FUNCTIONAL AND DEVELOPMENTAL STUDIES OF THE PRIMATE TESTIS

By

SHAHZAD IRFAN

Department of Animal Sciences
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad, Pakistan
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BY

SHAHZAD IRFAN

DEPARTMENT OF ANIMAL SCIENCES
FACULTY OF BIOLOGICAL SCIENCES
QUAID-I-AZAM UNIVERSITY
ISLAMABAD, PAKISTAN
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CERTIFICATE

This dissertation "Functional and developmental studies of the primate testis**"** submitted by **Shahzad Irfan** is accepted in its present form, by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Doctor of Philosophy.

Supervisor:

_____

Dr. Muhammad Shahab

1. External Examiner:

_____

Dr.

2. External Examiner:

_____

Dr.

Chairman:

_____

Dr. Irfan Zia Qureshi

Date: _____

DECLARATION

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

Shahzad Irfan

DEDICATION

I dedicate my thesis to my loving parents and my mentor

Professor Dr. Muhammad Shahab

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List of abbreviations

GnRH	**Gonadotropin Releasing Hormone**
LH	**Luteininzing Hormone**
FSH	**Follicle Stimulating Hormone**
HPG axis	**Hypothalamus Pituitary Gonadal axis**
GABA	**γ-Amino Butyric Acid**
NPY	**Neuropeptide Y**
GPR54	**G-protein Coupled Receptor- 54**
Kiss1	**Kisspeptin**
Kiss1r	**Kisspeptin receptor**
TGFβ	**Transforming growth factor Beta**
IGF	**Insulin like growth factor**

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General Abstract

Primate testis acts as the main source for androgen and sperm under the functional regulation of pituitary gonadotropins. The understanding of the functional regulation of adult primate testis as well as the developmental aspects of maturation during puberty in primates is still not complete. The present thesis aimed to assess the localization and functional significance of kisspeptin signaling in adult non human primate testis as well as to characterize the pubertal and testicular development in a New World monkey. Rhesus macaque (*Macaca mulatta*) and common marmoset (*Callithrix jacchus*) were employed as primate models. To sort out the functional significance of kisspeptin signaling in the primate testis *in vivo* pharmacological approach was used by employing chemical hypophysectomy by using acyline (a GnRH receptor antagonist) in adult rhesus monkeys (n=4). Animals were given iv boluses of kisspeptin (50µg) and kisspeptin (50µg) with hCG (50IU). Blood samples were taken and testosterone and LH was analyzed in the plasma by specific RIAs. To find out the testicular localization of kisspeptin receptor immunocytochemistry was performed on paraffin embedded testis tissue from adult rhesus (n=2) and marmoset monkey (n=2) whereas for testicular localization of kisspeptin immunocytochemistry was performed on paraffin embedded testis tissue from adult rhesus monkey (n=2). 12 sections from each testis were used for immunocytochemical analysis. To characterize the pubertal testicular development, immature common marmoset (n=48) were observed for a period of 13 months for monthly changes in body weight, testis volume and serum testosterone. At the end of the study animals were sacrificed and testis tissue was collected and processed for paraffin embedding. PAS staining was conducted. Histological and morphometric data were determined. Results of the experiments demonstrated that kisspeptin, given as an intravenous bolus enhanced stimulated plasma testosterone in pituitary clamped adult male rhesus monkeys whereas the same bolus had no effect on the basal levels of plasma testosterone. The immunocytochemical localization revealed that kiss1r positive areas were present at the periphery of the seminiferous tubules in the adult rhesus testis. Same

pattern of localization for kiss1r was found in adult marmoset testis tissue. Whereas the kisspeptin like immunoreactivity was found to be present in the interstitial area of the adult rhesus testis tissue. The detailed examination revealed that the kiss1r localization was present around the spermatogonial stem cells (A spermatogonia) which are present at the periphery of the seminiferous tubules. While the kisspeptin like immunoreactivity was observed in the peritubular myoid cells, Leydig cells and underlying layers of basement membrane. Developmental studies showed that the start of pubertal activation in common marmoset occur around 7 months of age and was characterized by sudden peaks of serum testosterone accompanied by a rapid increase in the testis volume. Morphometric analysis revealed that the pubertal activation created a lumen in the center of seminiferous tubules. The germ cell compliment was observed to divide mitotically after the initial increase in serum testosterone depicted by presence of B spermatogonia in the seminiferous tubules after 7 months of age. The complete spermatogenesis characterized by the presence of sperm in epididymis was first observed around 12 month of age in the common marmoset testis. Although testis volume continued to grow after 12 months of age, the qualitative maturity of testicular functions was achieved at one year of age in the male common marmoset.

The present findings implicate that an active paracrine kisspeptin-kisspeptin receptor signaling is present in the adult primate testis; where the kisspeptin can enhance stimulated testosterone thus hinting towards an indirect action on the Leydig cell. The finding of the tubular immunolocalization of the kisspeptin receptor in primate testis where Sertoli cells were found to be positive further extends our ***in-vivo*** results and affirms the assumption of an indirect action of kisspeptin on Leydig cell via the Sertoli cells. However to understand the true nature of this signaling cascade further investigations at the cellular level are required. The developmental kinetics of the process of puberty including pubertal activation and testicular development in common marmoset mimics higher primate like pattern. Although the endocrine correlates for these developmental events need to be further substantiated in common marmoset.

Chapter 1

General Introduction

General Introduction

Reproductive functionality in primates is achieved by the endocrine communication between hypothalamus, pituitary and gonads. This interplay of hormones imposes a functional regulation which ensures the maintenance of reproductive capacity. Gonadotropin releasing hormone (GnRH) a decapeptide, is synthesized in hypothalamic neurons called GnRH neurons, and is released in the pituitary portal circulation (Silverman et al., 1977; Silverman et al., 1982). At the pituitary, GnRH acts on the gonadotrophs which expresses GnRH-receptor (GnRH-R). The GnRH-GnRH-R coupling at the gonadotroph results in synthesize and secretion of gonadotropins into the portal circulation (Knobil et al., 1980). Two types of gonadotropins are secreted in response towards a specific tone and frequency GnRH release namely luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Kamberi et al., 1971; Neill et al., 1977). LH and FSH once released from pituitary reach gonads via peripheral circulation and acts on the Leydig cells and Sertoli cells, respectively. LH ensures the synthesis and secretion of sex steroids (steroidogenesis) from Leydig cells (Ascoli et al., 2002). FSH on the other hands is critical for proliferation of the developing Sertoli cell and affects the spermatogenesis quantitatively (Hackert and Griswold, 2002; Plant and Marshall, 2001). Testosterone is the primary steroid synthesized and released by the Leydig cells in response to the LH (Eik-Nes and Hall, 1965). Constant high levels of serum testosterone down regulates hypothalamic pituitary axis in terms of LH and the low serum levels of testosterone acts as a positive inducer of GnRH-LH secretion in male primates. Apart from the endocrine regulation a particular type of local intercellular communication also exists in the primate testis known as paracrine signaling. Paracrine signaling ensures the local interaction of testicular cells in delivering coordinated state of action to achieve the fine balance between the processes of steroidogenesis and spermatogenesis (Schlatt et al., 1997).

The functional competence of an adult primate testis depends upon the developmental events during fetal and postnatal life. The postnatal phases of

development in higher primates include infantile, juvenile, pre-pubertal and pubertal and adult stages (Plant and Witchel, 2006). The major developmental events occur in infantile, juvenile and pre-pubertal phases whereas the main maturational events occur during the pre-pubertal and pubertal phase (Marshall and Plant, 1996; Waites et al., 1985). Developmental events include cellular proliferation and rearrangement (Simorangkir et al., 2012). The maturational events include the initiation of the functional capacity of the testicular cells. The functional capacity is in turn characterized by the expression of nuclear receptors and the secretory response towards particular intratesticular stimuli (Griswold, 1988; McKinnel et al., 2001; Majumdar et al., 2012). The testicular developmental and maturational periods are generally characterized by intensive cellular proliferation, cell movement, cellular rearrangement in the juvenile phase of development whereas the attainment of functional capacity is observed after the onset of puberty (Schlatt et al., 1995). All these above mentioned events lead to a considerable increment in the testicular size during pubertal development and are anatomically depicted by a significant increase in the testicular volume (Plant et al., 2005).

The current thesis focuses on functional and developmental aspects of primate testis and accordingly the next sections will focus on the functional anatomy of the adult primate testis, the primate pubertal development, involvement of kisspeptin-kisspeptin receptor signaling in puberty and the postnatal and pubertal testicular development in primates.

Primate testis

Primate testis serves as a fundamental source for the production of male gametes (sperm) and testosterone through the process of spermatogenesis and steroidogenesis, respectively, under the influence of pituitary gonadotropins. Anatomically primate testis is divided into two functionally distinct compartments namely tubular and interstitial compartment (Schlatt et al., 1997). Tubular compartment contains Sertoli cell and developing spermatogonia whereas the interstitial compartment bears Leydig cells,

macrophages, sympathetic innervations and blood vessels. Sertoli cell serves as nurse cell for the developing spermatogonia whereas Leydig cell acts as the main source of steroidogenesis. Sertoli cells and Leydig cells are functionally regulated by FSH and LH, respectively. LH acts on Leydig cells through LH receptors (LHR) and initiates the process of steroidogenesis **(**Ascoli et al., 2002). Testosterone is the principal androgen synthesized through the side chain cleavage of cholesterol in the mitochondria of Leydig cell by the steroidogenesis (Preslock and Steinberger, 1977). The principal action for FSH is the control of Sertoli cell proliferation during development and maintenance of spermatogenesis quantitatively during adulthood (Plant and Marshall, 2001). Testosterone is the prime regulator of the testicular microenvironment during pubertal development and is also responsible for the maintenance of spermatogenesis qualitatively (Marshall et al., 1986; Schlatt et al., 1995; Weinbauer et al., 1988).

Apart from endocrine regulation of testicular functions via pituitary gonadotropins, a distinctive intercellular communication exists between the testicular cells. This intra-testicular intercellular communication which is also known as paracrine signaling, plays an important part in the testicular development as well as the normal functioning of the adult testis (Schlatt et al., 2007). The Leydig cells inside the interstitial compartment release testosterone, the Sertoli cells which are present in the tubular compartment express androgen receptor (Suarez-Quian et al., 1999). In order to act on the Sertoli cell, the testosterone must reach the tubular compartment by crossing peritubular myoid cells and basement membrane. Spermatogenesis, a testicular process through which male spermatogonial stem cells, which are present at the periphery of the seminiferous tubules, are transformed through mitotic and meiotic division into motile sperm with the capacity to fertilize the ovum (de Kretser et al., 1998). During spermatogenesis, the developing spermatozoa are in direct communication with the Sertoli cells and the developmental requirements of the germ cells are met through the secretory activity of the Sertoli cells (de Kretser et al., 1998). Sertoli cell expresses androgen receptor as the key events during the process of spermatogenesis are dependent and/or regulated by testicular androgens indirectly through Sertoli cells (Rey et al., 2009). Testosterone and dihydrotestosterone (DHT) are the main androgens types which are present at high concentration inside the seminiferous tubules ((Preslock and Steinberger,

1977). This high intra-tubular concentration of androgens is mainly achieved by androgen binding protein secreted by Sertoli cells (Ritzen et al., 1982; Galdieri et al., 1984).

The functional aspect of compartmentalization in the primate testis is to provide the proper environment and suitable conditions for the successful completion of steroidogenesis and spermatogenesis separately (Dym and Fawcet, 1970; Schlatt et al., 1997). Although these two compartments are present in close proximity, a cytological barrier exists between these two compartments which not only separates them functionally but is also responsible for highly sensitive inter-compartmental communication through the exchange of chemicals via an active transport mechanism (Pelletier and Byers, 1992). The cell types and extracellular structure responsible for this barrier include peritubular myoid cells, basement membrane and Sertoli cells. Peritubular myoid cell acts as the first layer of this barrier and completely encircles the tubular compartment or the seminiferous tubules. Peritubular myoid cells are followed by basement membrane and Sertoli cells. Adjacent Sertoli cell creates junctional complexes and thus form another barrier known as blood testes barrier (Lui et al., 2003; Setchell, 2008). Sertoli cell performs multiple functions in the tubules and is the main cell type responsible for establishing specific intra-tubular environment. Sertoli cells along with peritubular myoid cells and basement membrane work together to achieve the highly sensitive equilibrium created by this barrier for the proper functioning of the testis (Dym and Fawcet, 1970).

Apart from testosterone, numerous proteins and growth factors have been shown to act as paracrine factors in testis. Like the Sertoli cell secreted factors which include transforming growth factor (TGF), insulin like growth factor (IGF), interleukines (IL), fibroblast growth factor (FGF) and stem cell factor (SCF) and peritubular myoid cell secreted factors like TGFβ, IGF, leukemia inhibiting growth factor (LIF) and peritubular factor that modulates Sertoli cell function (PModS) (Mullaney and Skinner, 1991; Skinner, 1991; Skinner et al., 1991). The proposed actions of these growth factors in the testis include modulation of cellular growth and proliferation as well as the modulation of steroidogenesis. Thus paracrine communication is highly important for the proper

functioning of the adult testis, if this inter-compartmental communication is disrupted or compromised; the adult testis ceases to function properly.

Primate puberty is a complex process of temporal growth involving integration of hypothalamus, pituitary and testis (Ojeda et al., 1980; Plant and Berker-Gibb, 2004). Pubertal testicular development significantly affects the future functional capacity of the testis during the adulthood. A timely progression into pubertal phase of development is vital for the normal functionality of the adult primate testis both qualitatively and quantitatively.

Puberty in male primates

Puberty is the temporal process of development which is characterized by the activation of hypothalamic GnRH neurons at a specific age during postnatal development (Ojeda et al., 1980; Plant, 2001; Plant and Berker-Gibb, 2004). The juvenile phase is characterized by a hypogonadotropic state. The level of GnRH and the GnRH mRNA in the hypothalamus of juvenile monkey were found to be similar to those in adult monkey brain implying that the release of GnRH, instead of the synthesis, in the juvenile period of development is compromised (Fraser et al., 1989). As the control of pulsatile GnRH release occurs within the hypothalamus (Plant, 1985). The neurobiological mechanism by which this restraint is implied is known as pre-pubertal hypothalamic restraining brake (Plant and Berker-Gibb, 2004). This brake is achieved by the combined action of many putative neurotransmitters and neuropeptides. γ-Amino butyric acid (GABA, an inhibitory neurotransmitter), neuropeptide Y (NPY) and glutamate (an excitatory amino acid) have been shown to play major role in establishing this pre-pubertal hypothalamic restraining brake on GnRH neurons (Terasawa and Fernandez, 2001).

Although it was found that the pubertal activation can be achieved by the chemical stimulation of the hypothalamus (Plant et al., 1989). The precise neurobiological trigger was yet to be discovered until 2003. In 2003, a neuropeptide named kisspeptin along with its G-protein coupled receptor was demonstrated to be

involved in the pubertal activation of GnRH neurons in humans and primates (Terasawa et al., 2013). It has been shown that the kisspeptin action, via a G-protein coupled receptor (GPR54) present on the GnRH neurons, results in the pulsatile release of GnRH (Shahab et al 2005). This mode of release of GnRH is entrained by a neuro-biological setup known as GnRH pulse generator. Activation of GnRH pulse results in the generation of corresponding LH pulses from anterior pituitary gland (Plant, 2008). By binding to LH receptors, present on the Leydig cell membrane, these LH pulses are responsible for the activation of the process of steroidogenesis inside the Leydig cells (Ascoli et al., 2002). The sudden rise in serum testosterone acts on Sertoli cells present in the seminiferous tubules. Sertoli cells are expressing androgen receptor during this specific time of pubertal activation (Majumdar et al., 2012). This intra-testicular action of testosterone is very significant as the Sertoli cells show rapid response by attaining functional activity which is characterized by an increase in the cytoplasmic area and the initiation of secretory activity. Secretory activity is characterized by fluid secretion from Sertoli cells (Sharpe et al., 2003). Expression of androgen receptor by Sertoli cells during the pubertal onset is very important as the levels of testosterone are also high during early postnatal period (neonatal period) but Sertoli cells do not express steroid receptors during that period (Majumdar et al., 2012). The formation of lumen and the rapid increase in the Sertoli cell size eventually increase the seminiferous tubule diameter (Plant et al., 2005). The first sign of puberty at the testicular level is the rapid increase in the testicular volume. In parallel the A-spermatogonia, the spermatogonial stem cells present at the periphery of the seminiferous tubules also start to divide mitotically and the presence of B spermatogonia are observed. These events at the testicular axis are subsequent to the hypothalamic activation of the GnRH neurons (Plant, 2008).

However, from testicular perspective the process of puberty is defined as the attainment of the ability of the testes to produce and release mature sperm. The process of spermatogenesis is characterized by mitotic and meiotic cell divisions of the spermatogonia. The process of meiosis needs to be performed in an immunologically controlled environment as the haploid sperm can initiate an immunogenic response from the macrophages present in the interstitial area of the testes (Meinhardt and Hedger, 2011). To create the immunologic barrier inside the seminiferous tubule, Sertoli cell

creates tight junctional complexes between adjacent Sertoli cell membrane, these tight junctions forms the blood testes barrier (Lui et al., 2003; Setchell, 2008). Testosterone is responsible for the formation of this blood testes barrier by the Sertoli cells (McLachlan et al., 2002). Until the establishment of the blood testes barrier, the process of spermatogenesis could not proceed beyond the first mitotic divisions. For these reasons after the hypothalamic GnRH pulse activation, the presence of sperm either in the tubule lumen or in the epididymis is not observed immediately (Schlatt et al., 1995).

Initiation of puberty in primates is triggered by resurgence of GnRH pulsatility which then leads to pulsatile LH release and gonadal development (Plant, 2001). GnRH pulsatility itself is regulated by a number of neuronal signals including glutamate, GABA and NPY. However recently, there has been much excitement about the central role of kisspeptin signaling which has been shown to be the most potent drive to the GnRH neurons (Terasawa et al., 2013). Therefore the next section represents a brief description on the basic biology of the kisspeptin-kisspeptin receptor signaling.

Basic biology of kisspeptin and kisspeptin receptor signaling

Kisspeptin is a 145- amino acid precursor peptide encoded by *KISS1* in humans. Endogenous forms of kisspeptin comprising 54, 14 and 13 amino acids in length have been isolated from human placenta (Murphy, 2005; Hilden et al., 2007). Chemically kisspeptins are included in RFamide peptide family; a structurally related group of peptides terminating with an arginine-phenylalanine-amide at their carboxy terminal (Dockery, 2004). The kisspeptin receptor in humans (KISS1R) is a 398 amino acid G-protein coupled receptor with a short extracellular domain, seven transmembrane domains linked by extracellular and intracellular loops and an intracellular domain (Lee et al., 1999).

