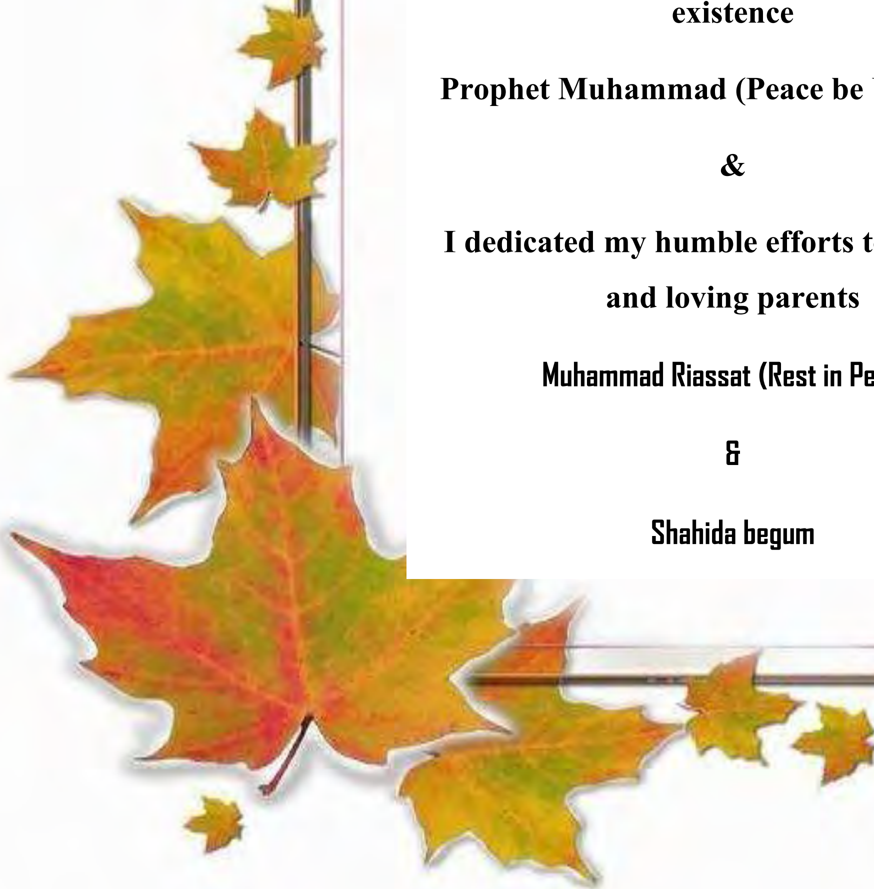
DECLARATION

I hereby declare that the material contained in this dissertation "Study of the role of NPY in the control of prolactin and growth hormone secretion through Y1 receptor subtype in juvenile male rhesus monkey (*Macaca mulatta*)'' is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Sonia Kanwal

Dedication



First of all, I dedicate my project to Allah Almighty

&

Dedicated to whom the world owes its existence

Prophet Muhammad (Peace be Upon Him)

&

I dedicated my humble efforts to my sweet and loving parents

Muhammad Riassat (Rest in Peace)

&

Shahida begum

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Allah Subhan Wa Taala says in surah Ahzab **“and that there is not for man except that (good) for which he strives”** Al-Ahzab 55:39

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LIST OF ABBREVIATIONS

Abbreviations	Full Form
<	Less than
>	More than
NPY	Neuropeptide Y
PYY	Peptide YY
PP	Pancreatic Polypeptide
CNS	Central nervous system
ARC	Arcuate Nucleus
DA	Dopamine
ATP	Adenosine Triphosphate
µIU/ml	Micro international unit /mililiter
^{0}C	Centigrade
Ala/Ser	alanine and serine
ANOVA	Analysis of Variance
Ant	Antagonist
Asn/ Ala	alanine and asparagine
BW	Body weight
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
COOH	Carboxyl group
CRF	Corticotropin-releasing factor
D2	Dopamine receptor
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
FSH	Follicle stimulating hormone
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GnIH	Gonadotropin inhibiting hormone

GnRH	Gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
hGH ELISA	Human Growth Hormone ELISA
HPG axis	Hypothalamic pituitary gonadal axis
HRP	Horseradish peroxidase
icv	Intracerebroventricular
im	Intramuscular
Iv	Intravenous
Ile/Leu	Isoleucine and leucine
Ile/Val	Isoleucine and valine
IR	Immunoreactive
IU	International Unit
K^+	Potassium ions
Kg	Kilogram
LH	Luteinizing hormone
MBH	Mediobasal hypothalamus
ME	Median eminence
mg	Milligram
min	Minute
mIU/L	Milli international unit per liter
ml	Milliliter
mm	Millimeter
MPOA	Medial preoptic area
mRNA	Messenger ribonucleic acid
NPY1R	Neuropeptide Y1 receptor
ng	Nanogram
OVX	Ovariectomized
PeN	Periventricular nucleus
PIF	Prolactin inhibitory factor

PL	Placental lactogen
PRL	Prolactin
PVN	Paraventricular nucleus
rpm	Revolution per minute
SEM	Standard error mean
SS	Somatostatin
SRIF	Somatotropin-release inhibiting factor
THDA	Tuberohypophyseal dopaminergic neurons
TIDA	Tuberoinfundibular dopaminergic neurons
TMB	3,3', 5,5 Tetramethylbenzidine
veh	Vehicle

ABSTRACT

Abstract

Background

Neuropeptide Y (NPY) plays an important role in controlling reproductive axis and food intake. Both prolactin (PRL) and growth hormone (GH) are important non-gonadotrophic hormones that are involved in many physiological processes. Many intrinsic and extrinsic factors are involved in modulating the secretion of PRL and GH. It is believed that NPY is also one of the factors involved in mediating the release of PRL and GH. Numerous studies in different animal models (rat, mice, sheep) have documented contradictory observations concerning NPY regulation of PRL and GH secretion via Y1 receptor subtype (NPY1R). However, the role of NPY in mediating non-gonadotropic hormones secretion by NPY1R has not been clearly defined in highly evolved non-human primates.

Aims and Objectives

The present study was undertaken to study the role of NPY in the control of PRL and GH secretion through Y1 receptor subtype in juvenile male rhesus monkey. For this purpose, highly specific NPY1R antagonist BIBO 3304 was used.

Materials and Methods

A total of four (n=4) juvenile male rhesus monkey (*Macaca mulatta*) were used to study the effect of NPY1R antagonist on GH and PRL secretion. These four juvenile monkeys were injected intravenously with either NPY1R antagonist 1ml of 2mg antagonist/animal or an equal amount of normal saline (1ml/animal) on two alternative days. A total of 3 boli of normal saline and antagonist in 4.8 % DMSO were injected with an interval of 60 minutes at 0, 60, and 120 min on two separate days of bleeding. On both days of bleeding, three blood samples (0.5-0.7 ml) were collected from animals at -60, -30 and 0 minutes. After taking 0-minute sample, animals were immediately injected with either vehicle or antagonist and then sequential blood samples with 30-minute interval up to 240 minutes were collected with the help of butterfly tube connected with 1cc syringe. Plasma was harvested and commercially available GH and PRL ELISA kit was used to measure GH and PRL concentration.

Results

Plasma PRL and GH levels in juvenile male rhesus monkeys treated with antagonist showed no statistically significant difference ($p>0.05$) as compared to vehicle group. Thus, current findings demonstrated that NPY signaling via NPY1R may not involve in regulating PRL or GH secretion in juvenile animals.

Conclusion

In conclusion, our study showed that there is no effect of NPY on PRL and GH release in juvenile male rhesus monkeys. However, contemplating previous data in different animal models, amount, and route of exposure of the drug are the key concerns. Therefore, non-involvement of NPY1R in mediating non-gonadotropic hormones secretion cannot be exclusively ruled out. Further investigations are required to ascertain plausible mechanisms by which NPY can regulate GH and PRL release. The study can be extended at the pituitary level to ascertain direct effect of NPY on somatotrophs and lactotrophs involved in GH and PRL release.

INTRODUCTION

INTRODUCTION

Neuropeptide Y (NPY) is a 36 amino acid tyrosine-rich neuropeptide that performs functions as neuropeptide, neuromodulator, or neurotransmitter depending on the circumstances. Almost 40 years ago, Tatemoto sequenced and identified NPY from porcine brain extracts in 1982 (Tatemoto et al., 1982a). NPY belongs to a family of structurally related peptides, which includes the hormones pancreatic polypeptide (PP) and peptide YY (PYY). NPY exhibits 70% structural homology with PYY and 50% homology with PP. NPY is abundantly present in brain areas whereas PP cause food absorption and it is released from pancreas in response to food intake (Joehl and DeJoseph, 1986) while PYY is expressed mainly in gut tissues (Tatemoto and Mutt, 1980). Allen and colleagues showed that all these structurally related peptides of the neuropeptide family have a common tertiary structure (hair pin like), that contains beta-turn connecting long alpha-helix and N-terminal poly-proline helix (Allen et al., 1987).

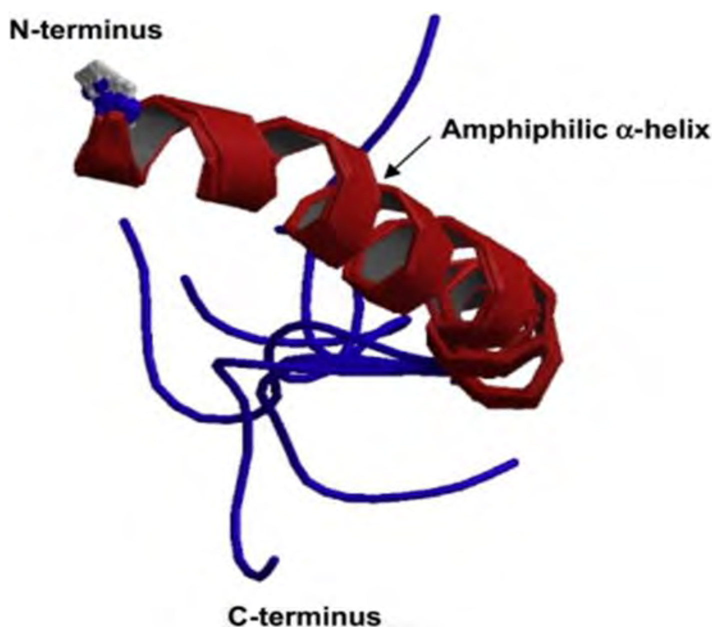

Fig i. NMR Structure of NPY

According to amino acids sequence of NPY depicted by Allen and colleagues for the rat and human origin, it has been shown that NPY primary structure is constituted by five tyrosine (Y) residues. This tyrosine resides at position 1, 20, 21, 27, and at 36 positions (Allen et al., 1987).

NPY is named as neuropeptide Y or neuropeptide tyrosine because it contains abundance of tyrosine in its structure. NPY, PP, PYY are grouped into same NPY family because of their terminal tyrosine in both carboxyl and amino terminals (Cerdá-Reverter et al., 1999).

NPY prepropeptide/gene locus

NPY shares remarkable sequence homology among some species and is evolutionarily well-preserved for over 450 million years (Larhammar, 1996). Although NPY is well conserved across vertebrate phyla, the position of NPY gene is different in different species. NPY encoding gene in rats is located on chromosome 04 and in humans on chromosome 07 at the locus 7p15.1 while NPY is located on chromosome 03 in rhesus macaques, each with 4 exons and 3 introns (Katsuya et al., 1993; Baker et al., 1995). The coding sequence of NPY consists of 291 bases (Minth et al., 1984). NPY genetic sequence was determined from human pheochromocytoma by in vitro translation of RNA. A 28 amino acid signal peptide, a 36 amino acid NPY molecule, and a 30 amino acids C-flanking peptide are present at two proteolytic cleavage sites in the inferred precursor amino acid sequence of NPY (CPON). A radioimmunoassay study for the predicted sequence of CPON acids shows that it is present in various body regions such as in adrenal gland, heart, and central nervous system with the highest concentration have been documented in the hypothalamic regions (Allen et al., 1985).

Central and peripheral expression of NPY

Several neurons in the central and peripheral nervous systems contain NPY. A thorough map of the NPY networks in the CNS has been created using specific antisera against NPY (Karla and Crowley, 1992). In addition to the anterior and medial preoptic hypothalamic areas, high-density NPY terminals can be seen across the suprachiasmatic, periventricular, paraventricular, and supraoptic nuclei (Chronwall et al., 1985). The arcuate nucleus (ARC) of the medial basal hypothalamus (MBH) is the area where abundance of NPY neuronal bodies is present whereas terminals of NPY are also projected in the median eminence area indicating that NPY can modulate neurosecretory activity of those neuronal populations the fibers of which culminating at ME. The internal and subependymal zones contain most of the NPY terminals, while fibers near the portal capillaries have also been discovered in the external zone (McDonald et al., 1987, Meister et al., 1989).

Allen et al. 1983 also discovered that the PVN nucleus of the hypothalamus is abundant with NPY immunoreactive bodies. The hippocampus and hypothalamus, particularly the ARC and dentate gyrus, have been reported to have the highest levels of NPY. Because of this, NPY is capable of controling the neuroendocrine release of many hypothalamic hormones, including luteinizing hormone and other hormones (Acuna-goycolea et al., 2005). As far as immunoreactive expression of NPY in human brain is concerned, it has been noted that basal ganglia, nucleus accumbens, limbic system and hypothalamus are the prominent areas where NPY is heavily concentrated. Among these areas, hypothalamus has gained special attention by the scientist to uncover the role of NPY in this area because this area is critically involved in appetite, energy regulation, homeostasis, and neuroendocrine control (Adrian, 1978).