Three concurrent discoveries revealed the natural ligand to this receptor as the peptide product of the metastasis suppressor gene *Kiss1*, termed kisspeptin (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). The minimum sequence necessary to

activate KISS1R is the common C-terminal decapeptide which is commonly shared by all endogenous forms of kisspeptin peptide having different fragment length. *Kiss1* is expressed in the CNS, pituitary, ovary, testis, and pancreas but is highly concentrated in placenta whereas *Kiss1R* is expressed in the CNS, testis, ovary, pancreas, intestine, liver, pituitary and placenta (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Funes et al., 2003; Terao et al., 2004; Mead et al., 2007). *Kiss1* is expressed by neurons in the hypothalamus and KISS1R is found to co-localize with GnRH (Irwig et al., 2004; Han et al., 2005; Kirilov et al., 2013). The stimulatory effect of kisspeptin on HPG axis is mediated via hypothalamic GnRH, as central or peripheral effects of kisspeptin are blocked by GnRH antagonist administration (Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Shahab et al., 2005).

The nomenclature for the kisspeptin and kisspeptin receptor has been officially recommended by the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification. Human kisspeptin gene was designated as *KISS1* where as non human kisspeptin gene was designated as *Kiss1*. The kisspeptin protein names for human and non human were given as KISS1 and Kiss1, respectively. For the kisspeptin receptor, *KISS1R* was designated for human gene whereas *Kiss1r* was designated for non-human gene (Kirby et al., 2010)

Kisspeptin-Kiss1r signaling in primate puberty

Role of kisspeptin-kisspeptin receptor signaling in primate pubertal activation was discovered in 2003, when it was shown that mutations in *KISS1R* (GPR54) gene caused idiopathic hypogonadotropic hypogonadism (IHH) in humans (DeRoux et al., 2003; Funes et al., 2003; Seminara et al., 2003). Humans with inactivating mutations (loss of function mutation) in *KISS1R* have low gonadotropin levels, lack LH pulses and fail to undergo puberty (Seminara et al., 2003). Since 2003, a number of studies have shown that IHH is caused by inactivating mutations of *KISS1R* in humans (Lanfranco et al., 2005; Semple et al., 2005; Pallais et al., 2006). On the other hand it has also been shown that activating mutations (gain of function mutations) of *KISS1R* cause precocious

puberty in humans, where the subjects experience puberty at much early age (Teles et al., 2008).

The hypothalamic *Kiss1* expression is increased during puberty in castrated non human primates and the intravenous (iv) administration of kisspeptin significantly enhances the serum LH levels in juvenile agonadal male rhesus monkeys (Shahab et al., 2005). The involvement of kisspeptin-Kiss1r signalling, as a mediator in the negative feedback action of the testosterone on the GnRH release in monkeys has also been shown and kisspeptinergic neurons upstream to the GnRH neurons are involved in the negative feedback action of testicular testosterone on LH secretion in the adult male rhesus monkey (Shibata et al., 2007). The structural interactions between kisspeptinergic and GnRH neurons in the mediobasal hypothalamus (MBH) of the adult male rhesus monkeys has been demonstrated (Ramaswamy et al., 2008). It has been shown that kisspeptin is secreted in a pulsatile manner in the stalk median eminence and also stimulates GnRH release in vivo and the pubertal increase in the kisspeptin is accompanied by GnRH release in a pulsatile manner (Keen et al., 2008).

Postnatal and pubertal testicular development in primates

Postnatal development of primate testes until the end of juvenile period is characterized by a slow and steady increase in Sertoli cell numbers which is then followed, in pre-pubertal phase, by a burst of proliferation and an increase in cytoplasmic volume, mostly referred to as functional maturation of Sertoli cells (Sharpe et al., 2003). Quantitative increase of Sertoli cells results in the significant lengthening of the seminiferous cords (Marshall and Plant, 1996). Further into puberty a dramatic rise in germ cells occurs resulting in an enlargement of seminiferous tubule diameter and consequently a rapid increment in the size and weight of the testis. Seminiferous tubule length and Sertoli cell number stabilize during this period (Plant et al., 2005). It is concluded that testicular volume presents a valid readout of testicular maturation during puberty. In rhesus macaques the Sertoli cell number increases from approximately 40 million at birth to 2,000 million in each testis in the adult (Sharpe, 1994), whereas the

undifferentiated spermatogonia increase from one million at birth to 600 million in the adult (Simorangkir et al., 2005). In human there is a 6-fold increase in testicular volume during the first year of life (Muller and Skakkebaek, 1983), whereas in new world monkeys a 13 fold increase is observed within the first 5 month (Abbot and Hearn, 1978; Rey et al., 1993). The distinctive 4-fold increase in Sertoli cell number in infancy, when gonadotropin levels are high, is then followed by an abated rate of proliferation in the juvenile phase of development when the serum levels of gonadotropins are very low (Plant et al., 2005). Until the onset of puberty, while the cords are slowly extending in length, no significant increase in the seminiferous cord diameter is detectable and the cords are devoid of a lumen. Thus the incremental rise in Sertoli cells number in the infancy and juvenile period, when Sertoli cells account for 95% of the seminiferous tubule mass, seems to be an innate feature of this period.

The next section reviews the general notion of developmental characteristics and regulation of different cell types present in the testis.

Sertoli cell

Enrico Sertoli, an Italian scientist first described the testicular cells in 1865 which bear his name. He described them as the large branched cells which are linked to the production of spermatozoa. The overall number of adult Sertoli cells establishes the upper limit of sperm production in the adult testis, and spermatogenic efficiency is highly associated with Sertoli cell supportive capacity, which in turn is the best indication of Sertoli cell function in the adult testis (Hess and Franca, 2005). Sertoli cells are columnar cells and extend from the basement membrane of the seminiferous epithelium to the lumen (Fawcet and Burgos, 1956). When fully differentiated they are mitotically arrested and act as nurse cells (Griswold, 1998). Forming a syncytium-like epithelial structure, Sertoli cells are embedding and holding the interconnected cohorts of germ cells (Russell, 1980). The Sertoli cells are interconnected by specialized junctional complexes which are responsible for establishing the blood testis barrier (Fawcet and Burgos, 1956; Dym, 1974; Russell, 1980). Approximately 40% of the Sertoli cell surface is in contact with the

surface of elongated spermatids (Griswold, 1995; Russell et al., 1986). In contrast, immature Sertoli cells form a simple columnar epithelium with limited cytoplasmic connections sharing the basement membrane with few A-spermatogonia. The immature Sertoli cell proliferates significantly in the infantile period owing to the high level of pituitary gonadotropins during this phase and thus creating nearly 95 % of the total seminiferous tubule cell mass along with germ cells which constitute 5 % (Rey et al., 1993; Simorangkir at al., 2012). This numerical density of Sertoli cells in seminiferous tubule cross section diminishes during pre-pubertal stage as the Sertoli cell cytoplasm volume increases considerably along with an increase in germ cell population (Dang and Meusy-Dessolle, 1985). During the infant and juvenile period Sertoli cells increase in number without any significant increase in size and morphology (Simorangkir et al., 2003). Apart from being mitotically active immature Sertoli cells are also characterized by their ability to secrete estrogens and anti-mullerian hormone (AMH) and presence of functional FSH receptors. At initiation of puberty a significant increase in cytoplasmic volume occurs apart from the mitotic proliferation (Marshall and Plant, 1996). This morphological change in Sertoli cells signifies an important first sign of puberty. An important and histological obvious feature is a re-arrangement of Sertoli cells at the beginning of puberty. While Sertoli cell nuclei are randomly distributed in the seminiferous cords prior to puberty, they are organized more epithelial-like with all nuclei being close to the basement membrane early into puberty. This epithelial re-arrangement as well as the increase in number are associated with longitudinal expansion of the cords and are augmented by an increase in the number and volume of peritubular cells (Fawcett, 1975; Marshall and Plant, 1996).

The pubertal onset is characterized by an intense proliferation of Sertoli cells and hence the testis size increases 10 fold (Marshall and Plant 1996; Plant et al., 2005). In the peri-pubertal time period, tubule diameters also increase to accommodate the growth of Sertoli cells during differentiation and the increasing number of germ cells. Now, Sertoli cells become closely interconnected and form the blood testes barrier. In consequence, the fluid accumulation occurs, which creates a lumen thus transforming the cords into seminiferous tubules (Fawcett, 1975) and evoking additional diametrical growth. These processes are coincident with the expansion of the germ cell population. At first,

spermatogonia populate the basement membrane below Sertoli cells. When meiotic differentiation starts, germ cells become engulfed in Sertoli cell extensions. The multiplied germ cells loose the contact to the basal lamina and move towards the centre of the tubules. The precedence of Sertoli cell proliferation prior to germ cell expansion is physiologically important. The maximum number of germ cells in the adult testis depends on the number of Sertoli cells because each Sertoli cell supports a limited number of germ cells (Orth et al., 1988). Therefore, adequate Sertoli cell expansion at the beginning of puberty is essential to provide the structural and functional basis in establishing the capacity of the testes to generate sperm later in life (Plant, 2005). In this respect the early phase of puberty is more relevant for future fertility than the second phase of puberty when germ cell differentiation starts after Sertoli cells have reached peak numbers and established the mitotic arrest.

The ability of Sertoli cells to respond to gonadotropins is achieved much earlier than the actual pubertal onset (Arslan and Qazi, 1976; Arslan et al., 1981; Arslan et al., 1993; Schlatt et al., 1995; Sameshima and Hamana, 1996; Ramaswamy et al., 2000). During the juvenile period male monkeys respond to exogenous FSH or hCG/testosterone treatment (Arslan et al., 1993; Schlatt et al., 1995). This response pertains to an almost immediate and robust proliferation and maturation of immature Sertoli cells along with an increase in A-spermatogonia. Thus a persistent low pre-pubertal level of gonadotropins is the limiting and critical factor maintaining the testes in a passive state. This signifies that immature Sertoli cells are highly dependent on gonadotropins to induce proliferation and differentiation and that the ability and potential of Sertoli cell to proliferate and differentiate is established through mechanisms independent from puberty and already established long before pubertal induction (Schlatt et al., 1995). On the other hand high levels of gonadotropins in infancy cannot induce germ cell differentiation revealing that some important changes must have occurred during the early juvenile period creating the capacity of the testes to not only expand its somatic components but to kick start spermatogenesis in the more mature seminiferous epithelium. These molecular and cellular changes are attributed to the lack of androgen receptor expression by the Sertoli cell during infancy (Majumdar et al., 2012).

Germ cell

Although many changes occur in the early stages of puberty in the seminiferous epithelium, the first emergence of meiotic spermatocytes after a peak of spermatogonial proliferation is considered often as a first sign of pubertal maturation of the seminiferous tubules (Plant, 2005). The increment in the total germ cell number before puberty is much slower as compared to Sertoli cells or longitudinal outgrowth of tubules. Consequently, the number of germ cells expressed as a function of Sertoli cell number or per tubule cross section decreases during juvenile period. This relative decline does not reflect the actual progressive increment in germ cells per testis before puberty (Chemes, 2001; Marshall et al., 2005; Albert et al., 2010). It is important to note that only unbiased morphometric procedures provide actual and valid values on cell counts. In contrast to adult animals in which changes in germ cell counts can be expressed in relation to a fixed number of Sertoli cells, it is mandatory to use more complex evaluation strategies in immature testes as all cellular components expand. The pubertal proliferation of germ cell results in a loading of the seminiferous tubules with high numbers of germ cells until at the end of the puberty the germ cell mass accounts for more than 90% of seminiferous tubule volume (Plant, 2005).

In primates the undifferentiated spermatogonia (Type A) have been classified into two categories, dark (Ad) and pale (Ap) based on their nuclear staining pattern (Ehmcke et al., 2005). Ad spermatogonia are mitotically quiescent in adulthood and hence designated as reserve stem cell. Ap spermatogonia on the other hand are mitotically active with self renewal capacity. Their clonal expansion and splitting is responsible for the generation of differentiated spermatogonia (type B) and maintenance of a progenitor pool (Ehmcke and Schlatt, 2006). In rhesus monkeys Ad and Ap spermatogonia are mitotically active in pre pubertal primate testis as evident by robust S-phase labelling of A-spermatogonia in the infantile and juvenile testis. This expansion of both spermatogonial subtypes occurs irrespective of a hypogonadotropic environment. The post-natal pattern of Ad spermatogonial proliferation is similar to Ap spermatogonial expansion (Simorangkir et al., 2005) which reflects the expansion of Ap spermatogonia

in hypogonadotropic adult monkeys (Marshall et al., 2005). The regulatory mechanisms of the gonadotropin independent proliferation of Ad (in immature monkeys) and Ap spermatogonia (in immature and adult monkeys) is still unclear but indicates an autonomous regulation often found in stem cell systems. Nevertheless, the juvenile testis can attain the potential to successfully undergo spermatogenesis well before the onset of actual puberty (Marshall et al., 1984, 1986; Arslan et al., 1993; Schlatt et al., 1995). Random but rare appearance of type B spermatogonia has also been reported in the pre-pubertal macaque testis (Cavicchia and Dym, 1978; Kluin et al., 1983). However, significant numbers of differentiated spermatogonia are only evident after the onset of puberty.

Leydig cell

Leydig in 1850 first reported masses of cells containing vacuoles and pigmented inclusions. These cells were later called after his name as Leydig cells. Leydig cells reside in the interstitial compartment of the testis and serve as the main source of testosterone production. Postnatal production of testosterone by the Leydig cell is regulated by the pituitary gonadotropin LH (Saez, 1994). The Leydig cell responsiveness towards pituitary LH is achieved early in primates i.e, during neonatal phase, as the gonadotropins levels are high during this phase and testosterone levels correspond to LH peaks (Frawley and Neill, 1979; Fouquet et al., 1984). Leydig cell number increases in a linear trend from birth towards puberty. The morphological differentiation of Leydig cells is a long process during pubertal development in primates, the peculiar correlation of pituitary LH and testosterone remains throughout this developmental period as after a long juvenile hiatus the rise in the levels of pituitary gonadotropin during the prepubertal period is followed by high levels of testosterone (Steiner and Bremner, 1981; Meusy-Dessolle and Dang, 1985; Bernstein et al., 1991; Plant, 2006).

The levels of testosterone are in turn correlated to the number and volume of Leydig cells. As shown by the Fouquet at al., 1984, rhesus monkeys show a strong positive correlation between Leydig cell number and plasma testosterone during postnatal

development. In rhesus macaques the post natal levels of plasma testosterone demonstrate four distinct phases, 1) a neonatal phase with moderate levels of testosterone and large Leydig cells, 2) a juvenile phase with very low levels of testosterone along with a significant reduction in Leydig cell size and volume, 3) a pre-pubertal phase with a rise in testosterone concentration along with a significant increase in Leydig cell size and volume and 4) a pubertal phase with increasing levels of testosterone but relatively unchanged Leydig cell volume (Steiner and Bremner, 1981; Fouquet at al., 1984; Meusy-Dessolle and Dang, 1985; Arslan et al., 1986).

The same pattern of postnatal testosterone secretion has been demonstrated in new world monkeys (Abbott and Hearn, 1978; Kholkute et al., 1983; Lunn et al., 1994; Rey et al., 1995; Kelnar et al., 2002; Chandolia et al., 2006). Immature and mature Leydig cells are present from birth to late puberty in the Cebus, a new world monkey. At birth and during infancy, immature Leydig cells represent the highest proportion, with mature Leydig cells comprising only 12%. The quantitative analysis of the postnatal testicular development in this monkey shows that in neonatal phase the interstitial tissue represents nearly half of the testicular parenchyma, with a progressive decrease during postnatal life to reduce to half of its size in late puberty. However, the absolute volume of interstitial tissue increases during development i.e. there is a 7 fold increase in the Leydig cell number during neonatal period which is followed by a stable period during juvenile until late puberty when there is a two fold increase. There are two periods of interstitial tissue growth, the first during the neonatal and early infantile periods, and the second, in mid to late puberty (Rey et al., 1991; Rune et al., 1994, 1996). As the pubertal activation of spermatogenesis is dependent on high intratesticular levels of testosterone, the physiological insensitivity of the neonatal testis towards high intratesticular testosterone is due to the absence of Sertoli cell androgen receptor expression during this period (McKinnel et al., 2001; Majumdar et al., 2012).

Rationale

Kisspeptin in primate testis

While the central role of kisspeptin signaling in the control of reproductive axis is thoroughly demonstrated, the functional role of kisspeptin at the primate testis is yet to be defined. The available data from human and non human primate studies indicates an active kisspeptin-Kiss1r signaling in primate testis (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Ramaswamy et al., 2007). The high expression levels of kisspeptin and moderate expression levels of kisspeptin receptor in adult human testis indicate a possible intratesticular role of kisspeptin signaling in primate testis. As the testicular paracrine signaling, featuring tens of molecules is a distinct feature of the adult primate testis (Schlatt et al., 1997) and it has been shown previously that hypothalamic neuropeptide, GnRH and GnRH receptors are present in the testis and affect steroidogenesis through paracrine signaling (Bambino et al., 1980; Bourne et al., 1980; Petersson et al., 1989). The possible involvement of kisspeptin in enhancing testicular steroidogenesis in adult male rhesus monkeys given an iv infusion of kisspeptin has been indicated, where the serum levels of testosterone were high for a given concentration of LH in these monkeys (Ramaswamy et al., 2007). The immunohistochemical localization of the kisspeptin and kisspeptin receptor in rodents and vertebrates testis has been reported recently (Anjum et al., 2012; Chianese et al., 2013; Hua et al., 2013), although the testicular localization in primate testis has not been reported to date.