In addition, NPY is also present in neuronal elements in the periphery (Emson and Dequidt, 1984). In the periphery, NPY is abundant in the sympathetic nervous system, where it usually acts with norepinephrine and adenosine triphosphate (ATP) to regulate cardiovascular and other functions. The primary source of circulating NPY is the adrenal medulla, (Cavadas et al., 2001), however, it was also found in platelets (Myers et al., 1988), megakaryocytes, bone marrow, spleen, blood cells, and thrombocytes of mice (Ericsson et al., 1987). Other tissues and organs which have NPY in abundance include the eye, thyroid, pancreas, ovary, kidney, skin, and spleen (Chen et al., 2005).

NPY difference from other peptides

NPY difference from PYY

NPY and PYY are identical at 25 positions in entire 36 residues. However, remaining 11 amino acids residues are non-identical, but all these 11 residues showed pairing with residues which show structural similarity. Among these variable positions, four positions are the replacements of Ser/Ala or vice versa, two exchanged positions for Asp/Glu, one position of exchange for Ile/Val and another position at which this replacement has been documented is Ile/Leu. In addition to these mentioned replacements, exchanges have also been observed at positions 7 and 13-14 for Asn/Ala and Pro-Ala/Ser-Pro, respectively. NPY and PYY show positional shift of Pro from position 13 to 14 like positional shift of avian PP and bovine PP. A greater number of exchanges has been observed between NPY and PYY, specially between Ser/Ala residues. These changes attribute significant variations in functional difference between these two

peptides. There is genetically single nucleotide replacement in 10 of the 11 non-identical residues of NPY and PYY, but two nucleotide changes were evident at position 7. The data observed about the similarity and difference between NPY and PYY show that a total of 12 base changes from total of 108 nucleotide positions are the prerequisite to change NPY into PYY or vice versa. These attributes lend credence to scientist to propose that a common ancestor may be present for both these peptides (Tatemoto et al., 1982b).

NPY and other PP difference

NPY shows 18 and 17 of its 36 residues similarity to porcine PP and bovine PP respectively. COOH-terminal region where 9 of the 13 residues at positions 24-36 are identical is responsible for such sequence homologies. In contrast, NPY and avian PP exhibit homology in 20 residues, with the most striking homology being in the central region at positions 8–17, where 8 of the 10 residues are similar. It has been reported that the brain contains a peptide that reacts with antisera raised against bovine and avian PP and hence, it further proves their structural similarity (Olschowka et al., 1981).

NPY receptors

From mammals, five NPY receptor subtypes have been cloned based on pharmacological profiles (NPY1R, NPY2R, NPY4R, NPY5R, and NPY6R), while multiple other receptor types, such as NPY3R, have been hypothesized (Berglund et al., 2003). Although an additional Y7 receptor (NPY7R) was cloned from chicken, its status is still unknown because it is ineffective in primates and does not exist in rat and human genome. NPY receptors are large transmembrane proteins coupled with G-protein belonging to the rhodopsin-like family. One of the gene groups with the greatest abundance in the human genome, accounting for more than 2% of all genes, is the G-protein-coupled receptor (GPCR) superfamily (Venter et al., 2001). All NPY receptors belong to G protein-coupled receptors (GPCRs) family, and there are over 800 GPCRs in the human genome. NPY and its receptors are widely expressed in the body. The spinal cord and the brain both express these receptors in various locations (Larhammar et al., 1992).

In 1992, the first human Y1 receptor was cloned (Larhammar et al., 1992). In 1995, two more subtypes of the receptor Y2 and Y4 receptors were cloned (Gerald et al., 1995; Rose et al., 1995). In the next year, another receptor, the Y5 receptor was discovered (Gerald et al., 1996;

Weinberg et al., 1996). Among these, four of the receptors (Y1, Y2, Y4, and Y5) are functionally active in humans while y6 is not functional in primates due to an inactivating mutation therefore its biological relevance remains to be clarified (Larhammar et al., 1992; Michel et al., 1998; Pedrazzini et al., 2003). In the 5q31 region of chromosome 5 in humans, the y6 receptor is an inactive pseudogene (Gregor et al., 1996; Matsumoto et al., 1996). The subtypes found in mammals possibly originated by means of gene duplication prior to the origin of vertebrates (Larhammar et al., 1992). Y1, Y2 and Y5 have been localized to the same chromosomal segment in humans, while Y4 and y6 have been mapped to separate chromosomes. All these subtypes have been cloned in mouse, human (Michel et al., 1998), pig (Wraith et al., 2000) and guinea pig (Sharma et al., 1998; Lundell et al., 2001). Each receptor has preference affinity for a particular ligand within NPY-PYY family of peptide (Magni, 2003). NPY1 receptor (NPY1R) and NPY5 receptor (NPY5R) prefer to bind with NPY and PYY, NPY2 receptor (NPY2R) has higher affinity for PYY, while NPY4 receptor (NPY4R) preferentially binds to PP (Tatemoto et al., 1982b). Pharmacological studies also suggested the existence of another receptor isotype designated as Y3 and it is controversial putative NPY preferring receptor (Gehlert, 1998; Lee and Miller, 1998). It is suggested that One or more NPY receptors may multimerize to form the putative Y3 receptor (Wahlestedt et al., 1992).

NPY1 receptor

NPY1R is the main type of NPY receptor, and it was the first to be cloned in early investigations using different peptide segments (Wahlestedt et al., 1986; Herzog et al., 1992). NPY1R has 384 amino acids and is pharmacologically unique from NPY2R by its ability to bind [Leu31, Pro34] (Herzog et al., 1992). In mammals 92-95% homology has been observed in NPY1R gene. NPY1R gene consists of 4 exons with two introns and its chromosomal localization is different in different species (Larhammar et al., 2001).

Central and peripheral expression of NPY1R

It has been shown that NPY receptors such as NPY1R and NPY2R appear in MPOA (Dumont et al., 1990; Aicher et al., 1991). In the human brain's hypothalamus, modest quantities of NPY1R mRNA were found inside the caudate, nucleus accumbens, putamen, ARC, amygdaloid nuclei, and paraventricular nucleus (Jacques et al., 1996).

According to the previously published NPY distribution and anticipated NPY binding locations (Adrian, 1978), NPY1R expression was discovered in the hypothalamus, different amygdaloid nuclei, the hippocampus, and multiple thalamic nuclei in the rat and mouse. These findings were made using immunocytochemical detection techniques (Eva et al., 1990; Kishi et al., 2005). NPY1R mRNA expression has also been noted in different peripheral tissues. Among these, adipose tissues (Yang et al., 2008), colon (Goumain et al.,1998), and pancreatic cells (Morgan et al., 1998) are the most prominent regions where NPY1R mRNA expression has been detected. The epithelial and mucosal nerves of the colon, kidney, adrenal gland, heart, and placenta in humans express NPY1R (Wharton et al., 1993). The NPY1R plays a role in regulating NPY-induced hyperphagia, multiplication of smooth muscle and neural precursor cells, vasoconstriction, as well as involve in avoiding ethanol tolerance (Bhisikar et al., 2009).

Other NPY receptors

All NPY receptors have diverse tissue expression profiles and hence have various binding affinities to NPY family peptides. The majority of Y2 receptor expressions occur presynaptically. Neurotransmitter release is inhibited because of their activation by the peptides NPY, and PYY. In addition, cleaved N-terminal product from 3-36 position of both NPY and PYY also inhibit neurotransmitter release (Wahlestedt and Hakanson, 1986; Smith-White et al., 2001). Adipocyte proliferation, differentiation, and angiogenesis within adipose tissue, all are facilitated by Y2 receptors (Kuo et al., 2007). With a picomolar range binding affinity, the Y4 receptor binds PP preferentially. This receptor is important in regulating reproductive activity (Sainsbury et al., 2010). This is due to involvement of this receptor in modulating the activity of reflex arc of vago-vagal in the area postrema which ultimately conveys PP anorexigenic signaling from peripheral circulation to higher brain center- hypothalamus (Lin et al., 2009). The hypothalamic paraventricular nucleus (PVN) is the site of expression for NPY5R, which cooperates with NPY1R to control energy balance (Gerald et al., 1996; Kanatani et al., 2000; Mashiko et al., 2009). Pharmacological studies in many tissues (Lee and Miller, 1998) have led to the hypothesized existence of NPY3R, although its cloned status has not yet been established. The y6 receptor is only a functioning receptor in rabbits and mice. According to Weinberg et al. (1996), NPY6R is expressed in the hypothalamic suprachiasmatic nucleus of mice, suggesting that these receptors may be involved in the control of diurnal rhythms and energy homeostasis.

NPY signaling mechanism

A series of G-protein coupled receptors is used by the NPY family of peptides to communicate. The activation of the NPY receptors link to inhibitory G-proteins subunits results in inhibitory responses. These inhibitory responses are manifested by downregulation of adenylate cyclase and subsequently inhibition of secondary messenger cyclic AMP (Herzog et al., 1992; Motulsky and Michel, 1988). Additionally, inhibition of potassium and calcium channels are also observed upon NPY activation (Aakerlund et al., 1990; Sun et al., 2001). The Y_1 receptor can also couple to other second messenger systems in control of direct Ca^{2+} influx (Prieto et al., 2000), thereby, imparting significant effects on cellular polarization and electrical signaling. When the NPY1R is activated, the metabolism of phospholipase C and inositol phosphate increases intracellular Ca^{2+} mobilization, whereas the NPY2R reduces the inflow of Ca^{2+}, further inhibiting the target cells' secretory activity (Ewald et al., 1988). Both NPY1R and NPY2R are adversely correlated with adenylcyclase (Kalra and Crowley, 1992). All NPY receptors mediate their effects via Gi protein-coupled receptors (Fig ii).

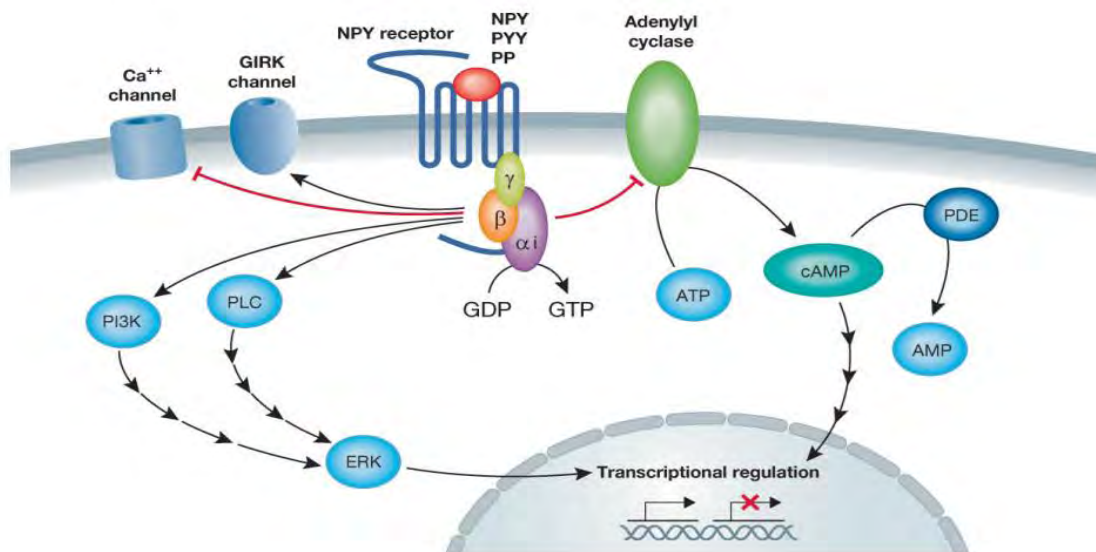

Figure ii. Typical NPY receptor intracellular signaling pathways. The alpha subunit of the Gi signaling cascade, which inactivates adenylyl cyclase, is related to all NPY receptors. There are several kinase cascades that the beta and gamma subunit triggers. Additionally, Ca^{2+} channel activity can be decreased, and G protein-coupled inwardly rectifying potassium (GIRK) currents can be increased when the G protein complex is activated (Acuna-Goycolea et al., 2005). The physiological effects of NPY are based on the activation of these cellular signaling cascades, which has a variety of impacts including the start of transcription or the stimulation or inhibition of hormone/neurotransmitter release.

Central and peripheral effects of NPY

NPY had a role in several physiological and pathological processes since its discovery. The peptide has been shown to have both central and non-central peripheral effects. In fact, NPY is the peptide that is most prevalent in the brain. Feeding behavior, anxiety, epilepsy, circadian rhythms, memory function, sleep, pain, and drug addiction are among the main effects of NPY. Numerous pieces of evidence suggest that NPY is essential for controlling hunger, body weight, and obesity (Silva et al., 2002). Humans and rodents have shown the peptide's anxiolytic qualities, and it may also play a role in depression (Heilig, 2004).

The cardiovascular actions of NPY's peripheral effects which are predominately located in sympathetic fibers include modulation of heart rate, coronary blood flow, ventricular function, and a vascular mitogen. Through certain postsynaptic NPY receptors, NPY also promotes atherosclerosis (Pons et al., 2004; Zukowska, 2005), and it may possibly be a candidate peptide for coronary artery disease (Shah et al., 2009).

NPY functions

Researchers started to hypothesize about the role of NPY in hypothalamic-mediated processes after NPY was isolated from the pig hypothalamus in 1982. NPY has been regarded as vital factor in adaptability of organisms under environmental stresses such as during predator assault, starvation, or infection. This adaptability helps the organism to survive under such stressful conditions by modulating their behaviors (Sokolowski, 2003). Studies revealed that NPY is a crucial energy homeostasis regulator in vertebrates and is frequently regarded as one of the strong hunger stimulants in both mammals and fish (Volkoff et al., 2005). Hypothalamic NPY increases appetite signaling in a leptin-dependent manner (Egan et al., 2017).

The NPY system has been found to be engaged in a wide range of physiological processes in animals, including the release of hormones in addition to its significant roles in food intake and energy balance (McDonald et al., 1985), obesity (Kuo et al., 2007), stress response (Morales-Medina et al., 2010), anxiety (Domschke et al., 2010), circulation of blood (Zukowska-Grojec and Wahlestedt, 1993), embryogenesis (Mathieu et al., 2002), sleep (Dyzma et al., 2010), sexual behavior (Kalra and Crowly, 1992), controlling energy balance regulation (Brady et al., 1990; Turton et al., 1997), memory retention (Borbely et al., 2013), and also in, secretion of

pituitary hormones e.g. gonadotropin, adrenocorticotropin, thyrotropin, growth hormone and prolactin hormone (Kalra et al., 1984; Contijoch et al., 1993; Peng and Peter, 1997; Carpio et al., 2006).

Role of NPY on the HPG axis

Although NPY is well recognized for its influence on appetite, numerous studies have shown that it also has a significant impact on the regulation of anterior pituitary hormones production, including that of reproductive hormones (Kalra,1993). NPY plays a pivotal role in regulating HPG axis by regulating GnRH release. NPY neurons in the arcuate region of hypothalamus have been shown to project to GnRH neurons in monkeys (Plant and Shahab, 2002) and in mice (Turi et al., 2003). GnRH neurons have been shown to express NPY1R (Li et al., 1999). NPY acts as neurobiological brake on GnRH pulse generator before the onset of puberty (El Majdoubi et al., 2000). At the hypothalamus level, NPY regulates GnRH release to control reproductive function (Khorram et al., 1987; McDonald et al., 1989; Kaynard et al., 1990).

Depending on the steroid levels, NPY either stimulates or inhibits the reproductive axis. For instance, NPY bolus injection stimulates luteinizing hormone (LH) release when oestrogen and progesterone levels are high, such as on the afternoon of proestrus. However, when gonadal steroids are low, as in the case of ovariectomized females or castrated male rats, NPY decreases the release of LH (McDonald et al., 1989; Bauer-Dantoin et al., 1992). In addition, chronic NPY infusion disrupts cyclicity in adult female rats and delays the beginning of puberty in prepubescent rats of both sexes, indicating that long-term exposure to high amounts of NPY acts as a suppressor for the release of LH (Catzeflis et al., 1993; Gruaz et al., 1993; Pierroz et al., 1995).

The release of reproductive hormones is regulated by modulatory substances such NPY, which is completely necessary for the regular female reproductive cycle. During the cycle, pulsatile release of GnRH occurs in conjunction with NPY in the hypophyseal portal blood vessels (Woller and Terasawa, 1992). This concomitant surge pattern of LH has been abolished after NPY deletion in mice (Xu et al., 2000). Likewise, immunoneutralization of NPY considerably reduces LH surge pattern (Sutton et al., 1988). NPY expressing neurons in the hypothalamus have been demonstrated in male monkeys (Plant and Shahab, 2002). The close association between NPY and GnRH neuronal entities has led to propose that NPY may regulate GnRH

release. However, interestingly, this juxtaposition between these two neuronal moieties observed to be higher in prepubertal animals as compared to adult animals conferring NPY as neurobiological brake on pulsatile release of GnRH during postnatal developmental period (Plant and Shahab, 2002). This conclusion was corroborated by the research showing that NPY administration centrally halt the GnRH induced pulsatile LH release in both sexes of agonadal monkeys achieved pubertal onset (Kaynard et al., 1990; Pau et al., 1995; Shahab et al., 2003). Postnatal developmental transition from infant to pubertal stage also showed inverse relationship between gene and peptide content of NPY and GnRH pulse generator activity as depicted by LH levels in male monkeys (El Majdoubi et al., 2000).

Additionally, it has been shown that NPY icv administration caused suppression in GnRH release in postpubertal adult monkeys mediated through NPY1R (Shahab et al., 2003). The critical involvement of NPY1R in mediating NPY based suppressive effect on LH release has been pharmacologically confirmed by using different antagonists. A variety of NPY1R antagonist have been tested to evaluate the role of NPY1R in inducing NPY dependent LH release. In this regard, in prepubertal non-human highly evolved primates have shown that icv administration of NPY1R antagonist enhanced GnRH pulse generator activity by diminishing NPY suppressive effect (El Majdoubi et al., 2000; Shahab et al., 2003). These data indicate that NPY acts as neurobiological brake in suppressing HPG axis activity during prepubertal stage.

Several studies indicate that NPY through NPY1R stimulates the release of LH and a selective NPY1R antagonist BIBP3226 when injected peripherally inhibited the release of proestrus LH and GnRH induction (Leupen et al., 1997) while It is interesting to note that chronic icv infusion of highly specific BIBP 3226 caused advancement in pubertal development in prepubertal female rats suggesting hiatus of GnRH before pubertal initiation is under the control of NPY arrest through NPY1R (Pralong et al., 2000). Since the antagonists used in primate studies were exhibiting both agonist and antagonist activity against NPY4R and NPY1R respectively, and antagonist used in rodent study was hazardous therefore the results are unclear as evident by pharmacological data regarding the involvement of the NPY1R in mediating NPY effect on GnRH release (Shahab et al., 2003).

Kisspeptin-GPR54 is a key neuroendocrine regulator of GnRH release (Plant, 2006; Tena-Sempere, 2006) according to genetic and pharmaceutical research. Kisspeptine-GPR54 not only had a significant impact on pubertal commencement (Han et al., 2005; Shahab et al.,

2005), but it also controlled the release of GnRH in animals belonging to order primate (Terasawa et al., 2013).

Both kisspeptin and NPY are shown to be potent stimulator and inhibitor of the HPG axis respectively, (Terasawa et al., 2013). Recently an intimate association between NPY and kisspeptin has been observed in ewes (Backholer et al., 2010; Polkowska et al., 2014) suggesting that NPY may regulate the HPG axis activity indirectly via kisspeptinergic pathway.

Moreover, recently a peptide known as gonadotropin inhibiting hormone (GnIH), which acts as a suppressor of gonadotrophin release, has been described in various mammalian models, including higher primates (Tsutsui, 2010). Close contact between NPY neurons and GnIH fibers has been also demonstrated in mice (Jacobi et al., 2013) which probably indicate that NPY based inhibition of GnRH pulse generator might be mediated through GnIH dependent intermediatory neuronal network. Hence, it is indicated that the HPG axis is highly susceptible to both internal and external influences for proper operation (Cameron, 1996; Wade and Jones, 2004).

Effect of NPY on prolactin hormone

NPY is a pleiotropic hormone involved in the control of food intake (Kalra and Crowley, 1992) as well as in the regulation of LH (Crowley and Kalra, 1984; Khorram et al., 1988; Bauer-Dantoin et al., 1992) and PRL (Ghosh et al., 1991; Wang et al., 1996) release. Although NPY has been shown to regulate prolactin (PRL) secretion, conflicting results have been obtained from different experiments involving different animal models.

The lactotrophs, which are specialized cells found in the anterior part of the pituitary gland, produce the polypeptide hormone, prolactin. PRL was first discovered in the late 1920s and given that name because it promotes lactogenesis in several animals and encourages the formation of mammary tissue (Trott et al., 2008). It is also referred to as luteotropic hormone due to its function in the maintenance and production of the corpus luteum.

Growth hormone (GH), prolactin (PRL), and placental lactogen (PL) genes, all descended from the same ancestral gene (Niall et al., 1971). It is a member of the PRL/GH/PL protein family and shares similarities with GH and PL in its amino acid sequence. About 400 million years ago, the GH and PRL lineages diverged (Cooke et al., 1980; Cooke et al., 1981). On

chromosome 6 of the human genome, there is only one prolactin-encoding gene (Owerbach et al., 1981). The gene encodes 5 exons and 4 introns and 10 kb (Truong et al., 1984). Single chain PRL molecule consists of 199 amino acids with six cysteine residues by three disulfide bridges (Cooke et al., 1981).

As the primary purpose of PRL is to encourage the production of milk, PRL was first identified and given that name based on this effect. However, a variety of prolactin-controlled processes have been identified over time. An early review identified 85 functions of PRL in the body (Nicoll, 1974), and this has subsequently been updated to over 300 identified functions (Bole-Feysot et al., 1998). PRL currently has a wide range of activities in the brain after being implicated in many new functions over the past ten years (Grattan, 2001). PRL affects sexual behavior and reproduction in both primates and non-primates in a variety of ways (Ben-Jonathan et al., 2008). Pleiotropic hormones, such as PRL, are those in which a single gene regulates a variety of diverse phenotypic consequences. It has a wide range of neuroendocrine effects, including suppression of fertility, activation of hypothalamic dopamine neurons to regulate its secretion, stimulation of maternal behavior, stimulation of myelination in the central nervous system, and the development of new neurons in the olfactory bulb and suppression of the stress response. This pleiotropic term perfectly captures these effects. In rats and primates, PRL is a key regulator of the luteotrophic complex, which controls LH and FSH (Richardson et al., 1985). PRL inhibits the luteinization of granulosa cells in humans (Adashi and Resnick, 1987). It stimulates prostate growth and increases the number of the androgen receptors on the prostate. Hyperprolactinemia reduces sperm fertilization and motility. On the other hand, hypoprolactinemia produces azoospermia. PRL is also involved in the maintenance of the Leydig cell morphology, probably via suppression of the gonadotropin secretion (Wang, 2001). Evidence suggests that both PRL and progesterone cooperatively operate together to enhance mammary gland growth in addition to segregated effects. In many animals, PRL plays a crucial part in several aspects of reproduction. Although PRL is frequently referred to as an adaptive hormone, there have been few attempts to explain why and how a single hormone may affect so many different and seemingly unrelated neuroendocrine activities.

Inhibitory regulation by dopamine plays a major role in the dual inhibitory and stimulatory hypothalamic control of PRL (Neill, 1974; Shin et al., 1987). Dopamine inhibits PRL activity. Dopamine is produced by tuberoinfundibular dopaminergic neurons (TIDA). Dopamine acts on the surface of lactotrophs by influencing the D2 (dopamine receptor) after being produced

from the hypothalamus into the hypophyseal portal circulation (Goldsmith et al., 1979; Holzbanct and Racke, 1985). In addition to TIDA neurons, another population of dopaminergic releasing characteristics called tuberohypophyseal dopaminergic neurons (THDA) residing in ARC also control dopamine release (Arbogast et al., 1989). Additionally, the release of PRL is impacted by catecholamine metabolism (Arimura et al., 1972).

Most of the research that have been published so far have shown that NPY has an inhibitory effect on PRL secretion. For example, Wang et al. 1996 found that NPY reduced PRL production from rat anterior pituitary cells when administered centrally. Opposite to the above cited observation, PRL secretion from dispersed estrogen-primed cultured pituitary cells was stimulated by NPY (Hill et al., 2004). While primary cultures of pituitary cells from ovariectomized rats treated with estradiol showed amount of the drug suppression of PRL release in response to NPY. Male rats' PRL release increased after endogenous NPY was neutralized by antibodies, demonstrating that the overall function of NPY is to suppress PRL secretion (Ghosh et al., 1991). However, another study showed stimulatory effect of NPY on in vitro PRL secretion from cultured pituitary rat cells (Baranowska et al., 1999).

NPY13-16 (a Y2 receptor agonist) icv injection increases the mRNA expression of PRL (Garcia et al., 1995). Since the anterior pituitary lacks Y2 receptors (Sheikh et al., 1998), the peptide's activity is most likely mediated through a primary hypothalamic action. Moreover, intracerebroventricular (icv) administration of NPY partially inhibits PRL secretion in male rats by increasing dopamine secretion (McCann et al., 1973; McCann et al., 1989; Kalra and Crowly, 1992). NPY icv administration increased tuberoinfundibular dopaminergic neurons activity in male rats (Fuxe et al., 1989).

During lactation, the mediobasal hypothalamus expresses more NPY (Ciofi et al., 1991; Pelletier and Tong, 1992; Smith,1993; Ciofi et al., 1993, Malabu et al., 1994). Since TIDA neurons typically do not express NPY, the discovery of immunoreactive NPY in these neurons is particularly unexpected. NPY promotes the release of PRL from randomly cycled rat cultured pituitary cells (Chabot et al., 1988). However, research from lactating or animals ovariectomized with estradiol-treatment demonstrates that NPY reduces PRL release from primary cultures of anterior pituitary cells in a concentration-dependent manner (Wang et al., 1996). PRL secretion is further inhibited by the peptide's inhibitory impact through dopamine (Wang et al., 1996). Like DA withdrawal, the peptide's removal from the culture media

promotes a rapid rebound in PRL output (Wang et al., 1996). Because NPY is present in TIDA neurons and their nerve terminals reside in the median eminence, it is possible for the two alternative mechanisms to take place. First, NPY may modify dopamine release presynaptically or second, dopamine directly acts in the pituitary to modulate PRL secretion (Wang et al., 1996). NPY administered intracerebroventricularly in male rats reduces PRL secretion (Rettori et al., 1990b). Inhibitory action of NPY on PRL secretion has been evident in intact male rats when antiserum against NPY injected centrally cause increase in plasma PRL levels (Ghosh et al., 1991). This shows importance of sex steroids to mediate central inhibitory action of NPY. The mediobasal hypothalamic expression of NPY greatly increases during lactation (Ciofi et al., 1991; Smith,1993; Malabu et al., 1994). However, despite being a lactation-dependent event, the increase in NPY expression does not require high plasma PRL levels (Pelletier and Tong, 1992). Thus, it is suggested that PRL feedback is not mediated by NPY in the hypothalamus.

Effect of NPY on growth hormone secretion

The 22,000-dalton protein known as growth hormone (GH), commonly referred to as somatotropin, is released by the somatotrophs from the anterior pituitary gland in mammals. GH belongs to a set of structurally related peptide hormones that includes PRL and placental lactogen (PL; chorionic somatomammotropin) (Baxter et al., 1979). GH consists of a single 191 amino acids polypeptide chain, containing two intrachain disulfide bridges. Remarkable structural similarity exists in GH of human and monkey (Li et al., 1986), differing at only positions 105, 107, 133, and 173 at four residues due to the mutation of only one codon nucleotide.