Pubertal testicular development in common marmoset (*Callithrix jacchus*) a New World monkey

The pubertal and testicular development in New World monkeys is not well characterized to date. Whereas the specific age of puberty, the prepubertal developmental pattern as well as the hormonal correlates of pubertal activation and the subsequent testicular developmental patterns are thoroughly depicted in old world monkeys (Plant,

2005). Despite the fact that common marmoset (*Callithrix jacchus*) is a representative New World monkey; a certain degree of controversy exists regarding its pubertal age and subsequent testicular development. Common marmosets have a unique reproductive endocrinology as compared with higher primates where the LH is replaced by a chorionic gonadotropin (CG) like molecule having a shorter half life as compared with LH (Muller et al., 2004) as well the testicular steroidogenesis utilizes Δ4 pathway whereas in higher primates Δ5 pathway is utilized (Preslock and Steinberger, 1977). Apart from these physiological diversity, the high individual variation in the timing of pubertal onset, the possible impact of social status on the pubertal onset and the subsequent testicular development in common marmoset has not been assessed systematically in a large number of cohorts. Apart from the above mentioned physiological variation, an important physiological aspect of adult common marmoset is the resemblance of its testicular tubular organization towards human testis. It has been shown that adult marmoset testis shows highly similar seminiferous tubule organization with human (Millar et al., 2000; Wistuba et al., 2003) as well as the fetal and neonatal germ cell development in common marmoset mimic human like pattern (Mitchell at al., 2008). While the use of common marmoset as a laboratory primate in toxicological and biomedical research has increased enormously in the recent years, owing to its small size, high breeding rate and longer life span in captivity (Carrion and Patterson, 2012; t' Hart et al., 2012), the need for a better understanding of pubertal and testicular development in common marmoset is mandatory.

Objectives

The present study was designed for three objectives:

1. To elucidate the possible involvement of kisspeptin in testicular steroidogenesis in adult rhesus monkeys (*Macaca mulatta*) through an ***in vivo*** approach.
2. To determine the immunohistochemical localization of kisspeptin and kisspeptin receptor in adult primate testis.
3. To characterize the pubertal onset and the subsequent testicular development in Common marmoset (*Callithrix jaccchus*) through in vivo and histomorphometric approaches.

Chapter 2

Intratesticular action of kisspeptin in adult rhesus monkey (*Macaca mulatta*): An *in vivo* study

Intratesticular action of kisspeptin in adult rhesus monkey (*Macaca mulatta*): An *in vivo* study

Abstract

Kisspeptin-kisspeptin receptor signaling in mammals has been implicated as an integral part of the reproductive cascade. Kisspeptinergic neurons upstream of GnRH neurons are involved in the activation of hypothalamic GnRH pulse generator during the pubertal onset. Thus, the major research focus either in rodents or in primates has been on the central effects of kisspeptin. The demonstration of the presence of *KISS1R* expression in human testes suggests additional, as yet unknown, physiological actions of kisspeptin-kisspeptin receptor signaling at the distal component of the male reproductive axis. In this study we tried to explore the impact of kisspeptin at testis in the adult male rhesus monkey, a representative higher primate. We employed the pituitary gonadotropin hypophysectomised monkey to assess the intratesticular actions of iv bolus of kisspeptin. Plasma testosterone and plasma LH levels were monitored in a group of four adult male monkeys. The peripheral administration of human kisspeptin-10 (50μg, iv bolus) caused a single LH pulse followed by robust increase in plasma testosterone levels which sustained for the next 180 minutes. This potent increase in testosterone was abolished when kisspeptin was administered to acyline (a GnRH receptor antagonist) pretreated animals (n=4) (Acyline: 60 μg/kg and 120 μg/kg BW, sc, morning and evening, respectively, 1 day before kisspeptin injection). However kisspeptin administration significantly ($p<0.005$) elevated hCG-stimulated testosterone levels in acyline-pretreated monkeys as compared with saline+hCG treatment. These results revealed a novel facet of kisspeptin-Kiss1r signaling at the testis, where kisspeptin administration significantly enhanced stimulated testosterone secretion in adult intact male monkeys suggesting a possible paracrine action of kisspeptin in amplifying the responsiveness of the primate Leydig cell towards luteinizing hormone.

Introduction

In male primates, GnRH pulses activate pituitary gland to release LH in a pulsatile fashion, activating the LH receptor present on the Leydig cell in such a manner that a response in the form of testosterone is produced. The pulsatile fashion of LH release is physiologically important as consistent high serum levels of LH down regulates the LH receptor on the Leydig cells which results in diminished testicular androgenic response. A single LH pulse will give rise to a corresponding testosterone peak. This LH-testosterone loop shows a diurnal rhythm in adult male rhesus monkeys (Schlatt et al., 2008). Rhesus monkey also shows seasonality in terms of reproductive efficiency. Levels of testosterone and testicular functionality are greatly reduced in non breeding season as compared with the breeding season (Wickings and Nieschlag, 1980).

Since the initial discovery of the role of kisspeptin-kiss1r signalling in primate puberty, most of the studies were focused on the central role of kisspeptin. Although the presence of kisspeptin and kiss1r transcripts in adult primate testis hints towards the possible role of kisspeptin signalling at the testis (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). On the other Ramaswamy found that in adult male rhesus monkeys (*Macaca mulatta*) the iv administration of kisspeptin given as a continuous infusion resulted in an increase in circulating LH levels which peaked after 2-4 hours of initial treatment and then followed by a decline (Ramaswamy et al., 2007). The interesting revelation made in this study was the disproportional relationship between circulating testosterone and LH, where testosterone levels were constantly high for a given concentration of LH during iv infusion of kisspeptin thus raising the possibility of an endocrine aspect of kisspeptin-kiss1r signaling within the primate testis. These results hint towards a possible role of kisspeptin signalling in regulating the testicular steroidogenic response towards LH.

In the light of the findings by Ramaswamy (2007), to further explore the possible role of kisspeptin at the testis, we decided to investigate the possibility of a kisspeptin iv bolus in enhancing basal testosterone levels in adult male monkey. As the kisspeptin has a central effect in enhancing hypothalamic GnRH release, the kisspeptin iv bolus would increase the serum levels of LH. This increased level of LH would enhance the testosterone levels in result of the kisspeptin bolus via the central mechanism.

In order to explore the role of kisspeptin in enhancing the basal levels of testosterone, we needed to establish an endocrine model which can assure the possibility of direct testicular effect of kisspeptin on the basal testosterone levels which is independent of serum LH and also the central effect of kisspeptin in enhancing the serum LH levels should also be compromised. In order to block the pituitary secretion of LH we used Acyline, a GnRH receptor antagonist (Shahab et al., 2005). Acyline competes with GnRH for binding with GnRH receptor present at the pituitary thus blocking the effect of GnRH at the pituitary levels. After achieving this chemical hypophysectomy in terms of gonadotropins, any effect observed in the basal testosterone levels after the administration of kisspeptin iv bolus can be positively attributed to the kisspeptin signaling at the testis level. We selected adult male rhesus monkeys to test our hypothesis in exploring the testicular effect of kisspeptin signalling.

Materials and Methods

Monkeys

Four, 4-6 years old, intact male rhesus monkeys (*Macaca mulatta*), weighing 6.0-8.0 kg were used in this study. Animals were housed in individual cages, under standard colony conditions, fed with monkey diet at 1300-1330 hours daily and supplemented with fresh fruits in the morning in the Primate Facility of the Department of Animals Sciences, Quaid-i-Azam University, Islamabad. Water was available *ad libitum*. The monkeys were trained for chair-restraint prior to the experiments in order to sample these animals without sedation or anaesthesia. Under ketamine sedation (5 μg/kg bw, im) animals were placed in primate chairs. After recovery from sedation the animals were allowed to sit on the chair for gradually increasing periods of time. The animals were habituated to chair restraint over several months. All experiments were approved by the Departmental Committee for Care and Use of Laboratory Animals and were performed in accordance with regulations of the Ethics Committee of the university.

Catheterization

To permit sequential withdrawal of the blood samples and iv administration of drugs, the animals were anesthetized with ketamine hydrochloride (Ketamax, Rotexmedica, Trittau, Germany, 5 μg/kg bw, im), and a teflon cannula (Vasocan Branule, B. Braun Melsungen AG, Belgium; 0.8mm/22G O.D) was inserted in the sephanous vein before initiation of sampling and the animals were restrained to the chair. The free end of the cannula was attached to a syringe via a butterfly tubing (20G diameter and 300mm length). Blood sampling and infusion of treatments were carried out when the animals had fully regained consciousness.

Pharmacological agents

Human kisspeptin-10 (112-121) (Phoenix pharmaceuticals, Belmont, CA. USA) and GnRH receptor antagonist acyline (Bioqual, USA) were kindly provided by Dr. Tony M. Plant. hCG (Pregnyl®, N.V Organon Oss Holland) and heparin (Rotexmedica, Germany) were purchased locally. Working solutions of kisspeptin and hCG were made in normal saline while acyline was dissolved in 5% aqueous mannitol. Ketamine hydrochloride (Ketamax, Rotexmedica, Trittau, Germany) was purchased locally.

Dosage

Based upon previously reported centrally effective iv kisspeptin doses (10μg, 30μg and 50 μg) in adult intact male rhesus monkeys (Ramaswamy et al., 2007; Wahab et al., 2008, 2011) the highest dose (50μg) of kisspeptin-10 was selected for exploring a possible direct testicular action in the present study. In our one of previous studies this dose of kisspeptin-10 was also found to be peripherally effective as it caused significant enhancement of plasma adiponectin levels in adult intact monkeys (Wahab et al., 2010).

In-Vivo experimental design and blood sampling

In order to examine our hypothesis of an intra-testicular action of kisspeptin-10 iv bolus in male monkeys, we used pituitary gonadotropin-clamped monkey model. The primary site for kisspeptin action is at hypothalamic GnRH neurons expressing kiss1r, this receptor coupling increase the GnRH pulse activity and subsequently increases the gonadotropin levels in blood circulation. To prevent this central action of kisspeptin administration being transduced at the pituitary level, chemical hypophysectomy was achieved by treating the animals with a potent GnRH receptor antagonist, acyline (Herbst et al., 2002; Shahab et al., 2005). Acyline competes with GnRH to bind with GnRH receptors at gonadotropes and blocking a secretory response from pituitary in response to GnRH and/or kisspeptin bolus.

The different sets of *in-vivo* experiments along with the treatments and pretreatments in this study are shown in Table 1. Same group of animals were used in each of these experiments. All the animals received the same treatments in each experiment. Animals were treated with acyline 24 and 12 hours before the experiment 1(c), 2(a) and 2(b) in the Table 1. The morning (24hr prior) and evening (12 hour prior) treatment was given subcutaneously with two different doses (60µg/kg and 120 µg/kg BW, sc, 9:00 am and 9:00 pm respectively). Blood sampling for all experiments started between 0900-0930 hours, and samples (2.5-3 ml) were obtained at 30-min intervals. First sample (-30 min) was collected 30 min before the treatment (vehicle/ kisspeptin/ hCG/ hCG + kisspeptin). Samples were taken in heparinised syringes and immediately transferred into culture tubes kept on ice. After completion of the blood sampling, these culture tubes were centrifuged at 3000 rpm for 30 minutes and plasma was extracted and stored at -15˚C until assayed. After taking every sample, approx 1 ml of normal saline containing 5IU of heparin was injected to compensate the lost blood volume to prevent hypovolumic shock to the animals and blood clotting.

Table 2.1 Details of the experiments.

Experiment #	Administration (iv)	Time of the year	Pretreatment	Animal number
1. (a) Vehicle Control	Normal saline (1ml)	**May**	None	4 Male monkeys
(b) Kisspeptin	**Kisspeptin-10** (50µg)	**May**	None	4 Male monkeys
(c) Kisspeptin	**Kisspeptin-10** (50µg) **hCG (50IU)** at 240min sample	**July**	**Acyline** (sc) 12 hrs and 24 hrs prior sampling	3 Male monkeys
2. (a) hCG+Saline	**hCG (50IU)** + Normal saline	**August**	**Acyline** (sc) 12 hrs and 24 hrs prior sampling	4 Male monkeys
(b) hCG+Kisspeptin	**hCG (50IU)** + **Kisspeptin-10** (50µg)	**August**	**Acyline** (sc) 12 hrs and 24 hrs prior sampling	4 Male monkeys

Effect of kisspeptin-10 on plasma testosterone and plasma LH in normal adult intact male monkeys

Impact of peripheral administration of kisspeptin-10 (50µg) on basal testosterone and LH was monitored in normal adult intact male monkeys (n = 4). Kisspeptin-10 or normal saline (vehicle control, 1 ml) was administered intravenously (iv) immediately after taking 0 min sample.

Effect of kisspeptin-10 on plasma testosterone in acyline pretreated adult intact male monkeys

Impact of kisspeptin-10 administration (50µg) on plasma testosterone was monitored in pituitary clamped monkey by the help of acyline pre-treatment (12 and 24 hr prior). Kisspeptin-10 (50µg) was administered intravenously (iv) immediately after taking 0 min sample and hCG was administered intravenously (iv) at 240 min sample.

Effect of kisspeptin-10 on plasma testosterone and plasma LH in acyline pretreated hCG challenged adult intact male monkeys

Impact of peripheral intravenous (iv) administration of kisspeptin-10 (50µg) on hCG stimulated testosterone secretion was determined in acyline pretreated adult monkeys (n = 4). Acyline pretreatment was given 12 and 24 hours before the start of the experiment to ensure the complete suppression of HPG axis. hCG (50 IU) and saline were given immediately after taking 0 min sample; and in the second set of experiment hCG (50 IU) and kisspeptin-10 (50µg) were administered after taking 0 min sample.

Plasma LH and testosterone measurement by specific RIA

Plasma LH concentrations were determined by double antibody radioimmunoassay (RIA) with reagents supplied by NHPP (National Hormone & Peptide Program). The standard preparation used was rec-moLH-RP-1, tracer was prepared from AFP-6936A and antiserum was AFP 342994. The tubes were counted in a Gamma counter (LKB Gamma Master 1277) for 1 minute. The sensitivity of the assay was 0.2 ng/ml and the intra- and inter assay coefficients of variation were 8.8 % and 5.8 %, respectively.

Plasma testosterone concentrations were determined by using specific solid phase competitive radioimmunoassay. The testosterone kits were purchased from Immunotech (Prague, Czech Republic). The assays were done according to the instructions given by the manufacturer. Tubes for testosterone were incubated at 37˚C. Tubes were then carefully decanted and placed in a Beckman Gamma counter (Gamma5500) for counting in-bound radioactivity. The counting time for each tube was one minute. The sensitivity of the assay was 0.025 ng/ml and intra- and inter assay coefficients of variants were 14.8% and 15%, respectively.

Statistics

Student's t-test was employed using Microsoft Excel to determine differences between means of hCG+saline and hCG+kisspeptin stimulated testosterone values. Statistical significance was set at $P<0.05$.

Results

Impact of peripheral administration of kisspeptin-10 on plasma testosterone and LH in normal adult intact male monkeys

Peripheral administration of 50µg kisspeptin-10 in acyline untreated group induced a potent increase in plasma testosterone levels (Fig. 2.1). Within 30 min after peripheral administration of kisspeptin-10, a single LH pulse was observed in all the animals. Subsequently plasma testosterone level increased 2 fold and remained elevated for the next 2 to 3 hours, while vehicle administration which served as a negative control did not influence the LH or testosterone levels (Fig. 2.2). The animals were not showing any LH pulsatile activity during the sampling hours (Fig. 2.2) mainly because of the quiescent hypothalamic pituitary axis in the morning hours in late pubertal animals.

Impact of peripheral administration of kisspeptin-10 on plasma testosterone in acyline pretreated adult intact male monkeys

In acyline pretreated group the kisspeptin treatment in all the animals did not induce an increment in the plasma testosterone levels (Fig 2.3), The testicular tissue was responsive in terms of testosterone synthesis in the acyline pretreated group as hCG administration caused a sudden increase in plasma testosterone levels in all animals (Fig. 2.3).

Impact of peripheral administration of hCG and hCG+kisspeptin-10 plasma testosterone and LH in acyline pretreated monkeys

Administration of hCG (50IU)+saline in acyline pretreated monkeys caused a moderate but sustained increase in plasma testosterone levels (Fig 2.4). Administration of kisspeptin-10 (50µg) + hCG (50 IU) amplified plasma testosterone levels as compared to the hCG induced increase in saline+hCG treatment in all the individual animals without effecting plasma LH values (Fig 2.5). Pituitary gonadotropin clamping was successful by Acyline treatment was obvious as kisspeptin (50µg) treatment could not induce a LH peak in the animals (Fig 2.5).

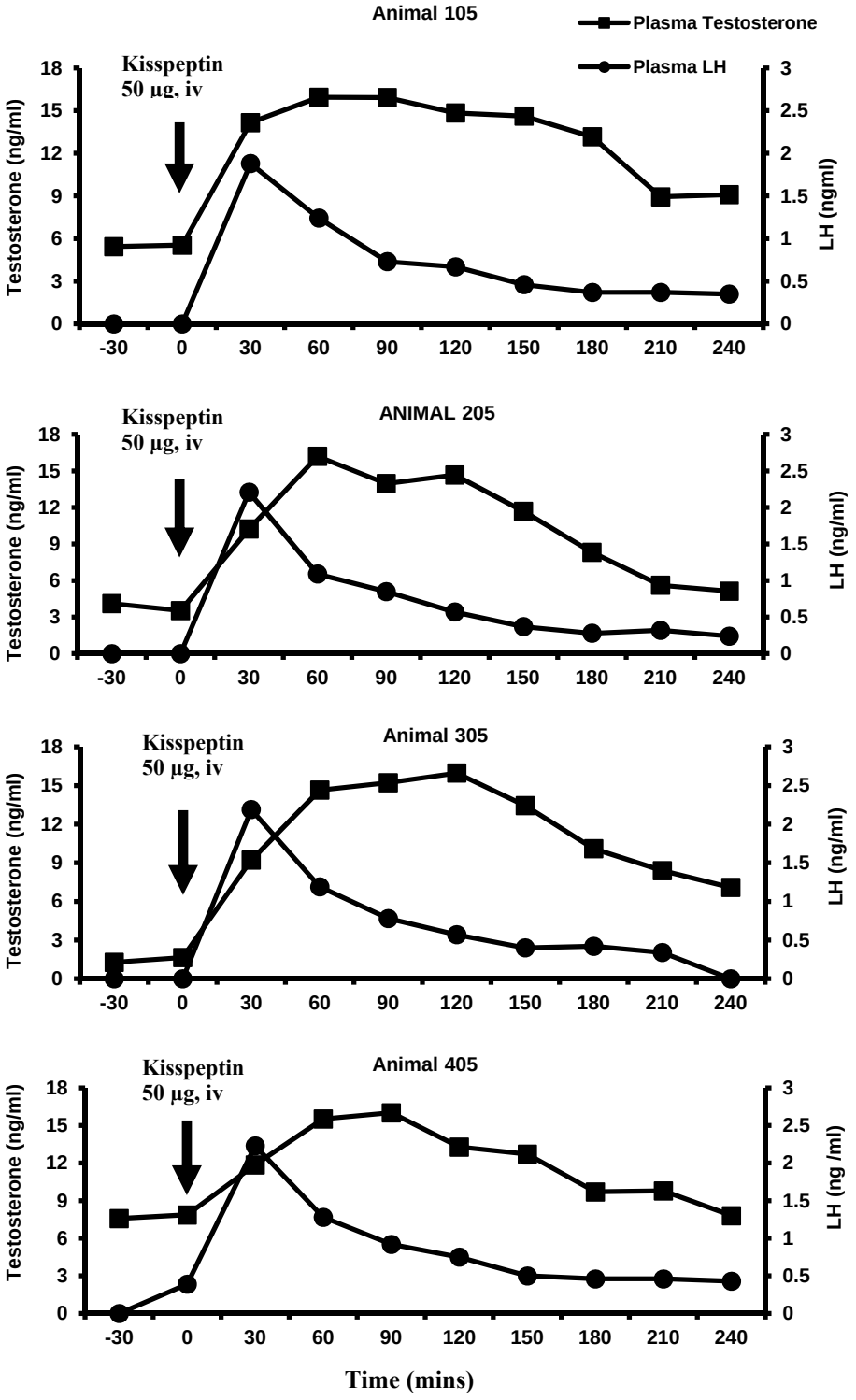


Figure 2.1 Individual plasma LH and testosterone concentration in adult intact rhesus monkeys before and after iv administration of kisspeptin (arrow).