Main job of GH is to encourage postnatal longitudinal growth. In addition to stimulating bone development, it controls the metabolism of carbohydrates, lipids, nitrogen, and minerals. It increases adipocyte lipolysis, which reduces body fat. It enhances muscular protein synthesis, nitrogen retention, muscle strength, and muscle hypertrophy (Bonert and Melmed, 2017; Troike et al., 2017). GH also has an impact on the cardiovascular system, immune system, central nervous system, neurogenesis, and aging (Brooks and Waters, 2010; Waters and Blackmore, 2011; Bartke et al., 2016; Gesing et al., 2017). Therefore, aberrant GH secretion or release may influence a variety of tissues and organs. GH overproduction results in childhood gigantism and adult acromegaly, whereas congenital GH signaling disruption results

in short stature. It is a well-known fact that a complicated neuroendocrine regulatory mechanism, specifically the interaction of two hypothalamic hypophysiotropic hormones, regulates GH secretion. GH is secreted in a pulse fashion, and its pattern depends upon the GH-releasing hormone (GHRH) and reciprocal action of somatostatin (SS) on pituitary somatotrophs (Tannenbanum and Ling, 1984). GHRH is released from the ARC, and it stimulates the secretion and synthesis of GH from the pituitary (Brazeau et al., 1982; Barinaga et al., 1983; Ibata et al., 1986). SS is primarily released from the periventricular nucleus (PeN) in the hypothalamus, and it inhibits the secretion of GH (Reichlin, 1983; Kawano and Daikkoku, 1988). Through a short-loop feedback system that controls the production and release of SS and GHRH, GH regulates its own secretion. Numerous studies indicate that GH inhibits the synthesis and secretion of GHRH from ARC neurons and promotes the secretion and synthesis of SS from neurons in the PeN (Chihara et al., 1981; Katakami et al., 1987; Chomczinski et al., 1988; Rogers et al., 1988).

Somatotrophic and gonadotrophic axes are closely associated with growth and sexual maturation. LH and FSH primarily control how the gonads operate during gametogenesis. GH is crucial for puberty, sexual differentiation, steroidogenesis, ovulation, and gonadal development (Zachmann, 1992). GH is typically but not always required for the timing of sexual development, as evidenced by the frequent correlation between GH deficiency and delayed or missing puberty and the GH injection inducing pubertal onset. The relevance of GH in triggering sexual maturity is demonstrated by the stimulation of sexual maturation in GH-deficient children (Darendeliler et al., 1990; Stanhope et al., 1992) and GH-depleted monkeys (Wilson et al., 1989) by exogenous GH. Growth hormone stimulation of the GnRH pulse generator led to the onset of puberty (Bartke et al., 1999) or the potentiation of androgen action (Ilondo et al., 1982).

Furthermore, preliminary findings indicated that NPY neurons in the ARC express the GH receptor, implicating that NPY also serves as crucial role in modulating feedback control of GH secretion (Chan et al., 1996). In sheep, central treatment of NPY is known to stimulate GH release via stimulating GHRH (Gładysz et al., 2001; Morison et al., 2003). On the other side, NPY inhibits GH by acting on somatostatin (SS) cells in the hypothalamic PeN (Hisano et al., 1990; Chan et al., 1996; Bluet-Pajot et al., 1998; Lanneau et al., 2000; McMahon et al., 2001; Gładysz et al., 2001; Wren et al., 2002, Park et al., 2005). The pituitary somatotrophs are affected by the GHRH and SS cells, which operate to either stimulate or inhibit GH release.

The GHRH and SS cells project to the external zone of the median eminence (Willoughby et al., 1995; McMahon et al., 2001). Intraperitoneal treatment of NPY caused a noticeable increase in the serum GH concentrations of goldfish (Peng et al., 1993). Catfish also exhibits the same stimulatory effects of NPY on GH secretion (Mazumdar et al., 2006). In addition, numerous studies showed that NPY may directly influence GH secretion in rat and pig pituitary glands (McDonald et al., 1985; Barb and Barrett ,2005). Furthermore, immunohistochemical analysis revealed that NPY neurons colocalized in GH immunoreactive cells in cattle (Ogasawara et al., 2008). Dual fluorescent immunohistochemistry of hypothalamic sections of humans shown that intimate association exists between NPY and GHRH neuronal systems (Deltondo et al., 2008). Thus, from the above cited data it is possible that NPY can directly regulate GH expression or indirectly by GHRH.

Interestingly, NPY prevented acromegaly patients' cultured pituitary adenoma cells from secreting GH. Therefore, it is possible that NPY's effect on the hormone release from neoplastic pituitary cells will be different from that on normal pituitary cells (Adams et al., 1987).

Although stimulatory effect of NPY on GH secretion has been documented in various animals under in vivo and ex vivo conditions, however, inhibitory effect of NPY on GH secretion has also been observed. In this context, in human pituitary cells NPY directly inhibited GH secretion in vitro (Adams et al., 1987). Indeed, prior research demonstrated that a rise in NPY levels under stress results in a decrease in GH and GHRH secretion (Barb and Barrett, 2005). In fasting diabetic male rats, pulsatile GHRH secretion has been observed to decrease, although NPY levels increased (Park et al., 2004). In fasting mice, GHRH secretion has been found to decline when a rise in NPY and CRF levels was observed (Luque et al., 2007). In addition, hypothalamic GHRH mRNA was significantly enhanced and NPY mRNA decline after refeeding (Luque et al., 2007).

From the above-cited data, it has been postulated that NPY plays a role in modulating the release of anterior pituitary non-gonadotropic hormones both GH and PRL. Previous studies showed the suppressive and enhancing effect of NPY on PRL and GH secretion respectively in various animal models, but the results were contradictory in different studies. However, the role of NPY in mediating non-gonadotropic hormones secretion by NPY1R has not been clearly defined in highly evolved non-human primates. Therefore, the present study was

undertaken to study the role of NPY in the control of PRL and GH secretion through NPY1R in juvenile male rhesus monkey by using NPY1R specific antagonist.

MATERIALS

AND

METHODS

MATERIALS AND METHODS

Animals

In the current study, four juvenile male rhesus monkeys (*Macaca mulatta*) were employed to study the effect of NPY1R antagonist on growth hormone and Prolactin secretion. These 10-14 months old monkeys weighing 1.8-2.2 kg (2 ±0.11 kg mean ± SEM) were used to conduct the experiment. All the animals were roomed in separate cages at the Departmental Primate Facility under semi-controlled settings (at room temperature 25±3°C and lights on from 06:00 to 18:00 h). At 10:00 h, the animals were fed with fresh seasonal fruits, at 12:30-13:30 h, they were provided boiled potato and egg, and 14:00 h bread was given. Water was available *ad libitum*. Additionally, vitamins were given as supplements. A few weeks before the start of the experiment, animals were trained to sit in the restraining chair. Restraint time was gradually lengthened to 4-5 hours per day until it was attained. For placing and removing of monkey from the chair, animals were tranquilized with ketamine hydrochloride (Ketler, Astarapin, Germany 5 mg /kg body weight, i.m). They were also provided with nuts and fruits during restraining. The Departmental Committee for Animal Care and Use approved all experimental protocols.

Pharmacological agent

Ketamine hydrochloride (Ketler, Astarapin Germany), heparin (Rotex media, Trittau, Germany), Normal Saline and DMSO (Dimethyl sulfoxide) were purchased from local pharmaceutical market. BIBO 3304 ((R)-N-[[4-(aminocarbonylaminomethyl)phenyl]methyl]-N^2-(diphenyl acetyl)-arginine amide trifluoroacetate) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

General experimental design

Four juvenile male rhesus monkeys (n=4) were utilized for sequential blood sampling on two alternative days. On the first day, animals were bleed for 1 hour before vehicle administration while eight blood samples with 30 minutes interval were collected after vehicle administration. After the gap of one day, second sequential bleeding was done. On the second day, three blood samples were collected before antagonist administration while eight blood samples were drawn

after the antagonist's administration. On both occasions serial blood sampling was done from 10:30-2:30 h, at the interval of 30 minutes. Details of sequential blood sampling were as follows.

Vehicle administration (Day 1)

A total of 11 blood samples were collected. Three blood samples (0.5-0.7 ml) were collected from animals at -60, -30 and 0 minutes. After taking 0-minute sample animals were immediately injected with vehicle (normal saline in 4.8% DMSO). A total of 3 boli of vehicle 1 ml /animal with an interval of 60 minutes at 0, 60, 120 minutes. A total of eight blood samples at 30,60,90,120,150,180,210,240 minute were collected after vehicle administration with the help of butterfly tube connected with 1cc syringe.

Antagonist administration (Day 2)

On the alternative day animals were injected with BIBO 3304 1ml of 2mg antagonist/animal. Before antagonist administration three blood samples at (-60, -30 and 0) were drawn. A total of 3 boli of antagonist in 4.8 % DMSO with an interval of 60 minutes at 0, 60, 120 min were injected. A total of eight blood samples were taken at (0,30,60,90,120,150,180,210,240) minutes from three animals while four blood samples were drawn from fourth animal after antagonist administration.

Venous catheterization

Under deep ketamine (5mg/kg body weight, i.m.) anesthesia, a teflon cannula (Vasocan Branule, 0.7 mm / 24 G O.D, B. Braun Melsungen AG, Belgium) was inserted in the saphenous vein. A butterfly tube (length 300 mm, volume 0-20 ml, 22 GX3 / 4 ″, JMS, Singapore) was attached to the cannula's distal end. Blood samples were drawn from the proximal end of the butterfly tube connected with a syringe (1cc -26G-X-1/2”). Blood sampling proceeded after the full recovery of the animals from anesthesia.

Blood sampling

Blood samples of 0.5-0.7 ml were collected on both occasions. Seven-hour sequential blood sampling was carried out at 30-minute intervals. Blood samples were collected in a 1ml syringe

(26G) and immediately transferred to eppendorf tubes. Samples were centrifuged at 3500 rpm for 15 minutes. Plasma was separated and kept at -20 ºC until used for hormonal estimation. After every sample, the assembly was flushed with heparin (5IU/mL) to avoid any blood clotting and an equal amount of saline was injected to prevent the animal from hypovolemic shock.

Prolactin assay

Assay principle

The prolactin (PRL) kit is based on solid-phase ELISA. The assay kit uses a mouse monoclonal antibody-enzyme (HRP) conjugate solution and one anti-prolactin antibody for coating microtiter plate wells for solid phase immobilization. The sample reacted with solid phase antibodies, and with the enzyme conjugate, forming sandwich PRL between the two antibodies. The addition of chromogen solution after incubation and washing resulted in the appearance of blue color. The color development was stopped with the addition of stop solution, changing color to yellow. Color intensity was measured at 450 nm, and it was directly proportional to the concentration of PRL in the plasma.

Assay procedure

Total plasma concentration of PRL was measured using a PRL ELISA kit (JTC Diagnose Mittel UG, Schulweg, Voehl, Germany Catalogue no. Pro-96). The standard protocol provided within the kit was followed .50 µl of sample, quality control, and standards were added. After that 100 µl of enzyme conjugate was added in every well and gently mixed by tapping then plate was kept at 22-24 ºC for 30 minutes. The microplate was rinsed five times with 300µl of working wash solution after being emptied of its contents into a waste container. The remaining volume was eliminated by smashing the plate on absorbent paper. 100 µl of chromogen-substrate solution was added to every well, covered and kept for 15 minutes at 22-24 ºC in dark. Then 100 µl of stop solution was added and within 30 minutes, a microplate reader (BIBIO-RAD) was used to read the absorbance at 450 nm. Intra and inter-assay coefficient of variation was <6% and <7%, respectively. The sensitivity of the assay was 15mIU/L.

Growth hormone assay

Assay principle

The solid phase sandwich hGH method is the foundation of the hGH ELISA. To the wells coated with streptavidin, the samples and conjugate reagent (anti-hGH biotin and HRP) were added. hGH in the sample binds to the matched pair of antibodies to create a sandwich complex, which is then immobilised by interactions between streptavidin and biotin. Through a washing stage, unbound protein and HRP conjugate were removed. The amount of hGH present in the samples was inversely correlated with the colour intensity following the addition of substrate. By connecting the absorbance to the hGH concentration, a standard curve is created.

Assay procedure

The total plasma concentration of GH was measured using a GH ELISA kit (JTC Diagnose Mittel UG, Schulweg, Voehl, Germany Catalogue no. HG377S). The standard protocol provided within the kit was followed. 50 µl of sample, and standards were added into appropriate wells. After that 100 µl of enzyme conjugate reagent was added in every well, gently mixed by tapping, and the plate was kept at 20-25 ºC for 60 minutes. The microplate was rinsed by flicking the plate contents in a waste container and then rinsed three times with 300 µl of working 1X wash solution. By smashing the plate against absorbent paper, all the remaining volume was eliminated. After that, each well received 100 µl of TMB-substrate solution, which was then added, covered, and left at room temperature in the dark for 15 minutes. Then 100 µl of stop solution was added to each well, and after 15 minutes, the absorbance at 450 nm was read using a microplate reader (BIO RAD). Intra and inter-assay coefficient of variation was <8% and <9%, respectively. The sensitivity of assay was 0.036µIU/L.

Statistical analyses

GraphPad Prism Version 9 was used to perform data analysis (GraphPad Software Inc., La Jolla, CA, USA) and all the data were expressed as mean with standard error mean (mean±SEM). Differences in mean plasma PRL and GH levels were determined by using a two-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison test.

Difference between vehicle and antagonist treated animals was analyzed using student's t-test. A value of $p > 0.05$ was considered statistically significant.

RESULTS

RESULTS

Effect of vehicle and NPY1R antagonist on plasma prolactin levels

Plasma PRL levels in individual juvenile male rhesus monkeys treated with vehicle (normal saline) at different time points are shown in Table 1. Blood plasma PRL levels in individual juvenile male rhesus monkey treated with antagonist at different time points are shown in Table 2**.**

Variations in plasma PRL levels related to different time points after vehicle (normal saline) and antagonist administration in individual juvenile male rhesus monkeys are represented in (Figure 1). No considerable individual variations in plasma PRL levels were evident in either vehicle administered, or antagonist treated monkeys. However, marked variations in prolactin levels are seen across few time points during vehicle regime however, such fluctuations are probably attributed by individual variations considering stress response.

Mean plasma PRL levels (mean±SEM) with respect to time course changes in vehicle or antagonist-treated animals also showed no variations in plasma PRL levels (Figure 2). Similarly, Two-way ANOVA followed by Tukey's multiple comparison test showed no statistically significant effect ($p > 0.05$) of antagonist administration on PRL secretion over different time points in juvenile male rhesus monkeys as compared to vehicle-administered monkeys. Neither time nor treatment effect was evident on PRL levels after antagonist administration in comparison to vehicle administration (Figure 3).

Mean plasma PRL levels (mean±SEM) were calculated for blood samples obtained at different time points i.e., pre-dose at three time points -60, -30, and 0, and post-dose at 30, 60, 90, 120, 150, 180, 210 and 240 minutes in both vehicle and antagonist treated juvenile male rhesus monkeys. Two-way ANOVA followed by Tukey's multiple comparison tests also showed no statistically significant difference ($p > 0.05$) in pre and post levels of plasma PRL levels in antagonist treated animals (Figure 4). However, fisher LSD showed significant difference ($p<0.01$) between pre and post values of vehicle treated animals. Such difference may be due to sample size. Unpaired t-test analysis showed no significant difference in overall mean plasma PRL levels in antagonist treated group as compared to control group of male juvenile rhesus monkeys (Figure 5).

Table 1: Plasma PRL concentrations (mIU/L) in individual juvenile male rhesus monkey following vehicle administration at different time points.

Time (min)	Mean Plasma Prolactin Concentration (mIU/L)				
	Vehicle (Animal number)				mean±SEM
	10M-21	11M-21	12M-21	13M-21	
-60	326.61	152.06	175.11	86.89	185.17±50.71
-30	109.54	133.94	167.33	66.12	119.23±21.30
0	60.75	67.98	173.80	82.21	96.19±26.25
30	44.76	85.25	126.13	96.75	88.22±16.85
60	28.54	240.44	293.92	48.78	152.92±66.99
90	28.29	267.43	134.19	57.94	121.96±53.37
120	27.55	105.74	111.21	48.78	73.32±20.78
150	24.53	64.02	35.65	39.03	40.81 ±8.33
180	40.70	40.70	76.37	28.05	46.46±10.40
210	53.96	23.77	43.33	15.26	34.08±8.85
240	58.64	80.34	21.47	37.58	49.51±12.78

Table 2: Plasma PRL concentrations (mIU/L) in individual juvenile male rhesus monkey treated with antagonist at different time points.

Time (min)	**Mean Plasma Prolactin Concentration(mIU/L)**				
	Antagonist (Animal number)				**mean±SEM**
	10M-21	**11M-21**	**12M-21**	**13M-21**	
-60	71.48	327.65	122.74	15.32	134.30±60.08
-30	60.75	129.54	82.44	36.13	77.22±19.84
0	46.89	101.00	87.59	15.05	62.63±19.59
30	51.14	85.25	102.89	66.82	76.52±11.21
60	93.93	64.02	113.84	57.24	82.26±13.20
90	101.00	61.68	119.12		70.45±16.95
120	64.95	88.76			38.43±11.90
150	89.93	93.93		32.73	72.20±19.76
180	85.01	49.72		45.47	60.07±12.53
210	56.77	55.37		36.38	49.50±6.57
240	78.01	53.49			65.75±12.25

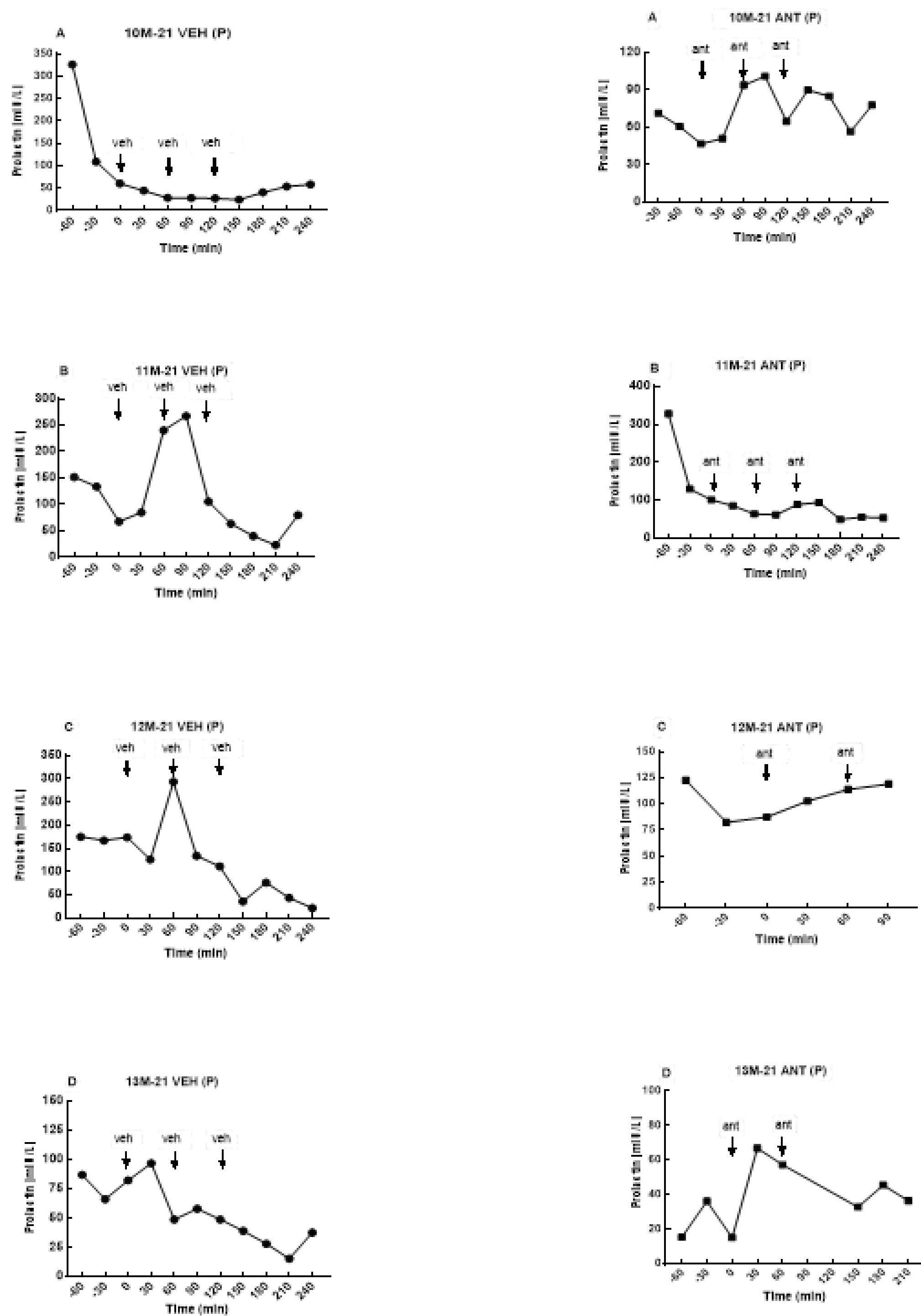

Figure 1. Individual variations in plasma PRL concentration in juvenile male rhesus monkeys before and after intravenous adminitrstion of vehicle and antagonist at time 0, 60, and 120 minute (arrows).

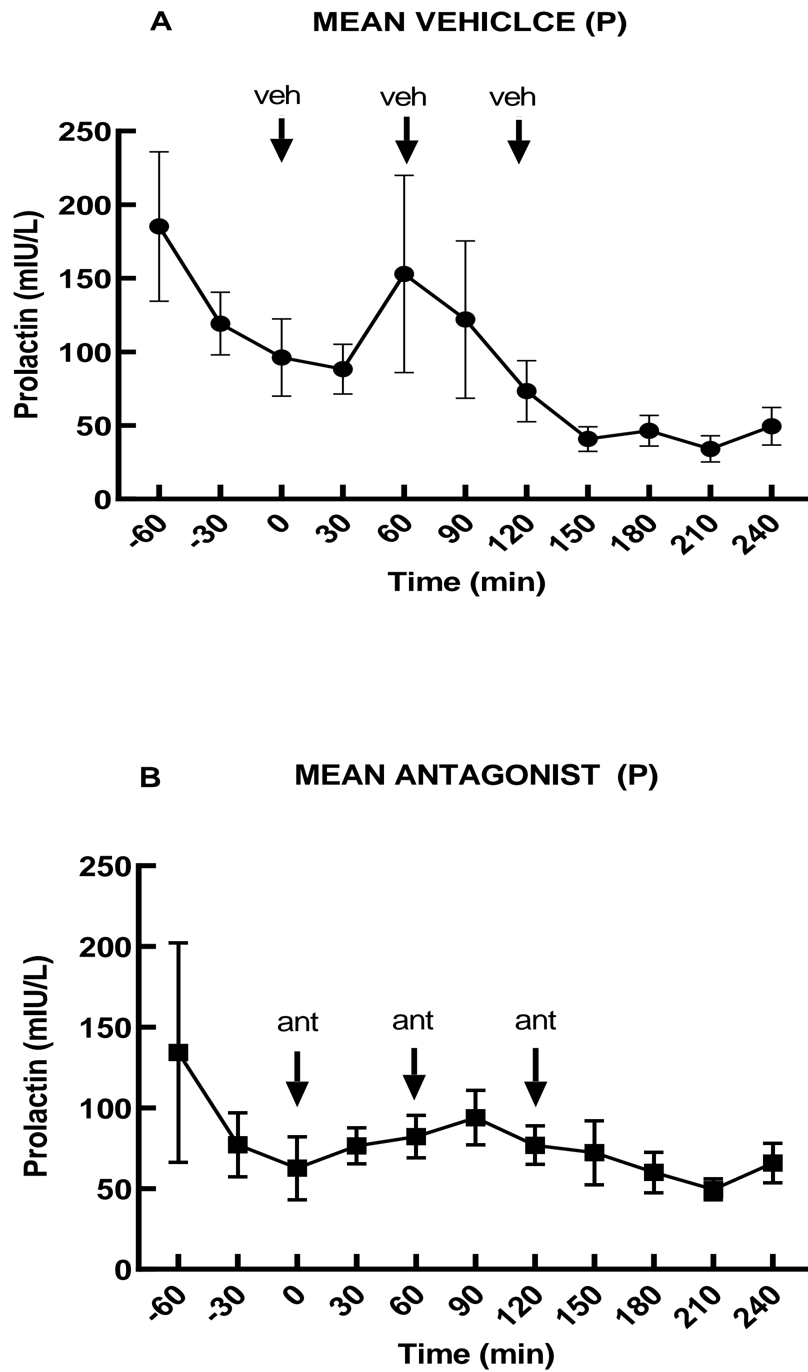

Figure 2. Time course changes in plasma PRL levels (mean±SEM) in juvenile male rhesus monkeys (n=4) before and after vehicle (A) and antagonist (B) administration.

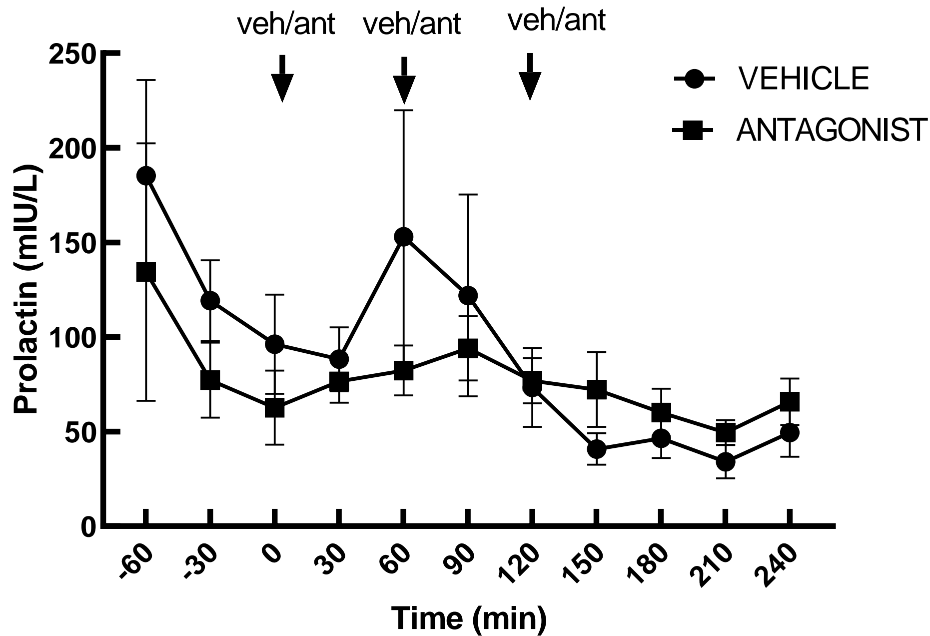

Figure 3. Time course changes in plasma PRL levels (mean±SEM) before and after intravenous administered vehicle and antagonist (arrows indicated) treated juvenile male rhesus monkeys. Two-way ANOVA followed by Tukey's multiple comparison test showed no significant ($p > 0.05$) difference of antagonist treatment on PRL secretion. Neither treatment nor time showed any observable difference in PRL secretion.