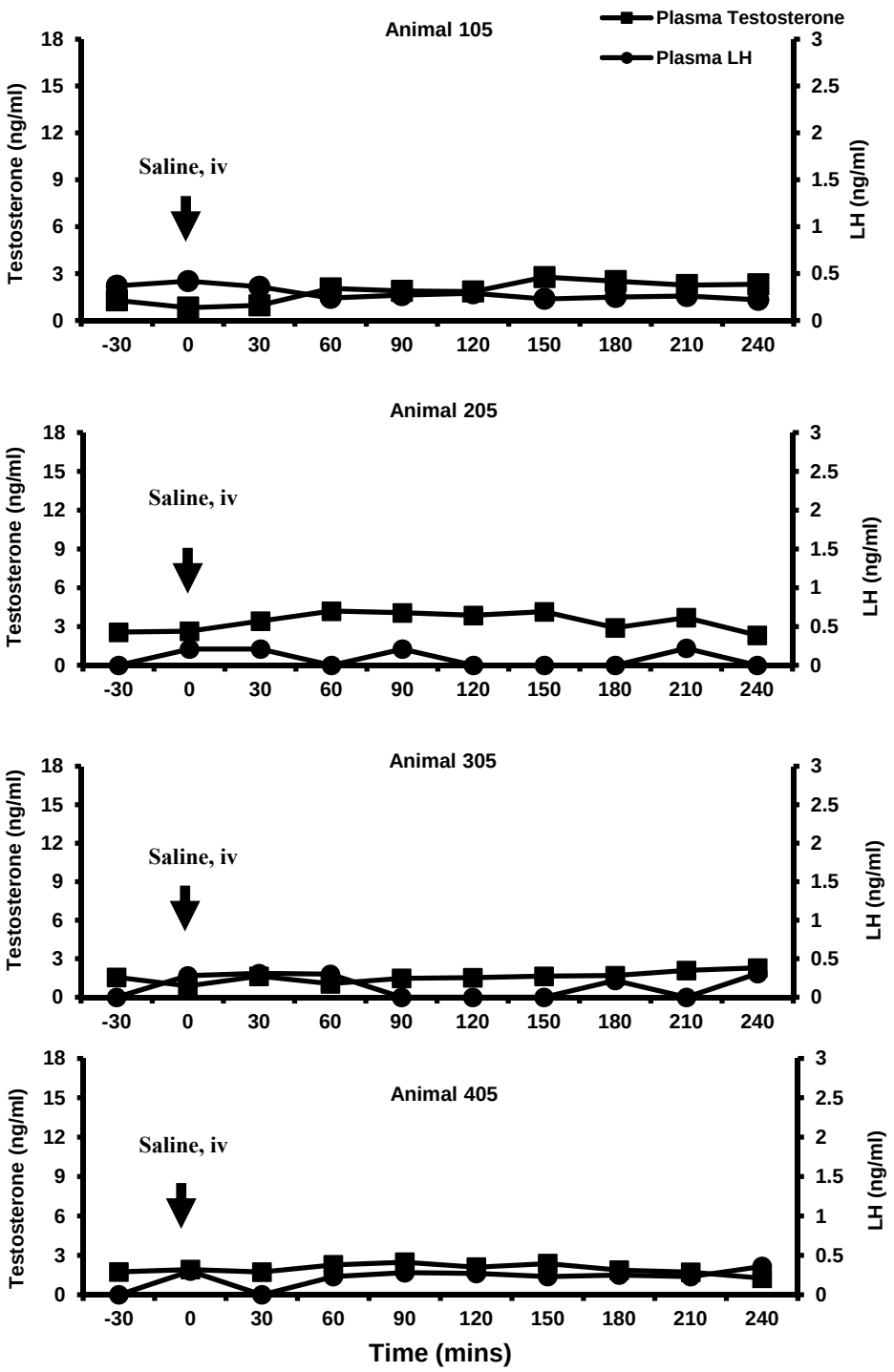


Figure 2.2 Individual plasma LH and testosterone concentration in intact rhesus monkeys before and after iv administration of saline (arrow).

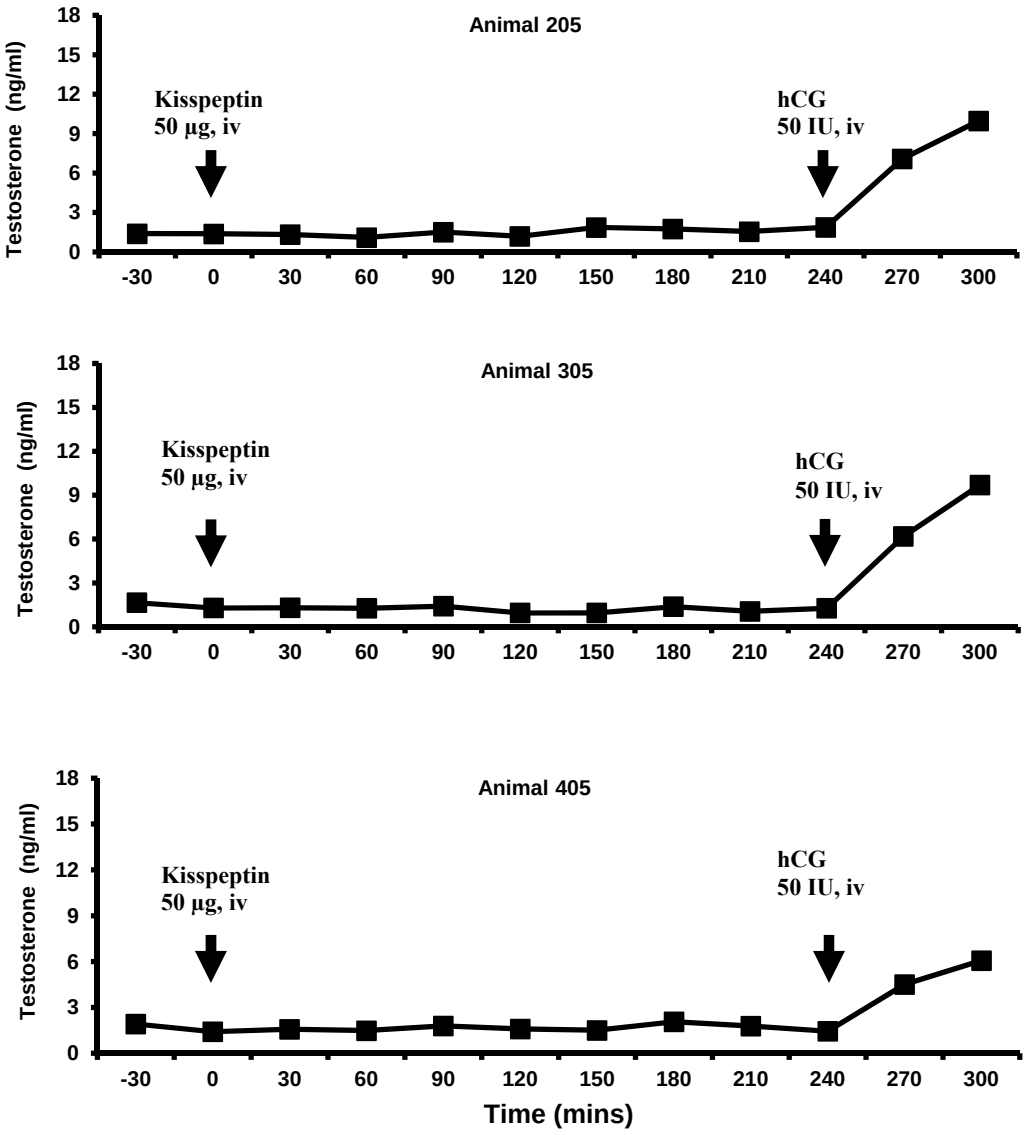


Figure 2.3 Individual plasma testosterone concentration in acyline pretreated adult intact monkeys before and after the iv administration of kisspeptin and hCG (arrows).

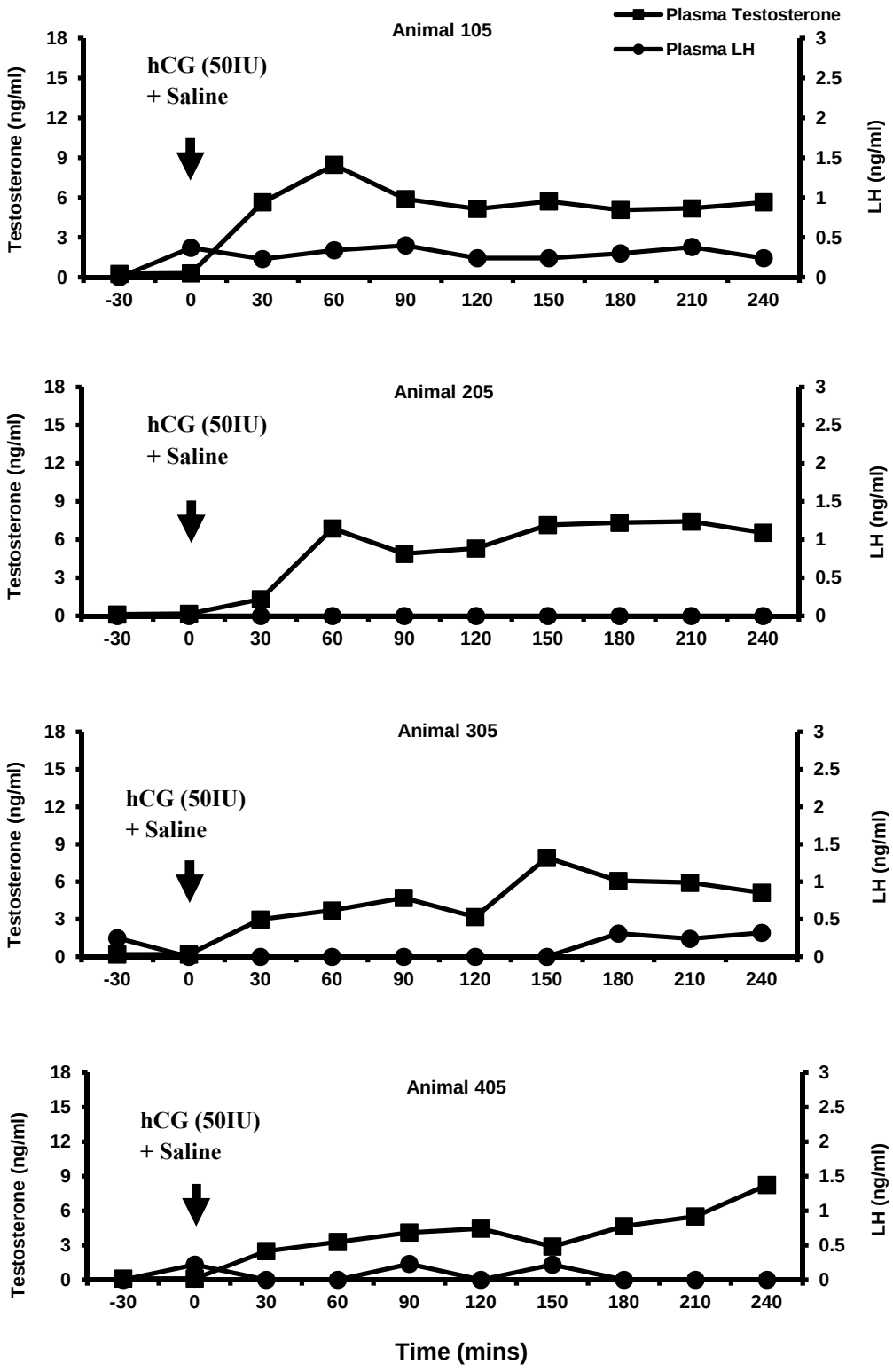


Figure 2.4 Individual plasma LH and testosterone concentration in acyline pretreated adult intact monkeys before and after iv administration of saline+hCG (arrow).

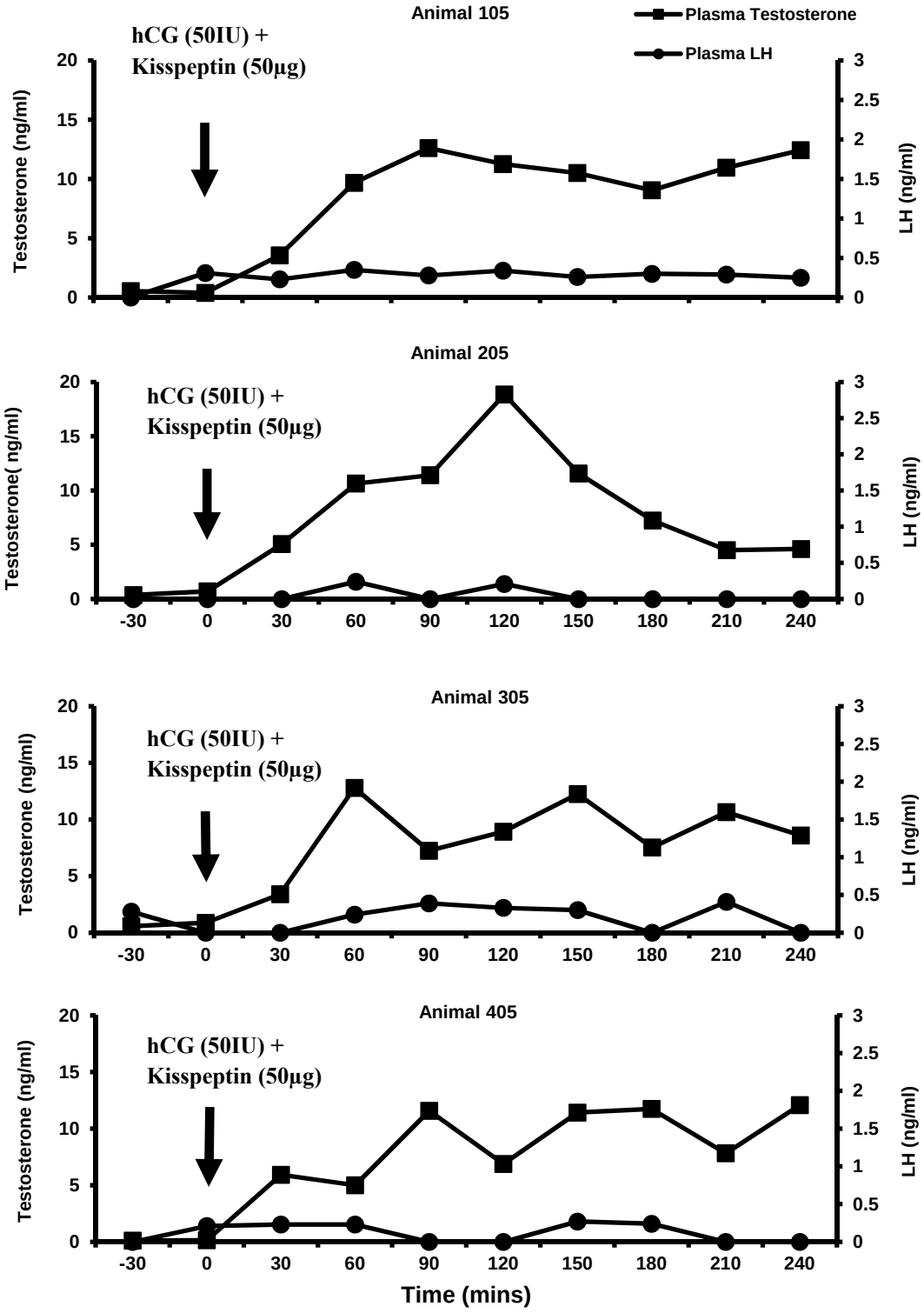


Figure 2.5. Individual plasma LH and testosterone concentration of acyline pretreated intact monkeys before and after iv administration of kisspeptin+hCG (arrow).

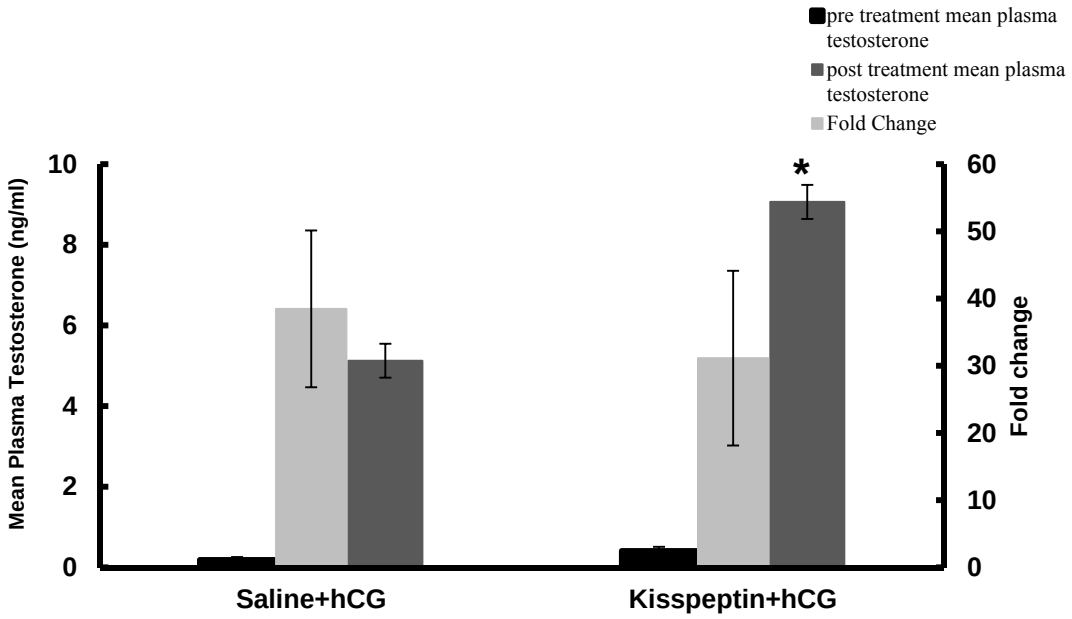


Figure 2.6 Comparison of the mean plasma testosterone values of pre and post kisspeptin+hCG and saline+hCG treatment as well as the fold change observed between pre and post treatment for kisspeptin+hCG and saline+hCG. Mean plasma testosterone concentration were significantly higher (*P<0.005) in post kisspeptin+hCG treatment as compared to post saline+hCG treatment. The fold change in plasma testosterone levels observed in saline+hCG and kisspeptin+hCG was not found to be significant.

confounding effect of acyline on kisspeptin testicular action was less likely in the present study.

Previous kisspeptin studies on primates have either utilized castrated animals (Shahab et al., 2005; Seminara et al., 2006) or the terminal signal for the HPG axis in males i.e., testosterone has not been assessed with regards to a direct testicular action of kisspeptin iv administration in higher primates (Plant et al., 2006). The observation of disproportional relationship between circulating testosterone and LH during continuous iv infusion of kisspeptin, when testosterone levels were constantly high for a given concentration of LH by Ramaswamy et al (2007) indicated about the possible action of kisspeptin within the testis. But the animals used in that study were not chemically hypophysectomised and were given a continuous kisspeptin infusion. The intratesticular action of kisspeptin can only be assessed by using chemical hypophysectomy as continuous kisspeptin infusion down regulates kiss1r (Seminara et al., 2006). We assume that the intratesticular action of kisspeptin can only be assessed by using gonadotropic hypophysectomised model given bolus injections of kisspeptin as performed in the present study. Our finding has implications for humans as the human testes have been shown to express *KISS-1* and *KISS1R* transcripts (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Clinical studies in humans are needed to explore the intratesticular role of kisspeptin during pubertal growth and senescence.

Our results demonstrate that kisspeptin exerts an intratesticular action in primate testes. This intratesticular action is in terms of an increased steroidogenic response towards plasma LH/hCG. Our results also demonstrate that kisspeptin cannot effect the basal androgen production in male rhesus monkey. While the mechanism through which kisspeptin enhance Leydig cell response to LH/hCG needs to be investigated at the cellular level. Immunocytochemical and cell culture approaches will help to further explain the peculiar pathway through which kisspeptin modulates Leydig cell's steroidogenesis in primate testes.

Discussion

Loci of kisspeptin action on levels other than hypothalamus have not been systematically assessed in primates. Our study aimed at assessing actions of kisspeptin at the testicular level in terms of testosterone production in the adult male rhesus monkey, a representative higher primate. In order to investigate the intratesticular action of kisspeptin without the influence of pituitary gonadotropic drive, we used pituitary gonadotropic clamped monkey model with pretreatment of acyline, a GnRH receptor antagonist. As the hypothalamic/pituitary influence on the testis is blocked in the acyline pretreated animals, seasonality does not play a role in our experiments. Despite the blockade of central influences at the hypothalamic pituitary level it was previously shown that seasonality does not affect the ability of the rhesus monkey testis to respond towards LH and the capacity of Leydig cells to secrete testosterone (Higashi et al., 1984; Wickings et al., 1981).

In the Acyline untreated animals a single bolus of kisspeptin resulted in testosterone discharge which is preceded by a single LH pulse. Although baseline pre-treatment levels differed the testicular testosterone response towards kisspeptin bolus was present in all four animals and showed similar amplitude (16ng/ml) with comparable kinetics with the maximum testosterone level reaching one hour after kisspeptin treatment. Regardless of basal testosterone levels our observation of an equivalent and analogous testosterone response in terms of the time and intensity towards a given dose of kisspeptin (50μg) is quite remarkable.

In acyline treated animals the lack of testosterone response towards kisspeptin-10 bolus affirmed the absence of pituitary responsive towards GnRH but also negated our hypothesis that kisspeptin might have a direct endocrine action on testes in terms of testosterone release. That the testicular tissue was responsive is confirmed by hCG bolus which resulted in testosterone release.

The significant amplification of hCG-induced testosterone release by co-treatment with kisspeptin in clamped monkeys indicates a novel indirect action of kisspeptin on testosterone release. The amplified testosterone secretion was evident in all the animals used in this experiment and this response sustained for almost 2 hours. This observation reveals a thus far unknown intratesticular effect of kisspeptin and indicates a functional kisspeptin receptor signalling cascade in the primate testis. The potential mechanism through which kisspeptin enhances stimulated testosterone would likely to involve a direct action on Leydig cell or an indirect action via Sertoli cells or germ cells. Such notions are supported by presence of a certain level of paracrine signaling in the primate testis (Schlatt et al., 1997). It has been shown that testosterone production can be enhanced by secretory factors from the tubular compartment (Johnson and Ewing, 1971). FSH treatment of hypophysectomized rats not only increased testicular LH receptor number but also augmented the steroidogenic response of Leydig cells to LH (Kerr and Sharpe, 1985). Sertoli cells have also been shown in-vitro to secrete factors which increase Leydig cell steroidogenesis and this effect is augmented in the presence of LH or hCG (Papadopoulus et al., 1987; Papadopolus, 1991; Sharpe, 1985). Studies on the testicular localization of kisspeptin receptor are needed in order to specify the site of action of kisspeptin in the primate testis. It is likely that kisspeptin through a direct or indirect action leads to enhancement of sensitivity of LH receptors towards LH/hCG on the Leydig cells.

It is important to mention that GnRH receptors are present in the testis and it has been demonstrated, though in a rodent model, that GnRH has a negative effect on *in vitro* testicular steroidogenesis (Bambino et al., 1980; Bourne et al., 1980; Petersson et al., 1989). This raises the possibility of a direct action of acyline in affecting testicular steroidogenesis in the current study and possibly confounding the testicular action of kisspeptin. However this notion appears slim because testicular action of acyline was expected to actually stimulate testosterone while in the current study no such effect of acyline was observed on basal testosterone levels in acyline pretreated monkeys. Therefore we tend to hold that there were no functional effects of antagonism of testicular GnRH receptors by acyline at least on testosterone secretion. Hence a

Chapter 3

Immunocytochemical localization of kisspeptin and kisspeptin receptor in primate testis

Immunocytochemical localization of kisspeptin and kisspeptin receptor in primate testis

Abstract

Hypothalamic kisspeptin-kisspeptin receptor signaling in primates ensures the successful progression of juvenile phase into puberty during development and maintenance of reproductive activity during adulthood. Human testis has been shown to express high to moderate levels of *KISS1* and *KISS1R* expression. We have recently demonstrated a novel intratesticular role of kisspeptin in enhancing stimulated testosterone in pituitary clamped adult rhesus monkeys. In the present study we aimed to sort out the immunocytochemical localisation of kisspeptin and kisspeptin receptor in adult primate testis tissue. Immunocytochemistry was performed on paraffin embedded testis tissue from adult rhesus monkey (*Macaca mulatta*), a representative higher primate (n=2) and common marmoset (*Callithrix jacchus*) a representative new world monkey (n=2). The immunolocalisation of kisspeptin receptor was found to be present inside the seminiferous tubules and concentrated around the peripheral areas rich in A spermatogonia. The localisation pattern for kisspeptin receptor was consistent in new world and old world monkey testis. The kisspeptin immunocytochemistry revealed an interstitial pattern of localisation where the Leydig cells along with testicular macrophages and peritubular myoid cells were found to be positive for the kisspeptin. These results demonstrate that primate testis is positive for kisspeptin receptor and kisspeptin localisation and imply that kisspeptin-kisspeptin receptor signalling might have an active role in primate testis involving the interplay of tubular and interstitial cells.

Introduction

We have demonstrated a novel intratesticular role of kisspeptin in primate testis in enhancing stimulated testosterone production in pituitary gonadotropin clamped adult male monkeys (Chapter 1). Our *in vivo* results promise the presence of an active kisspeptin-kisspeptin receptor signaling at the testis. The specific localization of kisspeptin and its receptor in the primate testis is vital to understand the precise cellular type through which kisspeptin exerts its possible role in the testicular microenvironment. The testis contains different somatic cell types where each cell performs different functions. The intercellular communications between somatic cells as well as the communications between the germ cells and somatic cells are key features of the normal testicular functionality. The kisspeptin and kisspeptin receptor localisation has been recently reported for rodent and vertebrate testis (Anjum et al., 2012; Chianese et al., 2013; Hua et al., 2013). In primate testis the cellular localisation for kisspeptin and kisspeptin receptor still need to be sorted out. The precise testicular cellular localisation would help further to delineate the role of kisspeptin-kisspeptin receptor signalling in primate testis. In the present study we aimed to carry out the immunohiostochemical localisation of kisspeptin and kisspeptin receptor in adult primate testis. In order to be concise e used testis tissue from Old World monkey (*Macaca mulatta*) and New World monkey (*Callithrix jacchus*) to assess the localisation pattern of kisspeptin and kisspeptin receptor across the primate testis.

Previously the presence of GnRH, a hypothalamic neuropeptide has been reported in the testis. GnRH peptide had been isolated from the interstitial fluid and the functional GnRH receptors have been found to be present on the Sertoli cells and functionally GnRH affect the testicular steroidogenesis in a negative manner (Bambino et al., 1980; Bourne et al., 1980; Petersson et al., 1989).

Materials and Methods

Animals

Two adult male rhesus monkeys (*Macaca mulatta*) were employed for testicular immunohistochemical analysis. These animals were obtained from the University of Pittsburgh Primate Facility's breeding colony and aged 6-6.5 years and weigh around 10 kg at the time of castration. Testicular tissues were obtained by castration via an inguinal incision. Surgeries were performed at the operation room of the facility. Anaesthesia was induced with Ketaject (Ketamine Hcl Inj., Usp; Phoenix Pharmaceuticals, MO, USA) via intramuscular injection and maintained by isoflurane inhalation. Animal husbandry and all experimental procedures involving the animals were performed in compliance with the University of Pittsburgh Guidelines for the Care and Use of Laboratory Animals and after IACUC approval were obtained.

Two adult male common marmoset (*Callithrix Jacchus*) were also employed for testicular immunohistochemical analysis. The animals were obtained from University Hospital Munster (UKM) animal facility and were aged 3-4 years and weigh 400-500 grams at the time of sacrifice. Animal husbandry and all experimental procedure were performed in compliance with the University of Munster and UniKilinikum Munster (UKM) guidelines.

Testicular biopsy and tissue fixation

Tissue samples from each testis were fixed overnight at room temperature in Bouin's fixative; tissues were then washed and stored in 70% ethanol. Subsequently, tissues were embedded in paraffin, and were sectioned to 3μm thick section employing a Leica SM 2000R microtome. A total of 12 sections were taken from adult rhesus and marmoset monkey testis.

Antibodies and chemicals

Primary antibody for kisspeptin (GQ2) was kindly provided by Dr. Stephan R Bloom. The primary antibody for KISS1R/GPR54 (Anti AXOR 12) was purchased from Phoenix Pharmaceuticals, Belmont, CA. USA. Chicken anti rabbit horse reddish peroxidase conjugated (Cat-Nr. sc 2955, Santa Cruz Biotechnologies, Heidelberg, Germany) was used as secondary antibody for kiss1r immunocytochemistry. Donkey anti sheep biotin was used as secondary and Streptavidin-HRP (horse reddish peroxidase) was used as tertiary antibody for kisspeptin immunocytochemistry. DAB (Diaminobenzidine, Cat.-Nr. D-4168) tablets were purchased from Sigma-Aldrich, Munich, Germany.

Dilutions and concentrations

KISS1R/GPR54 primary antibody (Anti AXOR 12) and secondary antibody (chicken-anti-rabbit-HRP-conj. Sc-2955) both were used at a dilution of 1:100. The antibodies were diluted in 25% chicken serum in 0.5% BSA in TBS. For kisspeptin the primary antibody (GQ2) was used at a dilution of 1:120,000 as previously described (Ramaswamy et al., 2008) and the secondary and tertiary antibodies were used at a dilution of 1:100 and 1:500 respectively. The primary, secondary and tertiary antibodies for kisspeptin were diluted in 25% donkey serum in 0.5% BSA in TBS.

Controls

Human placental tissue was used as a positive control and secondary antibody omission was used as negative controls for kisspeptin and kiss1r. Human placental tissue was kindly provided by the Gynaecology Clinic of the University Hospital Muenster, Germany. Placental tissues were fixed in sera fixative, embedded in paraffin and were sectioned to 3µm thick section employing a Leica SM 2000R microtome.

Immunocytochemistry for kisspeptin receptor (kiss1r)

A total of 6 sections were used from each testis from adult rhesus and marmoset monkeys were used for kisspeptin receptor immunocytochemistry. The tissue sections were deparaffinated in paraclear, rehydrated in an ethanol row, washed in running tap water and rinsed with distilled water. Antigen retrieval was performed using citrate buffer (pH 6.0) and heating in a microwave to approx. 80°C for 2 minutes. Tissue sections were washed in tris buffered saline (TBS), incubated in 3 % H_2O_2, washed in distilled water then again washed in TBS. Blocking of unspecific binding sites was performed using 25 % chicken serum and 0.5 % bovine serum albumin (BSA) in TBS for 30 mins. After blocking the sections were incubated with primary antibody Anti AXOR 12 (anti-GPR54; Cat.-Nr. 375-398, Phoenix Pharmaceuticals, Belmont, CA. USA, dilution 1:100) in humid chamber at 4°C over night. Next morning sections were washed in TBS, incubated with secondary antibody (chicken-anti-rabbit Horse raddish-peroxidase conjugated, Cat.-Nr. sc 2955, Santa Cruz Biotechnologies, Heidelberg, Germany; diluted at 1:100) for 1 hour in wet chamber. Sections were then washed with TBS and then stained with DAB was performed as specified by the manufacturer. Sections were then counterstained using Mayer's hemalaun. After counterstaining sections were dehydrated and mounted using permanent mounting medium.

Immunocytochemistry for kisspeptin

A total of 6 sections from each testis from adult rhesus and marmoset were used for kisspeptin immunocytochemistry. Tissue sections were treated the same way as mention in the previous sections. The blocking was performed using 25% donkey serum in 0.5% BSA in TBS for 30 mins. Sections were then incubated with primary antibody (GQ2, dilution 1:120,000) in humid chamber at 4°C overnight. Next morning sections were washed in TBS and then incubated with secondary antibody (Donkey- anti-sheep biotin, dilution 1:100) for 1 hour in wet chamber. Section were again washed with TBS and incubated with tertiary antibody (Streptavidin-HRP conj, dilution 1:500) for 30 mins. Section were then washed with TBS, stained with DAB as per manufacturer's

instructions, counterstained using Mayer's hemalaun, dehydrated and mounted using permanent mounting medium.

Microscopy

Slides were analyzed using an Olympus BX61 microscope (Melville, NY, USA) with an attached Retiga 4000R camera (QImaging, Surrey, BC, Canada). All images were acquired digitally using QCapture imaging software (QImaging, Surrey, BC, Canada).

Results

Testicular immunocytochemistry for kisspeptin receptor

Immunohistochemical localisation for kisspeptin receptor showed a positive staining in the all the primate testis tissue used (Fig 3.1 & 3.3). Slides revealed a strong signal at the outer periphery of the seminiferous tubules in rhesus and marmoset testis (Fig 3.1 & 3.3). Human placenta was used as positive control (Fig 3.2). Positive control along with the negative controls clearly demonstrates the specificity of the antibody (Fig 3.2). Detailed examination revealed a peculiar localization pattern which is quite remarkable. The interstitial tissue was devoid of any localisation in all the testicular sections employed. The specific peripheral pattern of localisation in seminiferous tubules is consistent in all the testicular samples used. Cytological analysis reveals that the kiss1r positive signal is present both on the Sertoli cell membrane and concentrated around A spermatogonia in the seminiferous tubules. The observation of a peripheral localisation pattern found in the seminiferous tubules of rhesus and marmoset testis tissue is a novel finding of the study.

In conclusion the immunocytochemical localisation of kisspeptin receptor in the adult primate testis is found to be in the seminiferous tubules. The immunoreactivity was evident only in the seminiferous tubules and no immunolocalization was found in the interstitial compartment. The results are consistent across human and monkey tissue samples. The peripheral area of the seminiferous tubules was positive for the kisspeptin receptor and this staining pattern is also similar in all the tissue samples used. Although Sertoli cell membrane harbouring undifferentiated spermatogonia exhibited strong staining as compared with those areas of Sertoli cell membrane which are devoid of any undifferentiated spermatogonia.