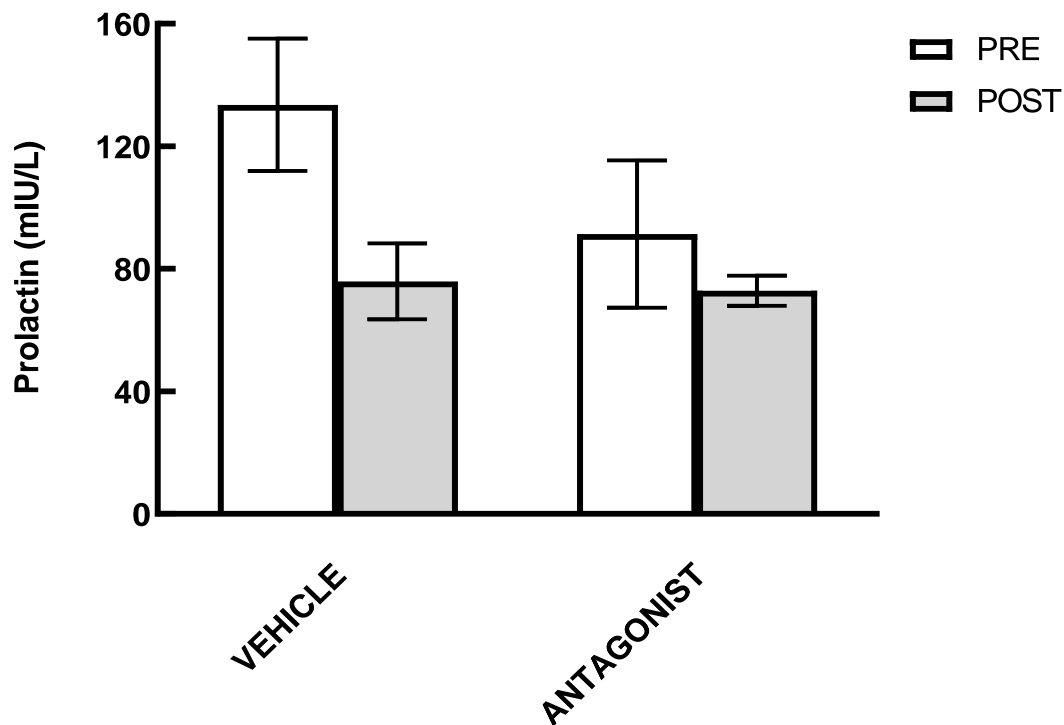

Figure 4. Comparison of mean plasma PRL levels (mean±SEM) in pre (-60, -30 ,0) and post (30, 60, 90, 120, 150, 180, 210, 240) vehicle and antagonist administered juvenile male rhesus monkeys (n=4). No change in plasma PRL levels was evident in pre and post antagonist treated animals. Two-way ANOVA followed by Tukey' multiple comparison test showed no statistically significant effect ($p > 0.05$) on plasma PRL levels in pre and post samples.

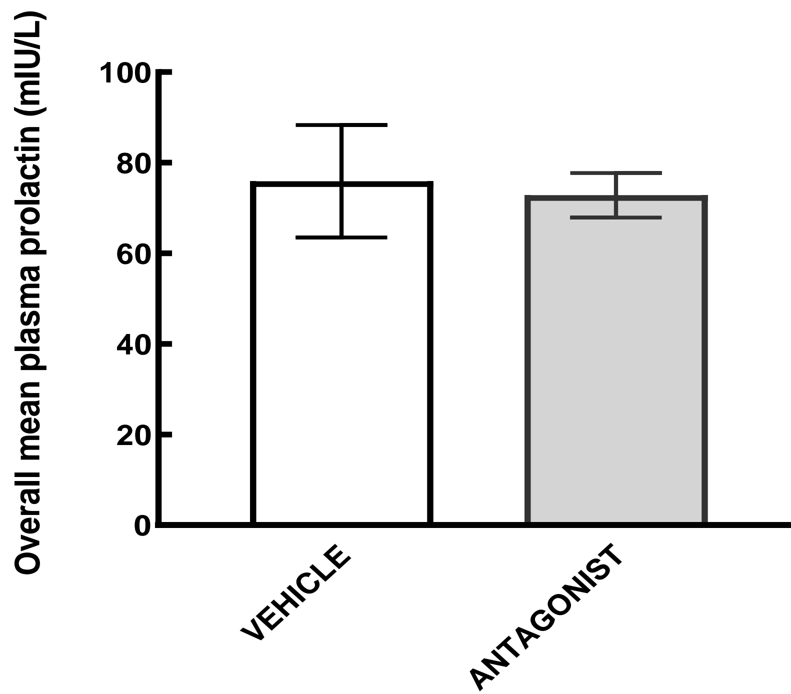

Figure 5. Comparison of overall mean plasma PRL levels (mean±SEM) in antagonist treated (n=4) and control group (n=4) of juvenile male rhesus monkeys. Unpaired t-test analysis showed no significant difference in plasma PRL levels in antagonist treated group as compared to control group.

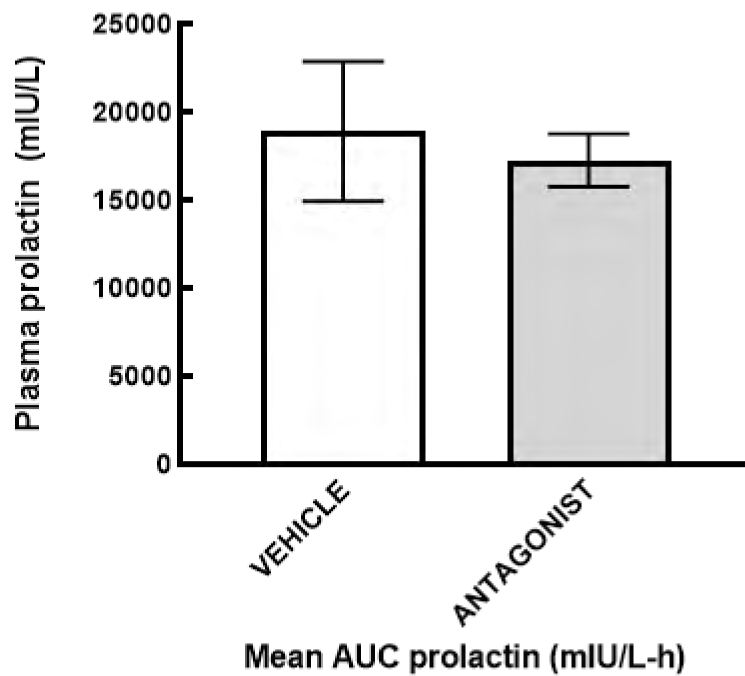

Figure 6: Comparison of mean±SEM area under the curve (AUC) for plasma PRL concentration during time 0-240 min between vehicle and antagonist treated monkeys (n=4).

Effect of vehicle and NPY1R antagonist on growth hormone levels

Plasma GH levels in individual juvenile male rhesus monkeys treated with vehicle (normal saline) at different time points are shown in Table 3. Blood plasma GH levels in individual juvenile male rhesus monkeys treated with antagonist were observed at different time points and are shown in Table 4.

Time-related changes in plasma GH levels in individual juvenile male rhesus monkeys after vehicle (normal saline) and antagonist administration at different time points are represented in Figure 7. No considerable individual variations in plasma GH levels were evident in vehicle or antagonist administered monkeys. Although after third injection of antagonist GH levels have shown to be high with respect to 30 min, however mean value of antagonist treatment did not change as compared to vehicle.

No observable changes in mean plasma GH concentrations were observed after antagonist or vehicle administration across different time points (Figure 8). Similarly, Two-way ANOVA followed by Tukey's multiple comparison test showed no statistically significant effect ($p > 0.05$) of antagonist administration on GH secretion over different time points in juvenile male rhesus monkeys as compared to vehicle administered monkeys. Neither time nor treatment effect was evident on GH levels after antagonist administration in comparison to vehicle administration (Figure 9).

Mean plasma GH levels (mean±SEM) were calculated for blood samples obtained at different time points i.e., pre-dose at three time points -60, -30, and 0, and post dose at 30 ,60, 90, 120, 150, 180, 210, and 240 minutes in both vehicle and antagonist treated juvenile monkeys. Two-way ANOVA followed by Tukey's multiple comparison test indicated no statistically significant difference ($p > 0.05$) in pre and post levels GH levels in antagonist treated animals (Figure 10). Unpaired t-test analysis showed no significant difference in overall mean plasma GH levels in antagonist treated group as compared to the control group of male juvenile rhesus monkeys (Figure 11).

Table 3: Plasma GH concentrations (µIU/ml) in individual juvenile male rhesus monkeys following vehicle (normal saline) administration at different time points.

Time (min)	**Mean Plasma Growth Hormone Concentration (µIU/ml)**				
	Vehicle (Animal number)				**mean±SEM**
	10M-21	**11M-21**	**12M-21**	**13M-21**	
-60	1.65	0.30	0.06	0.06	0.52±0.38
-30	4.94	0.03	0.23	0.45	1.41±1.17
0	0.93	0.03	0.59	0.50	0.51±0.81
30	0.07	0.62	0.13		0.27±0.17
60	0.07	0.07	3.05	0.21	0.85±0.73
90	28.45	3.63	0.22	0.04	8.08±6.83
120	2.40	1.63	0.31	0.89	1.31±0.24
150	0.14	0.17	0.41	0.64	0.344±0.45
180	32.88	0.03	1.58	0.33	8.71±8.06
210	14.51	0.03	1.94	0.31	4.20±3.46
240	1.26	3.69	12.86	0.92	4.68±2.79

Table 4: Plasma GH concentrations in individual juvenile male rhesus monkeys treated with antagonist at different time points.

Time (min)	**Mean Plasma Growth Hormone Concentration (µIU/ml)**				
	Antagonist (Animal number)				**mean±SEM**
	10M-21	**11M-21**	**12M-21**	**13M-21**	
-60	2.89	0.06	24.47	0.22	6.91±5.89
-30	0.92	0.14	1.56	0.11	0.67±0.34
0	0.27	5.75	1.04	0.03	1.77±1.34
30	18.43	25.17	14.72	4.01	15.58±4.42
60	1.08	8.51	17.20	1.41	7.05±3.79
90	17.58	4.20	6.00	0.45	7.06±3.69
120	22.04	0.38	19.71		10.53±6.86
150	3.40	3.12		0.36	2.3±0.97
180	2.97	0.47		0.32	1.25±0.85
210	1.13	0.03		1.07	0.74±0.35
240	0.12	0.64		0.62	0.46±0.17

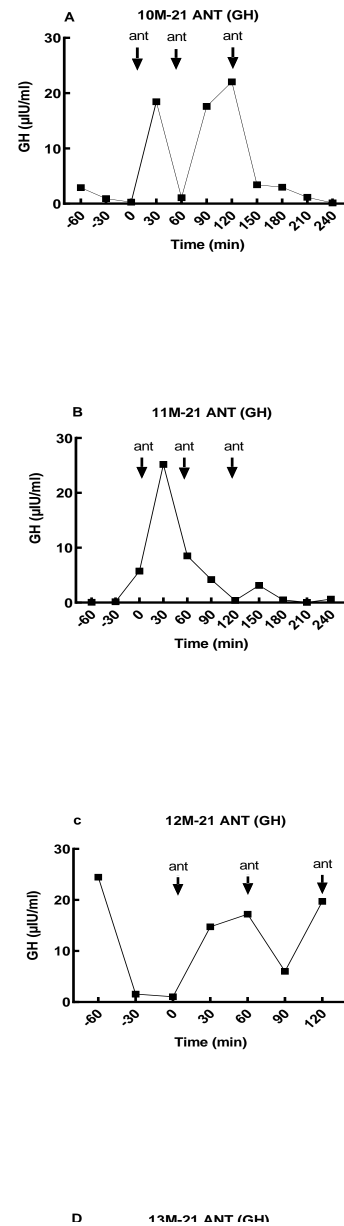

Figure 7. Individual variations in plasma GH concentration in juvenile male rhesus monkeys before and after intravenous adminitrstion of vehicle and antagonist administration at time 0, 60, and 120 minute (arrows).

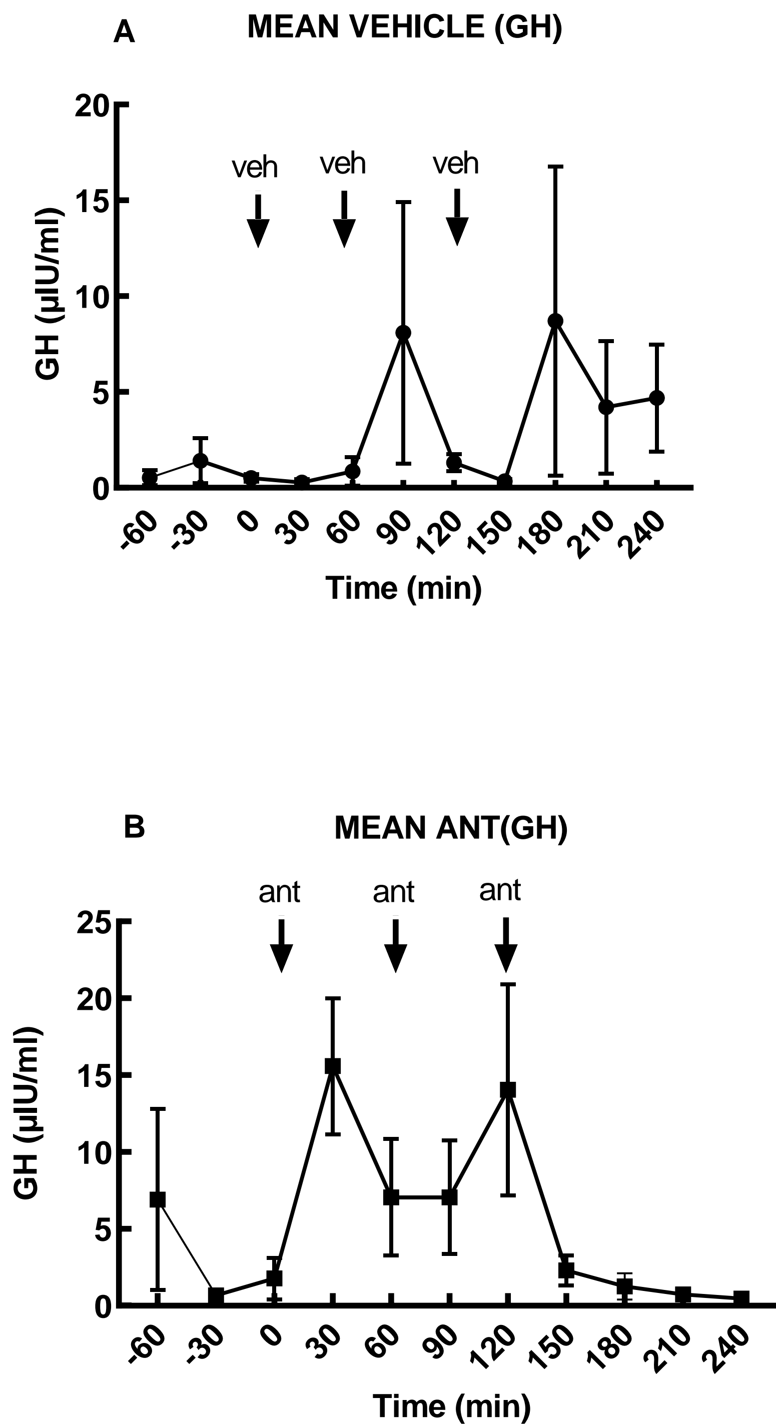

Figure 8. Time course changes in plasma GH levels (mean±SEM) in juvenile male rhesus monkeys (n=4) before and after vehicle (A) and antagonist (B) administration.