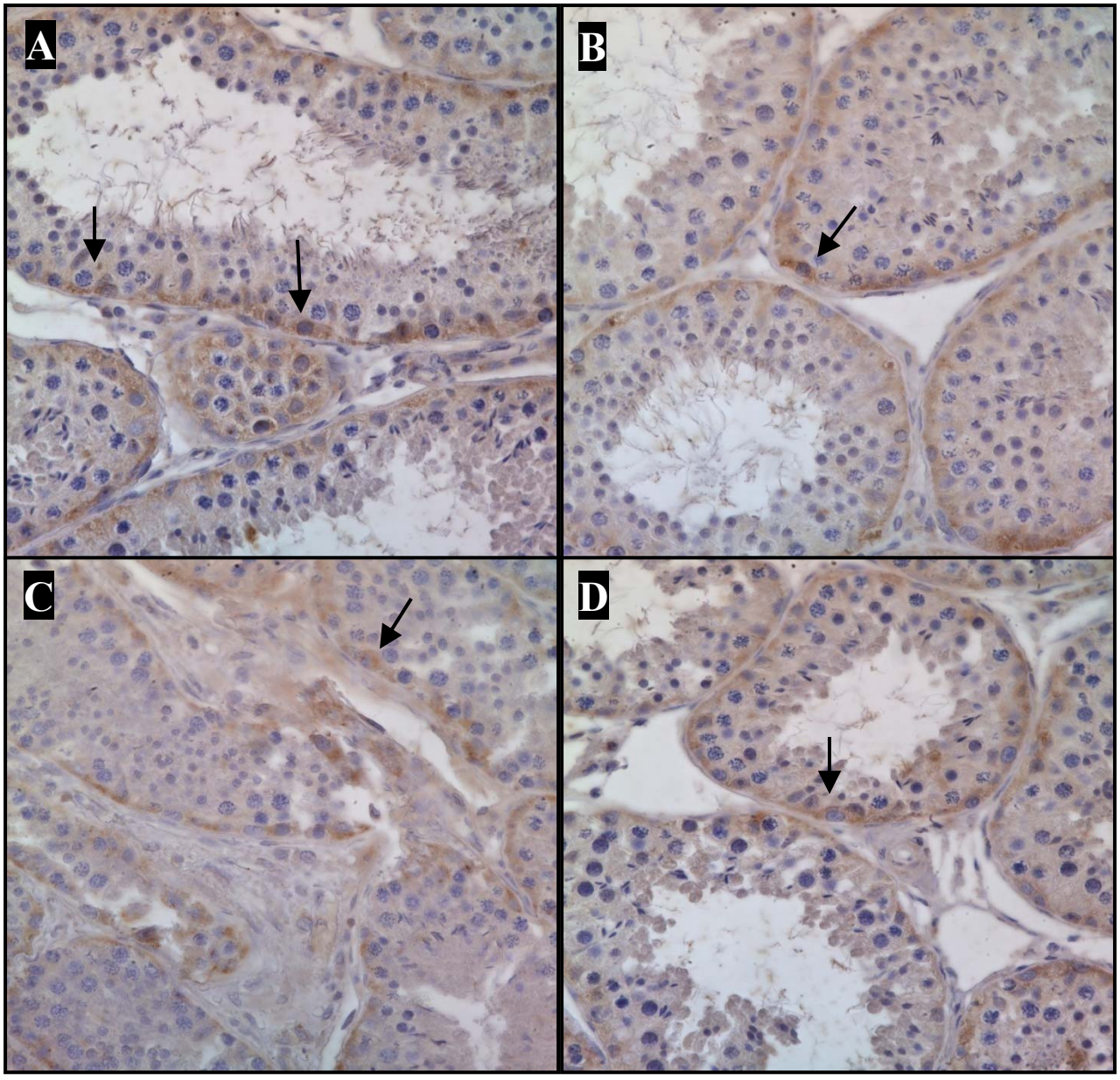


Figure 3.1 Immunocytochemistry for kisspeptin receptor (Kiss1r) in adult rhesus monkey testis sections (40x) (A,B,C,D). Positive kiss1r-like immunoreactivity at the periphery of the seminiferous tubule (arrows).

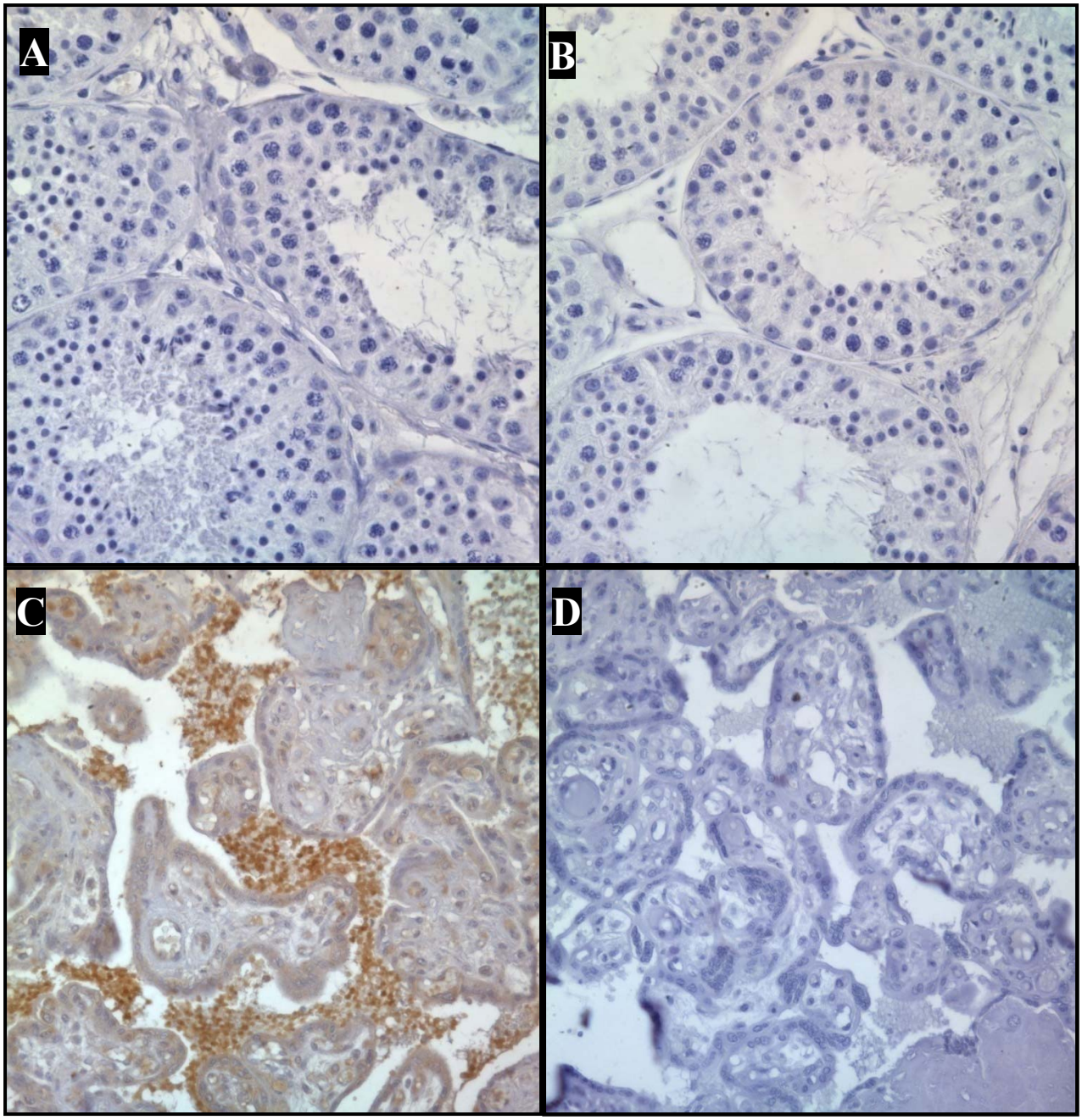


Figure 3.2 Controls for kisspeptin receptor immunocytochemistry. A) Primary antibody omitted negative control, adult rhesus testis (40x). B) Isotype control, adult rhesus testis (40x). C) Positive control, human placenta (40x). D) Primary antibody omitted control, human placenta (40x).

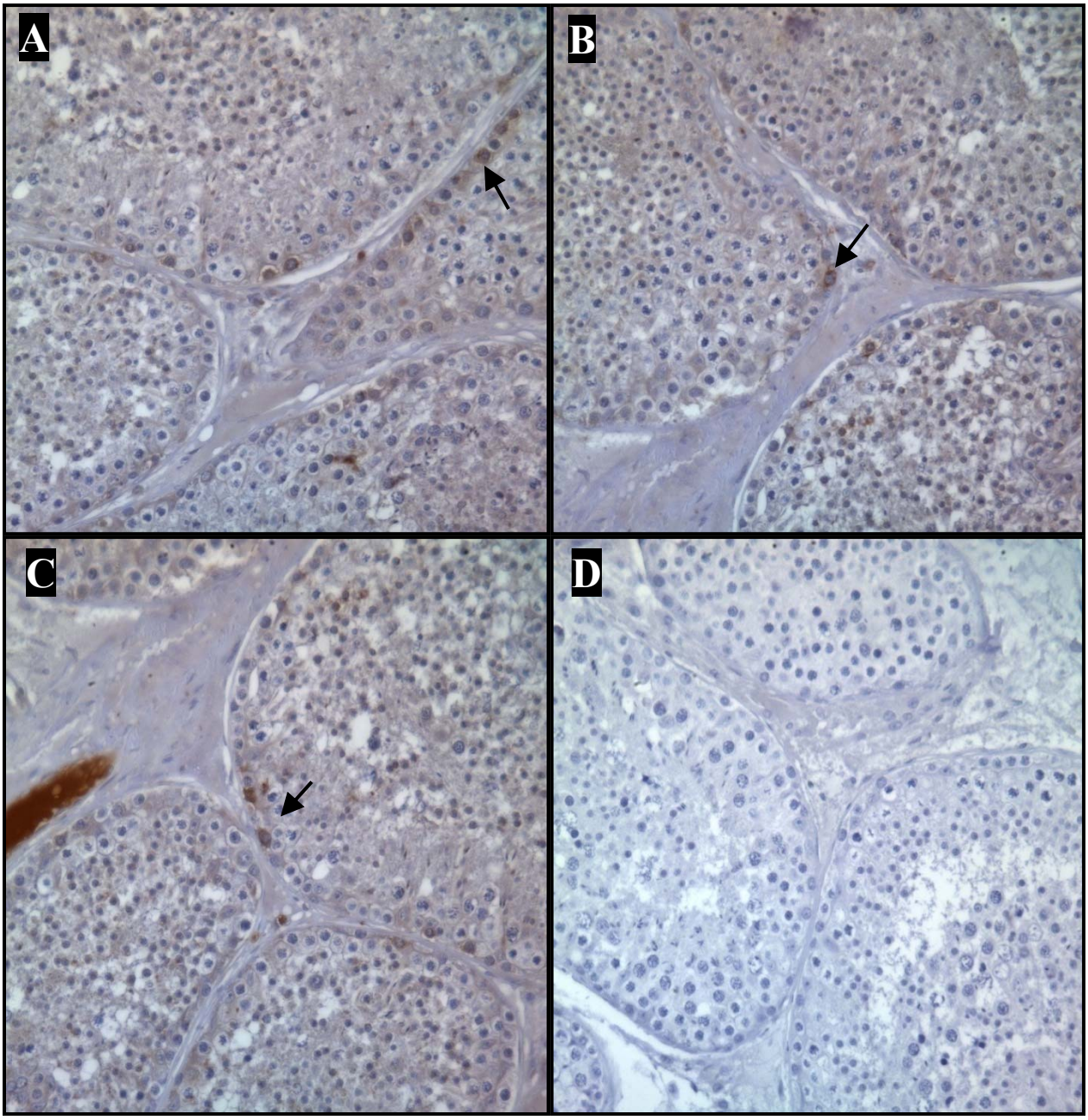


Figure 3.3 Immunocytochemistry for kisspeptin receptor (Kiss1R) in adult common marmoset testis (A, B and C) (40X). Primary antibody omitted negative control (D) (40X). Note the peripheral localisation of the kiss1r-like immunoreactivity at the periphery of the seminiferous tubules (Arrows).

Testicular immunocytochemistry for kisspeptin

The interstitial area was found positive for kisspeptin immunolocalisation in adult male rhesus monkey testis. The different cell types in interstitial area were found positive for the kisspeptin. Based on the location and the shape of these cells, we concluded that the most persistent and strong localisation was shown by the peritubular myoid cell along with the underlying layers of the basal lamina which forms the basement membrane around the seminiferous tubules (Fig. 3.4, Thin arrows). The other interstitial cells types were also seem to show positive kisspeptin-like immunoreactivity (Fig 3.4, thick arrows). However the precise identification of the cell type of these positive cells needs further labelling. Surprisingly the tubular area failed to show any immunoreactivity for kisspeptin in adult male rhesus testis.

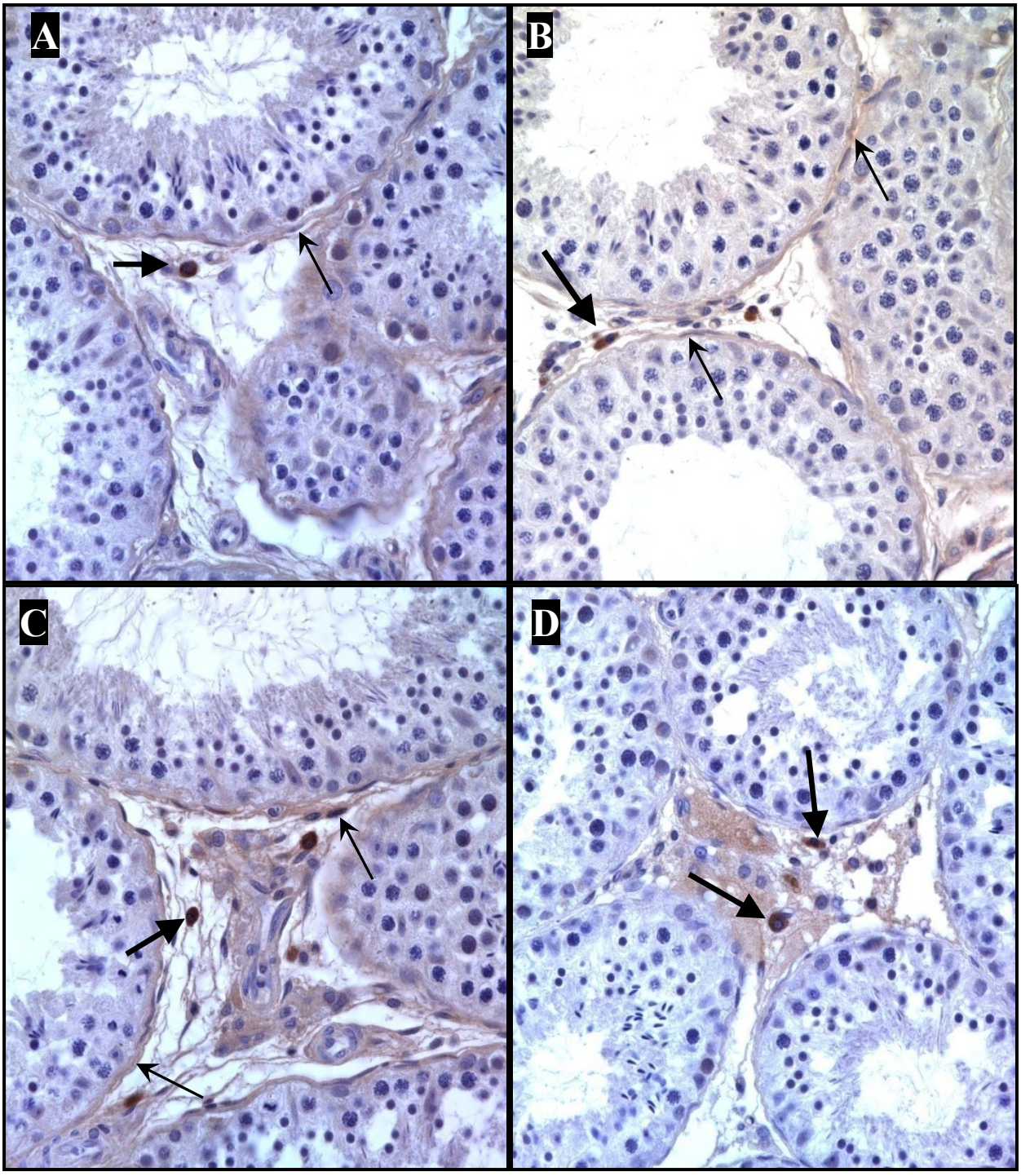


Figure 3.4 Immunocytochemistry for kisspeptin in adult rhesus testis A, B, C, D. (A, B, D 40x) (C, 60x). Peritubular myoid cells and basement membrane (Thin arrow), Interstitial cells (thick arrows).

Discussion

Previously a novel in vivo intratesticular action of kisspeptin in enhancing Leydig cell steroidogenesis in the presence of LH/hCG in the pituitary gonadotropin clamped adult monkey model has been demonstrated (Chapter 2). In the current study we aimed to sort out the cellular localisation of kisspeptin and kisspeptin receptor in the adult primate testis. Immunocytochemistry as a tool was employed to identify the peculiar testicular cell type positive for kisspeptin and its receptor. The Sertoli cell localisation for kisspeptin receptor (kiss1r) was the novel finding of this study. The same pattern of localisation for kiss1r was observed in adult rhesus and marmoset testis. According to our *in vivo* data (Chapter 2), the finding of Sertoli cell localisation for kisspeptin receptor makes sense at least from an androgenic perspective. The Sertoli cell is known to secrete factors which enhance Leydig cell steroidogenesis in the presence of LH (Saez et al., 1989). It seems that kisspeptin indirectly enhances Leydig steroidogenesis via Sertoli cells instead of a direct endocrine action at the Leydig cell membrane.

On the other hand the kisspeptin positive areas were found to be present in the interstitial region of the adult rhesus monkey testis. Surprisingly all the interstitial cell types were found positive. The cells which were found positive for kisspeptin like immunoreactivity included Leydig cells, testicular macrophages and peritubular myoid cells along with layers of basement membrane. The finding of non tubular localisation for kisspeptin in the current study is not surprising as it has been shown recently that kisspeptin like immunoreactivity is present in the interstitial compartment in mouse and frog testis where Leydig cells are found positive for kisspeptin like immunoreactivity (Anjum et al., 2012; Hua et al., 2013; Chianese et al., 2013). Although human sperm were shown positive for the kisspeptin like immuoreactivity (Pinto et al., 2012), but in our experiment we failed to observe any tubular areas positive for kisspeptin like immunoreactivity.

It seems that the interstitial compartment of the primate testis is responsible for the kisspeptin secretion which in turn acts on the Sertoli cells and induces the release of factors. These factors acts back on the Leydig cells and enhance the responsiveness of the LH receptor present on the Leydig cell membrane towards the LH. This idea is supported by previous studies where Sertoli cell produced factors that influenced testosterone secretion from the Leydig cell or affect Leydig cell response towards gonadotropins (Lejuene et al., 1992).

Apart from the androgenic perspective, another possible functional role of the kisspeptin-kisspeptin receptor signaling in the primate testis could be its involvement in defining the spermatogonial stem cell niche environment. As the peripheral area of the seminiferous tubules which was found positive for kisspeptin receptor like immunoreactivity in adult primate testis, harbours A spermatogonia which are also known as the undifferentiated spermatogonia or spermatogonial stem cells (Ehmcke and Schlatt, 2006; Ehmcke et al., 2006). This same site is involved in aiding the mitotic multiplication of the Ap spermatogonia to become B spermatogonia as well as the self renewal activity of A spermatogonia. This anatomical area had been suggested as the spermatogonial stem cell niche region because of its fundamental involvement in the initiation of spermatogenesis and spermatogonial stem cell renewal activity (de Rooij, 2009). The niche region is defined as the area which provides all the required factors needed by the spermatogonial stem cells for their mitotic multiplication and self renewal activity. The specialised niche region in testis is anatomically composed of Sertoli cell membrane encircling the A spermatogonia. It seems that kisspeptin through its receptor might be involved in playing a role in this niche region. Parenthetically, kisspeptin was initially found to be involved in the metastatic cancer as an anti metastatic peptide (also known initially as metastin) where the primary action of kisspeptin was suggested to either block or stop metastasis (Lee et al., 1996; Lee and Welch, 1997; Murphy, 2004). It has been shown that the antimetastatic effects are achieved by kisspeptin in metastatic cancer by inhibiting chemotaxis and cell migration (Hori et al., 2001; Yan et al., 2001; Harms et al., 2003; Navenot et al., 2009).

During the postnatal testicular development, the germ cells migrate from the centre of the seminiferous tubules to the peripheral area. During their migration these cells are called gonocytes and once they establish a physical contact with the basement membrane, these cells are known as A spermatogonia or spermatogonial stem cells (Culty, 2009). A spermatogonia do not show any migratory property in the primate testis. The peculiar localisation of kisspeptin and kisspeptin receptor in adult primate testis leads us to propose that kisspeptin via kisspeptin receptor signaling might be involved in hindering the migratory property of A spermatogonia in the adult testis. The migratory male germ cells known as gonocytes in postnatal primate testis must reach their niche regions in order to exhibit self renewal and mitotic capacity (Culty, 2009). The kisspeptin signaling by restraining the germ cells inside the niche regions might assist in establishing the self renewal ability and aid in establishing the spermatogenic capacity of the primate testis. Further studies employing *in vitro* tissue cultures on juvenile, pubertal and adult testis tissue is required to sort out the physiological significance of the presence of kisspeptin signalling in the primate spermatogenic dynamics.

Nonetheless the present study highlights a novel testicular site of localization of both kisspeptin and its receptor in higher primates. Present findings also suggest a role for kisspeptin-kisspeptin receptor signalling in the primate testis relevant to testicular endocrine action or possibly spermatogenesis, which might be supplementary to already well established central kisspeptin signalling relevant to control of the reproductive axis.

Chapter 4

Pubertal and testicular development in common marmoset (*Callithrix jacchus*): a New World monkey model

Pubertal and testicular development in common marmoset (*Callithrix jacchus*): a New World monkey model

Abstract

The common marmoset (*Callithrix jacchus*) became an attractive non-human primate model due to its small size and long life span in captivity. Adult marmoset share high testicular organizational similarity with the human testis but the details of pubertal testicular development in this monkey has been poorly explored. Aims of the present study were to describe onset and kinetics of pubertal testis growth and function in common marmosets. Immature common marmosets (n=48) were observed for 13 months either starting from birth or at an age of less than 12 months. Monthly changes in body weight and testicular volume were recorded. Testosterone was assayed by radioimmunoassay in serum samples. At the end of the study the testis tissue was collected, fixed and embedded in paraffin. Histological and morphometric data were determined. In the first six months a rapid rise in body weight but not in testis volume was observed. At this point some animals entered puberty (rise in testis volume (TV), increase in testosterone) reaching adult testis size (> 200mm³) already at 10 month of age. Others showed delayed entry into puberty until 12 or 13 months. While testosterone levels corresponded with early or late onset of puberty, body weight did not. At 15-18 months of age, body weights were similar in all monkeys but testis size and testosterone levels were highly variable. Histological analysis reveals that the onset of puberty is accompanied by an increase in the tubule diameter and the formation of a tubular lumen. Increase in the diameter and length of seminiferous tubules leads to a continuous increase in tubule weight. Motile sperm were first observed in the epididymis at 12 months of age. Marmoset puberty begins earliest at 6 month of age. In contrast to a uniform increase of

body weight, pubertal onset is highly variable between individual animals. Onset of testicular growth and steroidogenesis are synchronized.

Our data indicate that the hypothalamus in marmosets controls growth and gonadal function independently. Whether body weight gain is a prerequisite for pubertal activation is unclear. The high variability of pubertal development appears to depend on the timing of the GnRH-pulse generator as morphological and functional parameters develop normally once initiated. Preliminary observations are indicative for a control of the hpg-axis during puberty via social factors. This observation needs to be substantiated in future studies.

Introduction

Common marmoset (*Callithrix jacchus*) a New World primate, has become an attractive laboratory animal in biomedical research and toxicology owing to its small size, high breeding rate and long life span in captivity (Abbott et al., 2003; Mansfield, 2003; Zühlke and Weinbauer, 2003: 't Hart et al., 2012). Common marmoset has been proposed as animal model in research areas which include aging, arthritis, multiple sclerosis, neuroscience and Parkinson's disease (Eslamboli, 2005; Austad and Fischer, 2011; Petersen and Yu, 2011; Carrion and Patterson, 2012). The benefit of a non human primate model over the rodent model is very important for medical research as the basic physiology of postnatal reproductive development is different between rodents and primates (Plant, 2012).

Apart from being a model animal for medical research an important physiological aspect of adult common marmoset is the resemblance of its testicular tubular organization towards human testis. It has been shown that adult marmoset testis show highly similar seminiferous tubule organization with human (Millar et al., 2000; Wistuba et al., 2003) as well as the fetal and neonatal germ cell development in common marmoset mimic human like pattern (Mitchell at al., 2008). These findings highlight common marmoset as an attractive non human primate for the study of testicular morphogenesis and fertility research (Gassei and Schlatt, 2007).

The process of primate puberty and testicular development on the other hand has been characterized on the basis of data generated in higher primates (Plant et al., 2005; Plant, 2006, 2008; Plant and Witchel, 2006). In comparison with Old World monkeys (2.5-3 years), the New World monkeys need a lot less post natal time to reach sexual maturation (12 months) (Plant and Witchel, 2006). Apart from a significant difference in the time period required for the sexual maturation the question that how similar is the process of puberty in terms of hormonal and gonadal parameters between Old World monkeys and New World monkeys has never been addressed.

On the contrary, the limited data available on the pubertal and testicular development in common marmoset have been controversially argued thus fabricating a perplexed picture in terms of the hallmarks of the pubertal testis development (Li et al., 2005). However recent findings in common marmoset points towards a unique reproductive endocrinology which involve the presence of a chorionic gonadotropin (CG) like molecule instead of LH in HPG axis interplay. This CG like molecule has a much shorter serum half life as compared with LH (Muller at al., 2004). The reason for the dissension regarding pubertal testicular ontogeny mainly arise due to the fact that nearly all of the reports regarding pubertal and testicular development in common marmoset were conducted well before the findings of the presence of CG like molecule instead of LH, except for Chandolia (2006). The unique reproductive endocrinology in the common marmoset is also present in terms of steroid hormone resistance where the unbound plasma testosterone in new world primates were found to be around 20-40 ng/dl as compared to 3-9 ng/dl in old world monkey and 10 ng/dl in humans (Puggeat et al., 1984). Adult common marmosets have also been shown to exhibit a strong social regulation of fertility both in male and females and to date it is unclear whether this phenomenon also impacts the timing of pubertal onset in common marmoset (Abbot et al., 1999; Baker et al., 1999).

The general notion to date is that in common marmoset the pubertal activation of HPG axis occurs between 6-12 months of age (Li et al., 2005). This high variation of time is critical for selecting animals for developmental and testicular research and might affect the outcome of these studies. While keeping the above mentioned background, we aimed to investigate the pubertal and testicular development in common marmoset by using a simplistic approach of observing immature common marmoset monthly for body weight, testis volume and blood samples for a period of 13 months. At the end of the study animals were sacrificed and testis tissue was collected, fixed and embedded in paraffin for histological and morphometric analysis.

Materials and Methods

Animals and sampling

Immature common marmosets (n=48) were observed for 13 months either starting from birth or at an age of less than 12 months. Animals were housed in the central animal facility of the University Hospital Muenster (UKM). Animals were either housed in their respective family cages or were housed in the cages where all animals were males and were of the same age approximately (± 1 month). Animals were fed fresh fruits in the morning and feed ration in the evening. Water was *ad libitum*.

Body weight and testicular volume samples were recorded monthly. Also the blood samples were collected from the femoral vein of each animal during the collection of body weight and testicular volume. All the samples were collected between 0800 and 1030. The serum was separated from the collected blood samples and stored at -20 C till assayed. For the testis volume the testis length and width was measured using vernier caliper and the testicular volume was calculated using an established ellipsoid formula.

Hormone assay

Testosterone (T) was assayed in serum samples using an in-house (CeRA, Muenster, Germany) developed RIA method. Serum testosterone was measured by a solid-phase, double-antibody RIA technique, using a commercially available iodinated tracer (testosterone-3-(0-carboxymethyl) oximino-2-[12T] iodohistamine, (Amersham International, Braunschweig, Germany) and an antiserum raised in rabbit against testosterone-3(carboxymethyloxime)-BSA. The bound/free separation was performed by addition of a solution of solid-phase antirabbit immunoglobulins (Immunobead Second Antibody, Biorad, Munich, Germany). The recovery after ether extraction was monitored by addition of trace amounts of [lß,2ß- 3H]testosterone (NET-187, NEN, Boston, MA) and the final results were corrected accordingly. The sensitivity was 2 pg/tube (0.07

nmol/l). In 10 consecutive assays the intra-assay coefficients of variation (mean ±SEM) were 8.43+1.42, 4.2+0.59 and 4.37+0.63% for control sera with low, middle and high testosterone concentrations, respectively. The corresponding inter-assay coefficients of variations were 16.62, 6.26 and 3.85%, respectively.

Tissue collection, fixation and histology

At the end of the study each animal was anesthetized with ketamine and body weight and testicular volume are measured, subsequently the animals were sacrificed by exsanguinations. The testis tissues were removed and weights of testes were recorded immediately. Testis tissues were then placed in Bouin's solution overnight. The next day the testis tissues were dehydrated in ascending grades of alcohol (70%, 80%, 90%, 100% for 1 hour each) and then two xylol washes each for one hour in histomaster. The tissues were then embedded in liquid paraffin and allowed to cool. 2.5-3 µm thick sections were cut. Total of six sections were used from the left testis of each animal. Periodic Acid Schiff staining was conducted.

Microscopy and histo-morphometry

Slides were analyzed using an Olympus BX61 microscope (Melville, NY, USA) with an attached Retiga 4000R camera (QImaging, Surrey, BC, Canada). All images were acquired digitally using QCapture imaging software (QImaging, Surrey, BC, Canada). Pictures were taken from five independent positions from each section at 20X (5 pictures/section) using the camera fitted on the microscope. A total of 30 pictures from each testis were used for point counting and morphometric measurements.

Volume densities, expressed as the percent of testicular parenchyma were determined by point counting. Randomness and sufficient sampling were ensured by the use of 6 sections from each testis. A total of 120 points were scored for each testis. Volume density of various components multiplied by testis weight yielded the total weight of that component per testis. For tubule diameter 60 measurements were taken from each testis (10 measurements from each section). Round tubules were selected for

measurements. For point counting square shaped grid was used and four corners of grid were observed in terms of their location on interstitium, tubule epithelium and tubule lumen.

Statistics

All data were expressed as mean ±SEM unless stated otherwise.

Results

Mean body weight, mean bi-testis volume and mean plasma testosterone

The mean body weight in immature common marmoset showed accelerated growth rise during the first seven months of age which was followed by a passive rise (Fig. 4.1). The mean testis volume shows an abated increase till the first six months of age, which is then followed by relatively sharp increase up till 16 months of age (Fig 4.2). The testis volume of the individual animal shows a period of quiescent developmental up till 24 weeks of age (Fig 4.3). The increase in testis volume was first observed around 28 weeks (7 months) which then followed a highly diverse individual pattern where some animals reach adult size around 44 weeks (9-10 months) while others show a slow rise in the testis volume. Mean serum testosterone levels of male common marmoset from birth till 18 months showed a sudden rise in serum testosterone at 7 month of age (Fig 4.4). The sudden rise in serum testosterone at 7 month of age is the first sign of the pubertal activation of hypothalamic pituitary gonadal axis and it also correlates with the rise in the testicular volume.

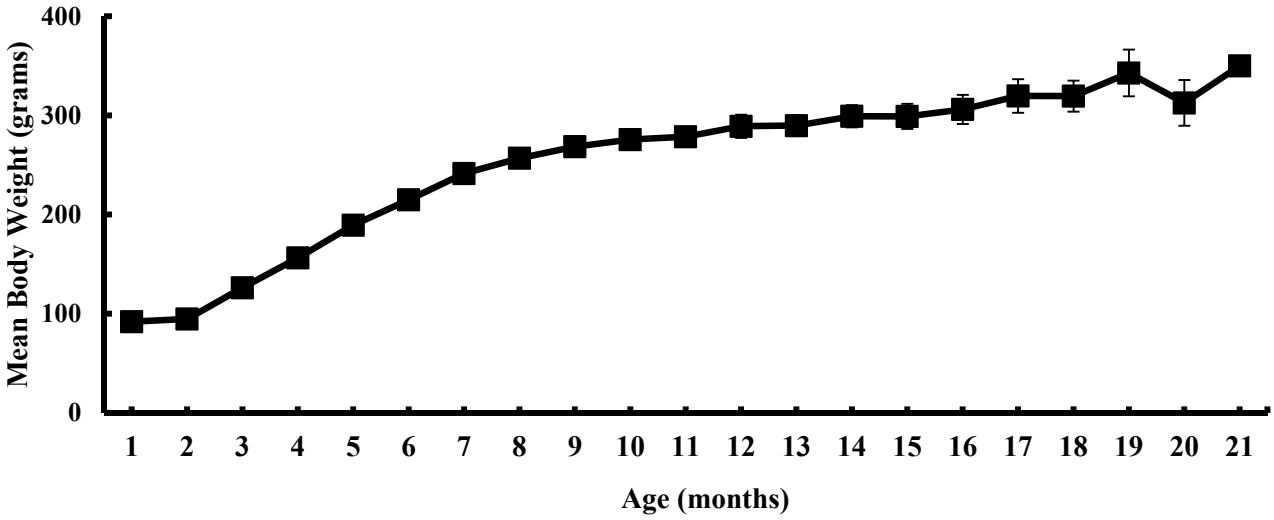


Figure 4.1 Mean body weight in common marmoset from birth till 21 months of age (n=48).

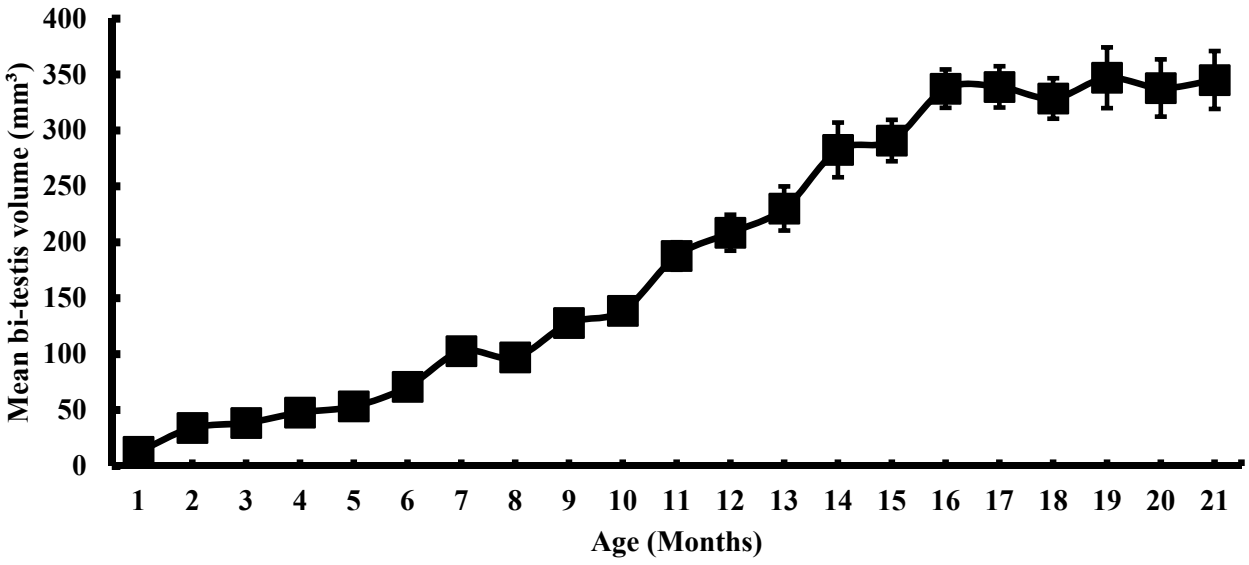


Figure 4.2 Mean bi-testis volume in common marmoset from birth till 21 months of age (n=48).