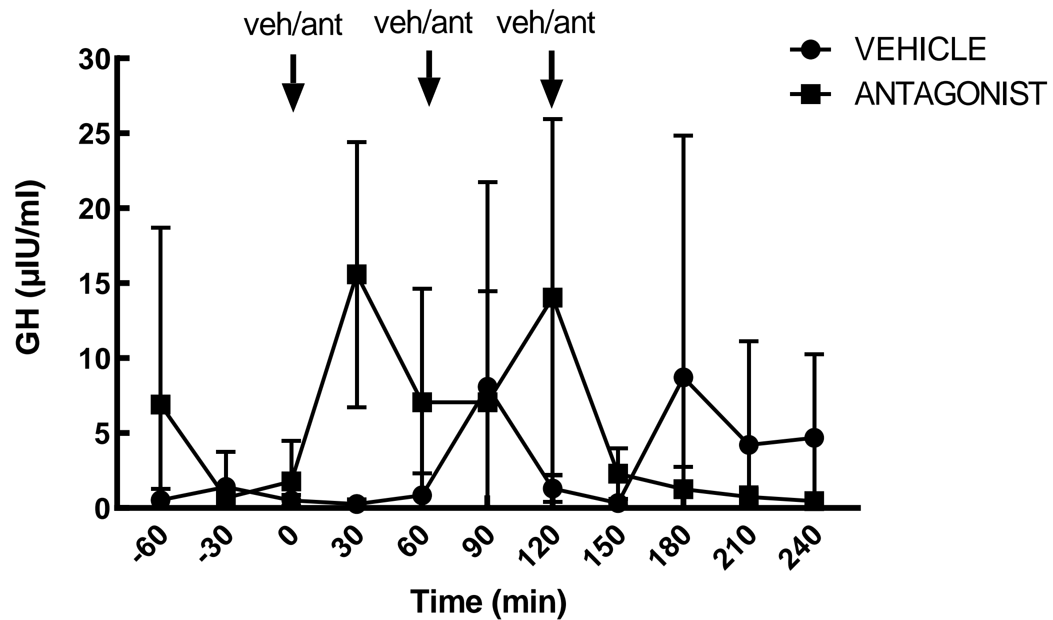

Figure 9. Time course changes in plasma GH levels (mean±SEM) before and after intravenous administered vehicle and antagonist (arrows indicated) treated juvenile male rhesus monkeys. Two-way ANOVA followed by Tukey's multiple comparison test showed no significant ($p > 0.05$) difference of antagonist treatment on GH secretion. Neither treatment nor time showed any observable difference in GH secretion.

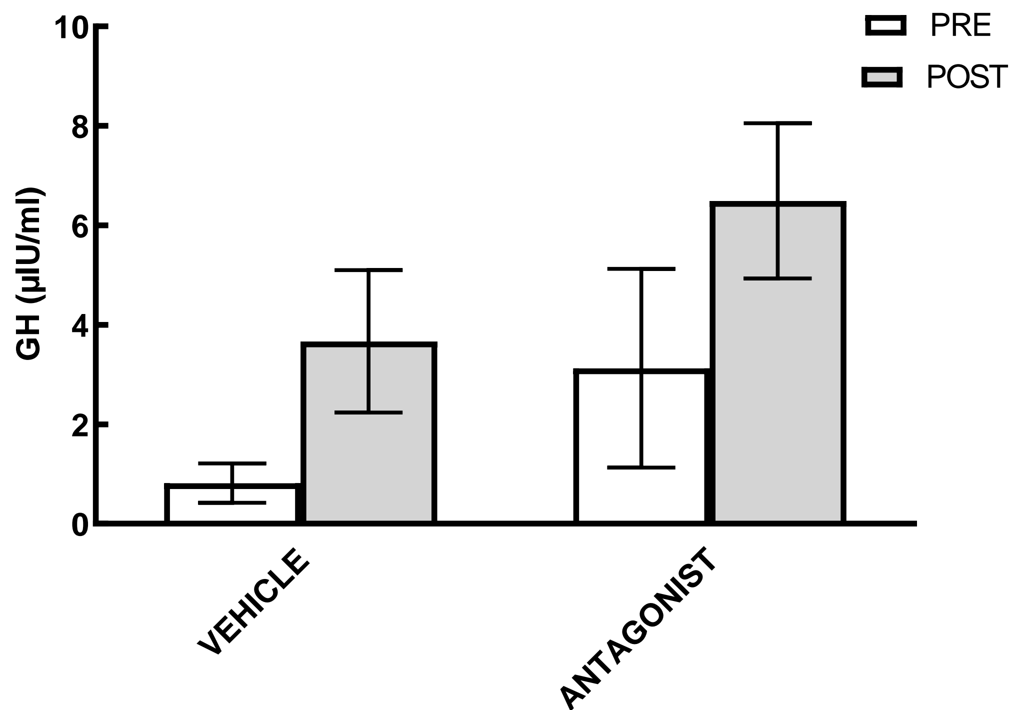

Figure 10. Comparison of mean plasma GH levels (mean±SEM) in pre (-60, -30 ,0) and post (30, 60, 90, 120, 150, 180, 210, 240) vehicle and antagonist administered juvenile male rhesus monkeys (n=4). No change in plasma GH levels was evident in pre and post antagonist treated animals. Two-way ANOVA followed by Tukey's multiple comparison test showed no statistically significant effect ($p > 0.05$) on plasma GH levels in pre and post samples.

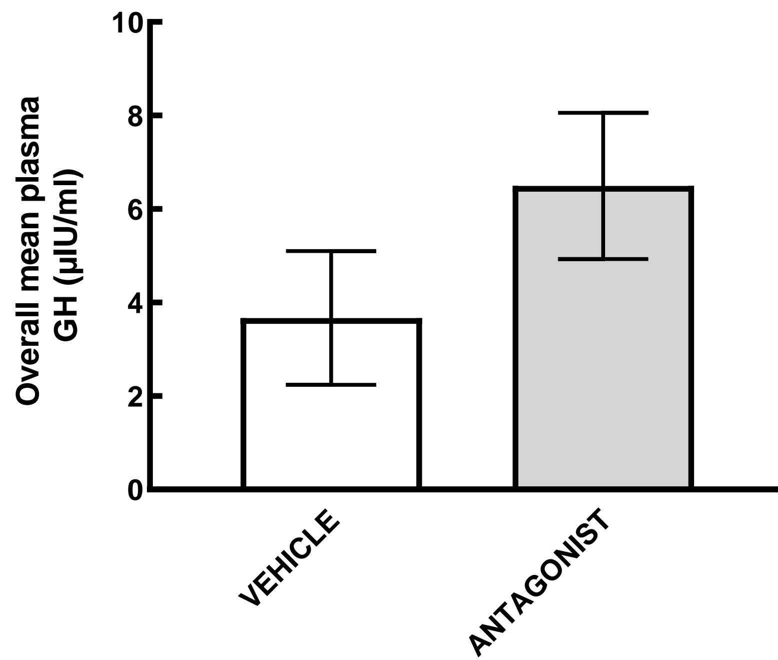

Figure 11. Comparison of overall mean plasma GH levels (mean±SEM) in antagonist treated (n=4) and control group (n=4) of juvenile male rhesus monkeys. Unpaired t-test analysis showed no significant difference in plasma GH levels in antagonist treated group as compared to control group.

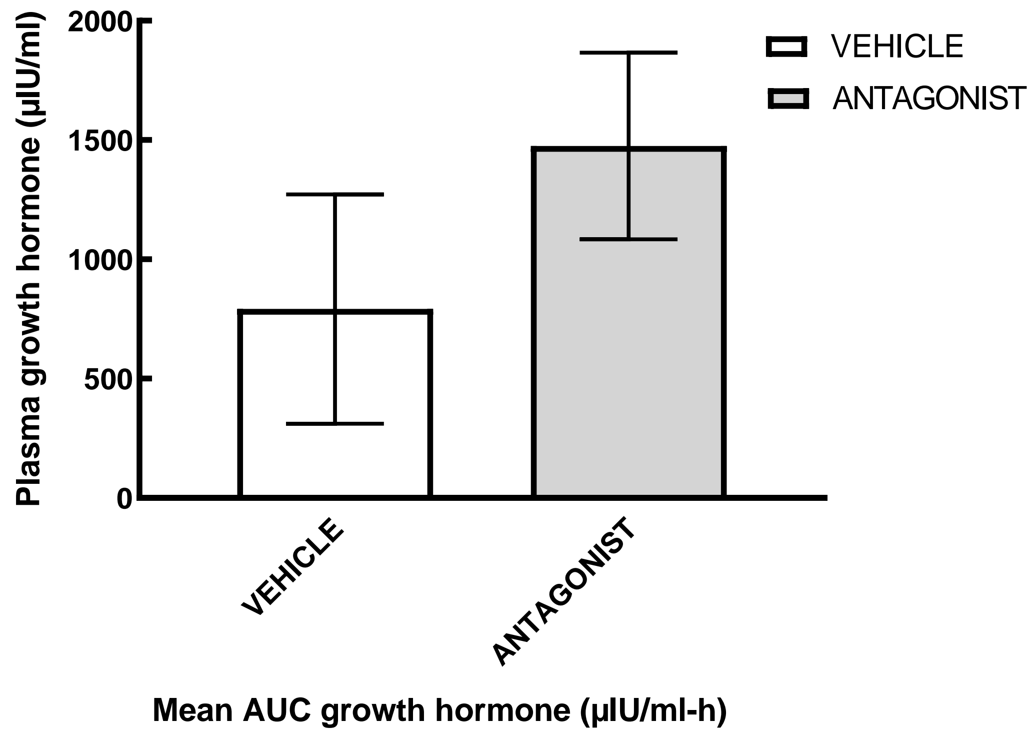

Figure 12. Comparison of mean±SEM area under the curve (AUC) for plasma GH concentration during time 0-240 min between vehicle and antagonist treated monkeys (n=4).

DISCUSSION

DISCUSSION

Data from the various studies under different physiological conditions have shown that NPY plays paradoxical role in regulating GH and PRL release. In earlier literature, the effect of BIBO 3304 on non-reproductive hormones (GH and PRL) has not been reported in juvenile primates. The current research was undertaken to find out the role of NPY in regulating PRL and GH secretion mediated via NPY1R in juvenile male rhesus monkeys.

The current findings demonstrated that NPY signaling via NPY1R may not involve in the control of PRL or GH secretion in juvenile animals. This is the preliminary study in juvenile male monkeys as no considerable studies are available in non-human primates to show comparison with the results of the present study.

To evaluate the role of NPY1R in the control of PRL and GH secretion BIBO 3304 was used. The novel Y1-selective arginine amide derivative BIBO 3304 (R)-N-[[4-(aminocarbonylaminomethyl)phenyl]methyl]-N^2-(diphenylacetyl)-argininamide trifluoroacetate) was synthesized and commercially available for research purposes. The antagonist was utilized in different studies due to its selectivity for NPY1R subtype (Wieland et al., 1998). BIBO 3304 displayed affinity in the nanomolar quantities for both the rat and the human Y1 receptor subtype. The cells stably transfected with the rat/human NPY1R cDNA or cells that are expressing the NPY1R endogenously showed sub- nanomolar affinity of the novel BIBO 3304 compound. BIBO 3304 showed 10-20 times higher affinity as compared to BIBP 3226 (another selective Y1 antagonist) for both the rat and human Y1 receptor subtypes. The novel BIBO 3304 compound is highly selective for the Y1 receptor subtype since it binds with more than 1000-10000-fold lower affinity to any of the other NPY receptor subtypes tested (Y2, Y4, Y5) both for rat and human species (Wieland et al., 1995).

Although BIBO 3304 is highly specific for NPY1R, no effect of this antagonist in modulating PRL and GH secretion in juvenile monkeys was observed in the current study. This in efficacy of the antagonist can be attributed either by considering two plausible reasons. First, the quantity of the dose used was insufficient. Second, the antagonist was unable to cross the blood brain barrier. Whatever the reason may be, further studies are required to explain.

In a previous study, novel BIBO 3304 compound was used to investigate the role of kisspeptin in GH release by utilizing NPY pathways in adult ewe and revealed that central administration

of kisspeptin (positive regulator of reproductive axis) activates arcuate nucleus NPY cells and stimulates NPY release. This in turn stimulates growth hormone releasing hormone (GHRH) which ultimately leads to increase GH secretion. By pretreatment of the animals with the selective NPY1R antagonist, kisspeptin-induced GH release was completely blocked (Foradori et al., 2017). Furthermore, the same study also argues that enhanced NPY plays dual role in stimulating GH secretion that is it can activate the GHRH neurons in arcuate while suppress the somatostatin (SS) cells in periventricular area (Foradori et al., 2017). This shows a positive effect of NPY on GH secretion. However, our results are contradictory to this study as our result displays no effect of NPY1R antagonism on GH secretion. This discrepancy in results might be due to species and age differences. It can also be speculated that the effect of NPY in inducing GH release may be dependent on kisspeptin stimulation. In this context, it is interesting to note that during juvenile stage, inverse relationship between kisspeptin and NPY exists both at morpho functional and physiological level in non-human primates (Bano et al., 2022). By comparing these observations with the current results, it can be speculated that kisspeptin dependent NPY stimulation of GH can only be viable in the post-pubertal period in monkeys. Therefore, it will be interesting to note the effect of kisspeptin administration on NPY dependent GH release in juvenile and adult monkeys. This is because reciprocal interaction between kisspeptin and NPY neurons in ewes has been documented (Backholer et al., 2010), while in case of rhesus monkeys, NPY projections onto kisspeptin neurons along with NPY1R expression on to kisspeptin neurons have been observed (unpublished observations of the lab). Nevertheless, it has been documented that intravenous administration of kisspeptin was failed to modulate GH and PRL levels in adult male monkeys (Ramaswamy et al., 2009). Keeping in view the discrepancy in results with previous studies, it is likely to interpret that different NPY neuronal populations may involve in regulating both somatotropic and gonadotropic axes and different pathways including intermediatory players may be involved to regulate both axes separately.

Although conflicting results regarding NPY regulation of GH have been documented, intravenous administration of NPY did not show any effect on GH release in normal humans (Watanobe et al., 1994) which shows coherency with the current results.

Contrary to the above observation, it was analyzed that NPY has negative influence on GHRH expression (Mano-Otagiri et al., 2006). NPY inhibited GH release in both sexes of rats (Rettori et al., 1990a). It has been shown that NPY inhibited the GH release from intact male rats and

sex steroid primed ovariectomized rats (Suzuki et al., 1996). These studies show the inhibitory role of NPY in regulating GH secretion. However, the inhibiting NPY tone by NPY1R antagonism did not show any change in GH secretion in the current study. Various factors can be attributed for the bimodal role of NPY on GH secretion. Among these factors, metabolic status, physiological levels of steroids, stress, and day length are involved. Moreover, various results obtained in different studies also pertained to specie difference. Therefore, future studies could be done during different developmental stages to evaluate the precise role of NPY on GH secretion in non-human primates. In addition, experiments could also be done under different metabolic conditions to discern the interplay between energy, growth, and reproductive status in different animal models.

The role of GH in the onset of puberty has been investigated in many previous studies. Shahab et al (2015) showed that in agonadal male monkey there is no change in any parameter (frequency, amplitude etc.) of GH secretion during the late pubertal period (final 6 months of juvenile development preceding the pubertal reactivation of LH secretion) and concluded that increase in GH during developmental stages in highly evolved primates has no impact on timing of puberty. These results were in accordance with a study in the juvenile rhesus monkey, in which the somatostatin analog administration impact neither the timing of the pubertal rise in LH secretion nor the age of menarche (Wilson et al., 2004). In another study data in boy's developmental changes also showed nonremarkable effect on GH secretion (Martha et al., 1996). Although growth parameters like bone age etc. which depend on GH release are considered to evaluate timing of puberty, however, data from the literature showed that GH does not play critical role in timing pubertal onset. This can also be suggested by the present data where no observable changes in GH secretion were noted. These findings corroborate the current findings that NPY1R antagonism has no effect on GH secretion in juvenile monkeys. Although it is important to note that NPY plays a critical role in pubertal onset (Plant and Shahab, 2002; Bano et al., 2022). NPY mediated signaling may be involved in the control of GH secretion has not been clearly defined. NPY1R antagonist treated animals showed no observable changes in GH secretion in juvenile monkeys which shows that NPY may not regulate GH secretion in juvenile animals.

It has been shown that NPY neurons make synaptic contact with GHRH neurons in arcuate and with SS neurons in the PeN by electron microscopic immunohistochemical studies (Hisano et al., 1990; Deltondo et al., 2008). This suggests that NPY neurons can influence the release

of GH secretion by acting on PeN SS neurons. Deletion of NPY neither alters hypothalamic GHRH and SRIF mRNA expression in ad libitum-fed mice nor GH pulsatility. Accordingly, NPY neurons did not modulate GH release under fed conditions. In addition, the reverse pattern of these results was also evident under fasting conditions (Steyn et al., 2011, 2012; Huang et al., 2014). It is anticipated from these observations that NPY regulates GH release via modulating activity of GHRH and SRIF neurons according to metabolic condition (Stanley et al., 2013). Potentially, NPY based GH secretion is mediated by NPY1R because NPY1R is abundantly expressed in ARC and PeN the two areas where GHRH and SRIF are residing (Zhang et al., 1999; Kishi et al., 2005). Interestingly it is to mention that NPY1R deletion had no effect in modulation of GH secretion in mice (Zhang et al., 1999). Our data also in line with this observation where NPY1R antagonist was unable to modulate GH levels in juvenile monkeys.

Before summarizing these these results, it is to mention that icv administration of NPY in juvenile male monkeys enhance GH secretion (unpublished observation as cited in Shahab et al., 2003), yet the peripheral administration of antagonist in the current study did not cause any changes in GH secretion in juvenile monkeys. Keeping in view the BIBO 3304 as the highly specific NPY1R antagonist, it can be perceived that the drug might not cross the blood brain barrier to halt NPY based signaling to GHRH neurons in higher hypothalamic center or it may raise the notion that NPY depend on GH regulation may be mediated by another subtype of NPY receptor. Hence, direct icv administration of the antagonist in future would be helpful to delineate the role of NPY in GH secretion.

Like GH, ambiguous observations are documented regarding NPY regulation of PRL release in different animal models. NPY stimulated PRL secretion has been observed cells cultured from anterior pituitary (Baranowska et al., 1999). Contrary to this, a significant decline in PRL levels 30 minutes after intraventricular injection of NPY for a longer time from castrated and normal male rats (Alexander et al., 1993). In another study, intraventricular administration of NPY at low doses showed reduced PRL releases in rodents (Härfstrand et al., 1987). The plausible mechanism of this PRL inhibition by NPY was in opinion that NPY could modulate TIDA neuronal activity hence alter dopamine levels which consequently regulate PRL secretion. This idea was speculated by the observations in which NPY with dopamine had shown additive effect in inhibition of PRL release (Wang et al., 1996). A similar observation was also presented by (Hsueh et al., 2002). Apart from hypothalamic action of NPY on

dopaminergic neurons, it was revealed that these neurons send their projections at median eminence and administration of NPY may influence dopaminergic system at median eminence hence subsequently modulate PRL secretion. Therefore, it can be assumed that NPY regulation of PRL secretion may involve some intermediatory pathway in juvenile monkeys which is required to be explored.

As far PRL release by NPY is concerned, no significant effect of 100 µg bolus injection of NPY on PRL levels in a patient with prolactinoma has been documented (Watanobe and Tamura, 1996). In another study, Chao et al. (1987) showed that there is no effect of in vitro dosages of NPY (0.01 to 100 nM), on PRL release in bovine pituitary cells. These findings corroborate with the current findings that NPY1R antagonism has no effect on PRL secretion in juvenile monkeys. NPY signaling involved in the control of PRL secretion has not been clearly defined. NPY1R mRNA antisera provoked PRL secretion in humans (Silveira and Franci, 1999) suggesting inhibitory effect of NPY on PRL secretion may be mediated by NPY1R. Therefore, NPY1R specific antagonist was used in the current study, however, NPY1R antagonist treated animals showed no observable changes in PRL hormone secretion in juvenile monkeys which shows that NPY may not regulate PRL secretion via NPY1R in juvenile animals.

Recent data concerning NPY regulation of PRL secretion in monkeys is scarce. Nearly fifty years ago, it has been shown that lactotrophs number and PRL release was higher in adult male monkeys as compared to juvenile animals (Herbert and Hayashida, 1974). Furthermore, steroid treatment in juvenile male monkeys increased number of PRL secreting cells and hence stimulated PRL release (Herbert, 1978). Since in juvenile stage low testosterone levels and high NPY levels are noted (Terasawa, 2019), it can be suspected that during juvenile phase steroid dependent NPY regulation of PRL pathway is present at minimal level and after pubertal onset when the testosterone enhanced it may regulate NPY dependent PRL release through some intermediatory pathway as suggested earlier about TIDA neurons involvement pf PRL release. Alternatively, before pubertal onset NPY neuronal pathway regulates gonadotropic axis and after pubertal onset NPY mediated prolactin pathway predominates.

Concerning non-effectiveness of NPY1R antagonist in modulating GH and PRL secretion in juvenile male monkeys, it should be noted that immunoneutralization of NPY did not show any fluctuations in both GH and PRL release in mature ewes (Barker-Gibb and Clarke, 1996;

Malven et al., 1995). Moreover, dual effect of NPY both inhibitory and stimulatory on PRL release has been documented in male rats by high and low dose respectively which shows the concern about dose of the antagonist used in the current study. Hence, different doses along with different routes would be helpful to understand NPY physiology of PRL secretion.

In conclusion, our study showed for the first time that there is no effect of NPY on two non-gonadotropic hormones (PRL and GH) release in juvenile male rhesus monkeys. Further investigations are required to ascertain other mechanisms by which NPY can regulate GH and PRL release. In this regard, other potential neurobiological candidates (kisspeptin, and GnIH etc.) of hypothalamic origin needs to be studied which can mediate NPY dependent GH and PRL secretion. Numerous factors like specie difference, route of exposure, amount of dose, and steroidal and metabolic milieu can modulate NPY effects on GH and PRL secretion. For instance, it is a matter of concern for this study that dose quantity, exposure site, developmental stage, and species difference must be considered before any conclusion to draw about these results. The study should be extended to the pituitary to ascertain the direct role of NPY in regulating somatotrophs and lactotrophs activity. This study can also be extended to cellular and genetic levels to explore the pathways which are used by NPY to affect GH and PRL release.

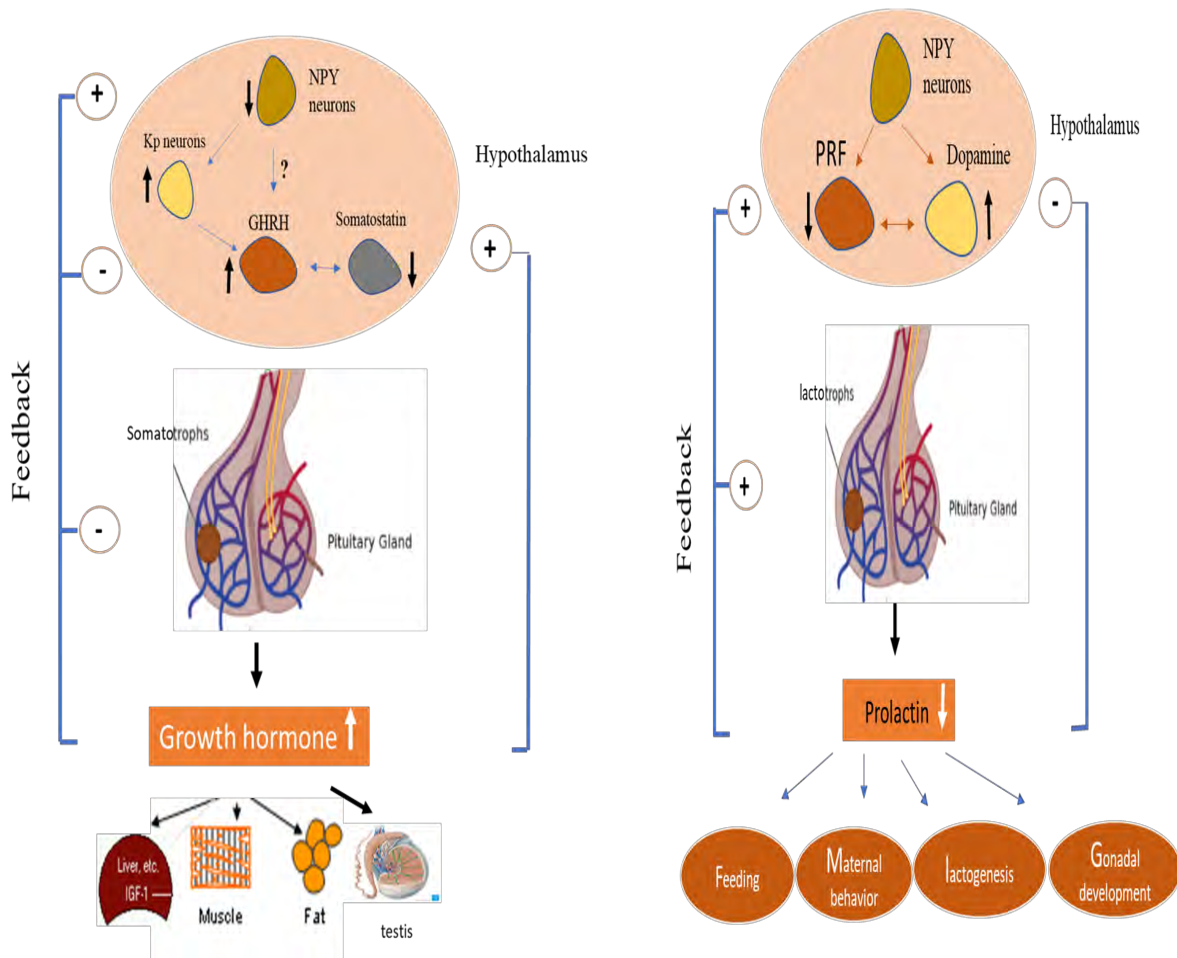

Figure 13. Proposed Model of current study showing different mechanisms of how NPY effect the release of Growth hormone and Prolactin. **NPY** (Neuropeptide Y), Kp (Kisspeptin), **GHRH** (Growth hormone Releasing Hormone), **PRF** (Prolactin Releasing Factor).

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