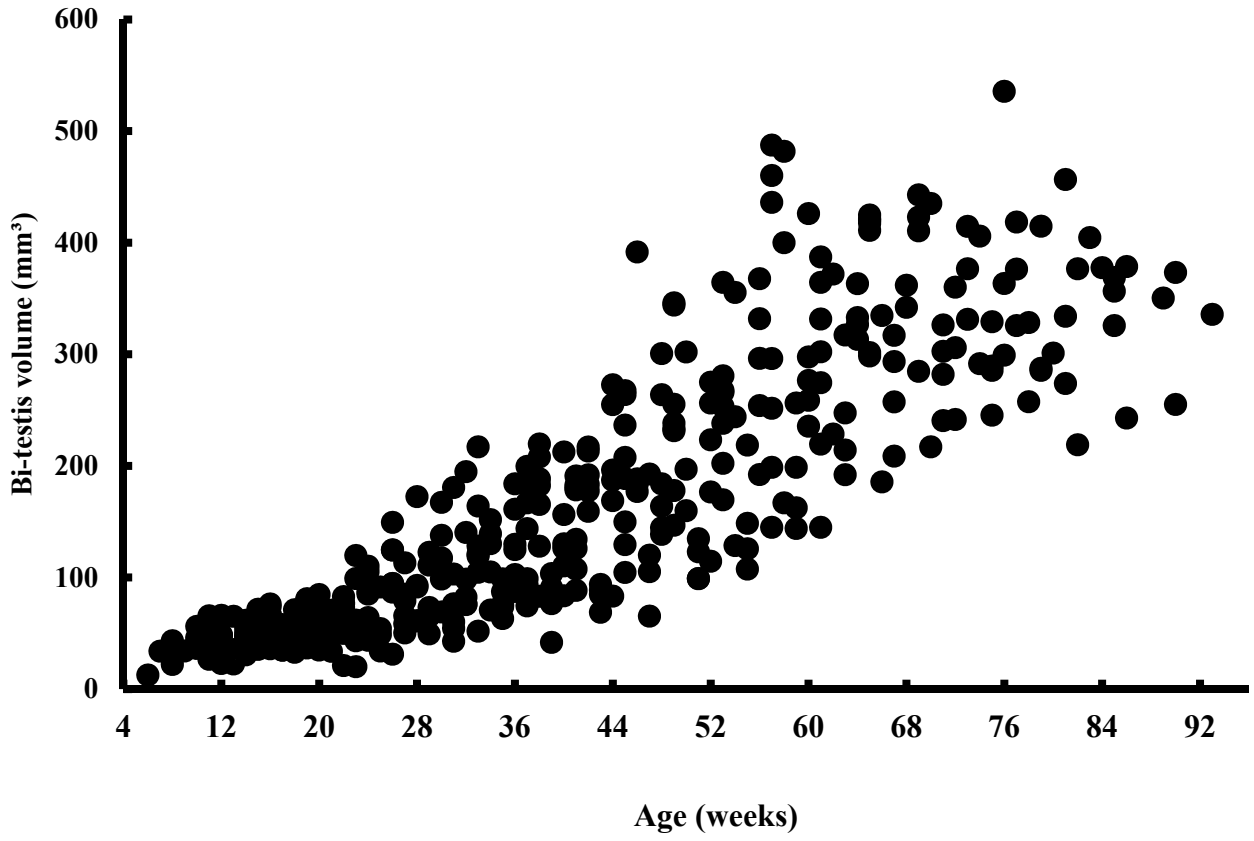


Figure 4.3 Bi-testis volume of the individual common marmoset (n=48).

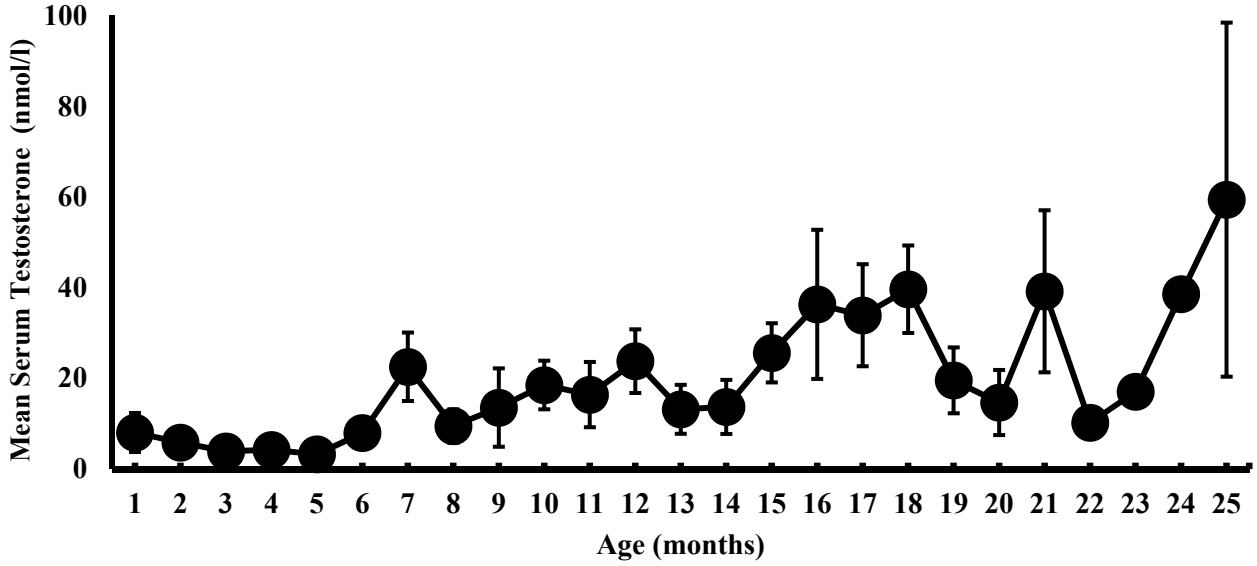


Figure 4.4 Mean serum testosterone levels from birth till 25 months of age in common marmoset (n=48).

Testicular histo-morphometric analysis

The seminiferous tubule diameter showed an increasing trend after 24 weeks (6 months) onwards up till 68 weeks of age (16 months) (Fig 4.5). The timing of the initial increase in the seminiferous tubule diameter correlates with serum levels of testosterone (Fig 4.4). The presence of a tubule lumen was also observed from 24 weeks (6 months) onwards (Fig 4.6 and 4.7) which in turn is the testicular tubular response towards the higher levels of serum testosterone. With advancing age the percentage of the seminiferous tubules exhibit a decreasing trend and the percentage of the lumen demonstrated an increasing trend (Fig 4.6), While the weight of the seminiferous tubules increased from 28 weeks onwards (Fig 4.7). Our testicular morphometric analysis completely correlates with increasing levels of serum testosterone observed from 28 weeks onwards in immature male common marmoset. Histological analysis for the Sertoli cell arrangement demonstrated that randomly placed Sertoli cell are observed up till 5 months of age, at 6 month Sertoli cells arranged themselves towards the periphery in epithelial like fashion around (Table. 4.1). Gonocytes were observed around 5 months of age while the A spermatogonia were first observed around 6 month of age and the occasional B spermatogonia were first observed around 7 month of age, whereas sperm were found in the epididymis at 12 months of age (Table. 4.1).

Our manual measurements of the testis volume in common marmoset were quite accurate as the testis weight and the volume were highly comparable as seen in the (Fig. 4.8). The relative testis weight also show an increasing trend after 28 weeks (Fig. 4.9). Note the sudden increase in the bi-testis weight and bi-testis volume in the age groups of 44 weeks and 52 weeks (Fig 4.9). This sudden increase in testis weight and testis volume is also observed in the seminiferous tubule diameter of the same age group (Fig.4.5). The reason for this two fold increase in the testis weight in these two groups might be due the spermatogonial expansion which was observed at 52 weeks (12 months) of age in terms of the presence of elongated spermatids as well as the epididymal sperm (Table 1).

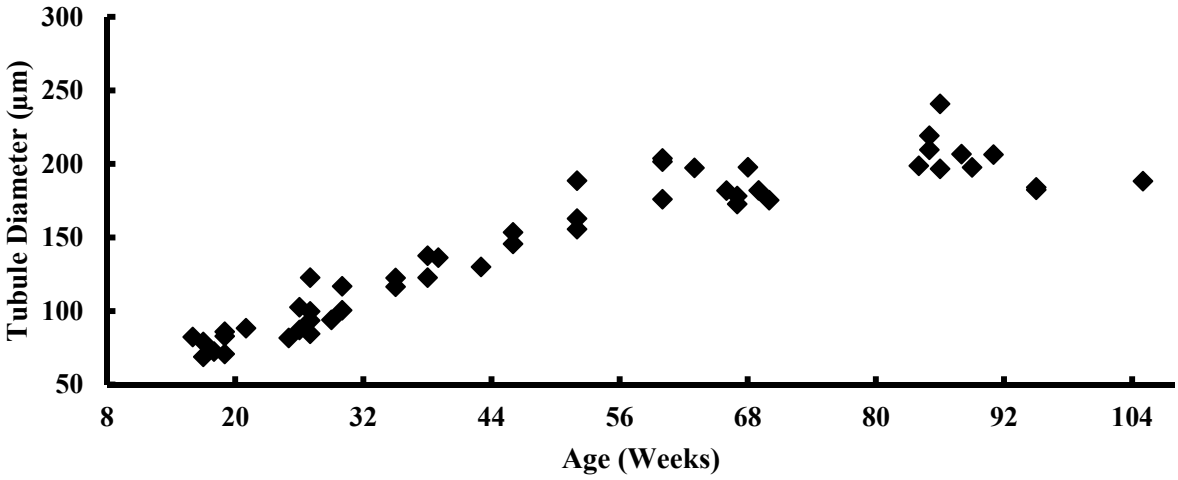


Figure 4.5 Seminiferous tubule diameter from 16 weeks onwards till 96 weeks in common marmoset (n=48).

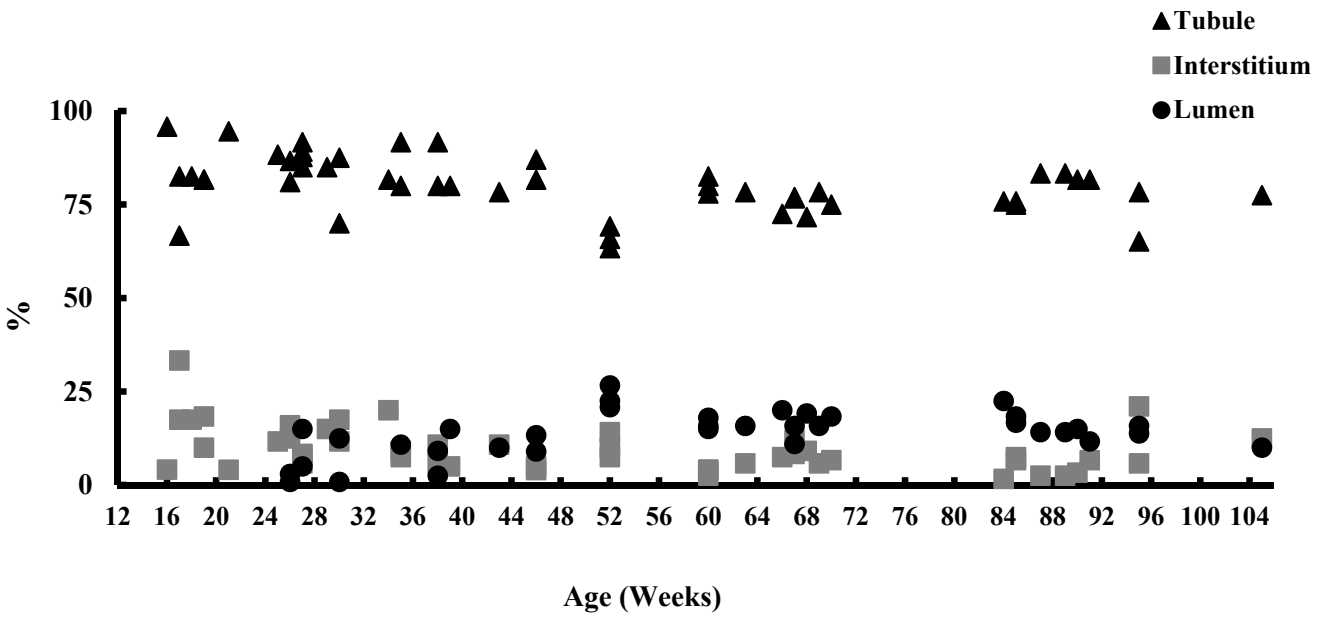


Figure 4.6 Percentage of seminiferous tubules, interstitial area and tubule lumen during development in common marmoset (n=48).

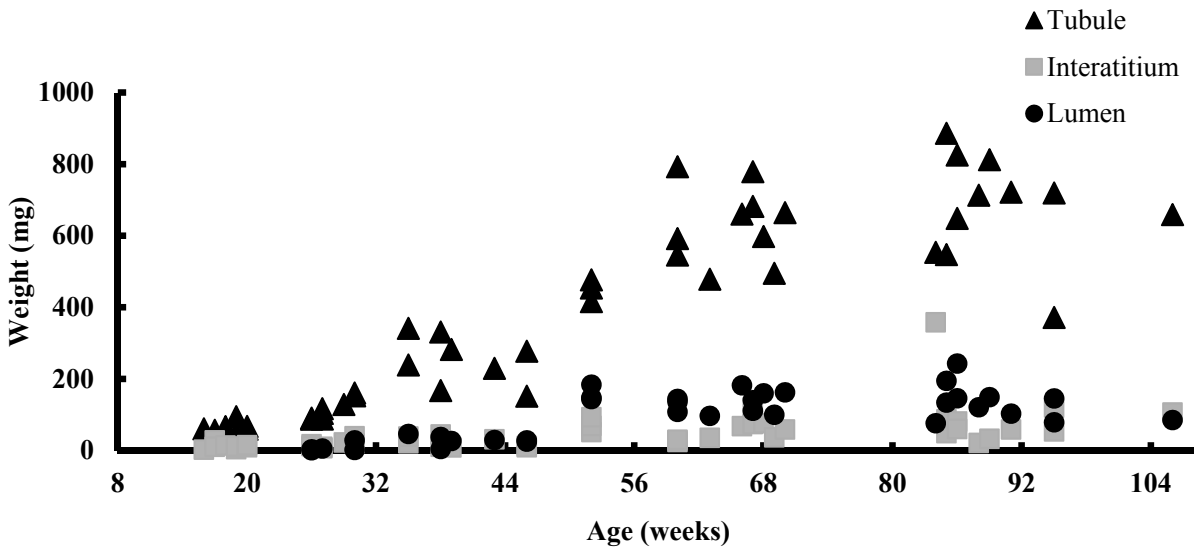


Figure 4.7 Calculated weight of seminiferous epithelium, interstitial area and the tubule lumen during development in common marmoset (n=48).

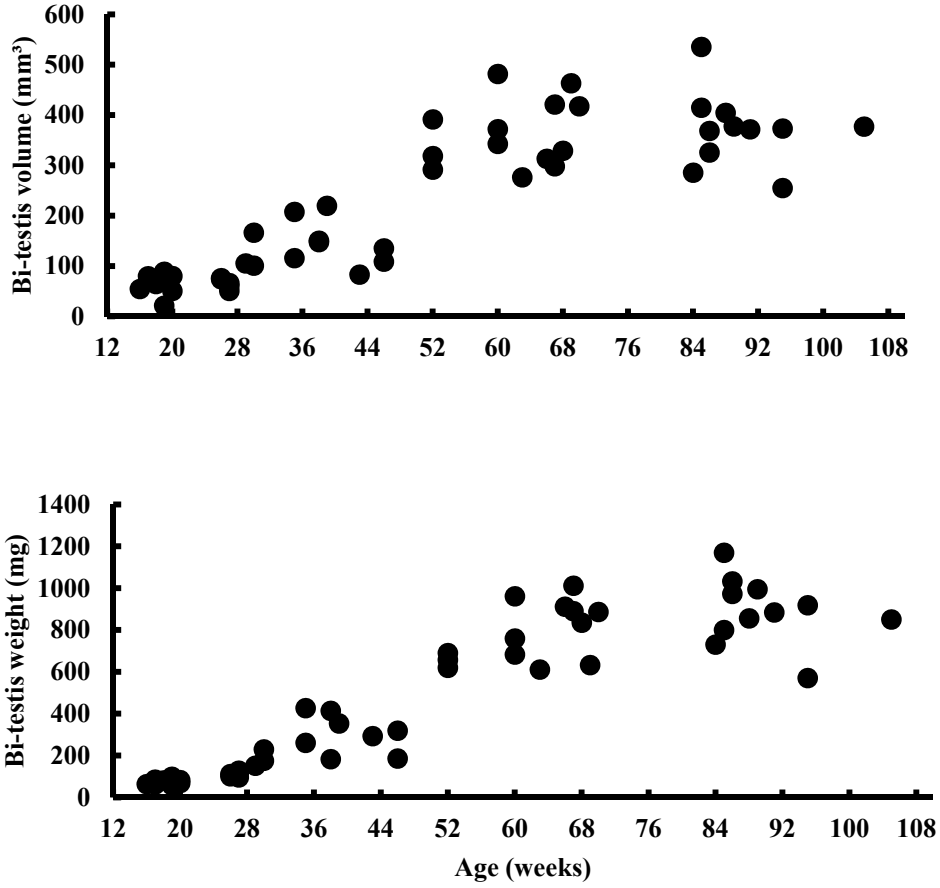


Figure 4.8 Bi-testis weight and bi-testis volume of the sacrificed common marmoset (n=48).

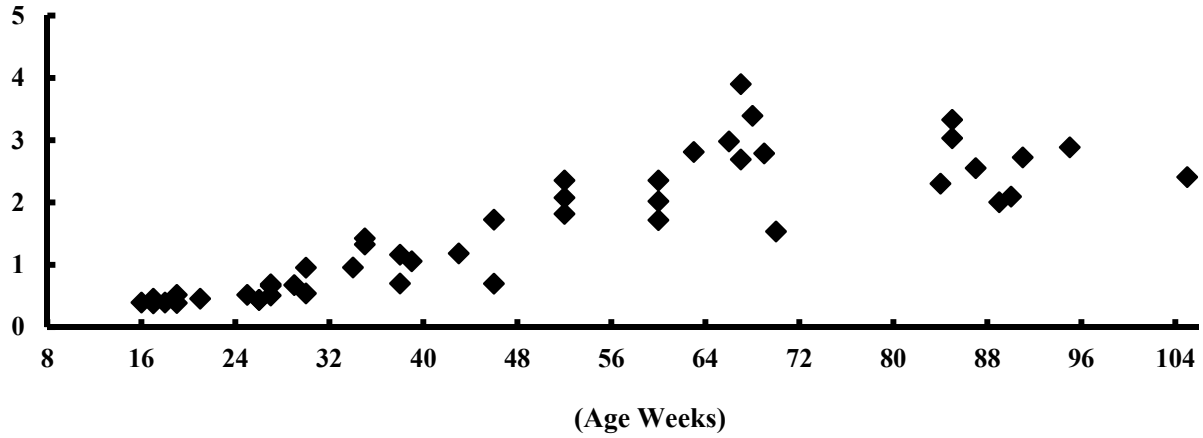


Figure 4.9 Relative testis weight of the sacrificed common marmoset (n=48).

Table 4.1 Description of advanced germ cell type, Sertoli cell arrangement and the presence or absence of sperm in the epididymis of common marmoset over the course of development (n=48).

Age	Most advanced Germ cell type	Sertoli cell nuclei arranegment	Epipdidymal sperm
4 Months *n= 6*	Gonocytes	Centrally placed Randomly arranged	No
5 Months *n= 3*	A spermatogonia	Centrally placed Randomly arranged	No
6 Months *n= 6*	A spermatogonia	Peripherally placed epithelial arrangement	No
7 Months *n= 3*	B spermatogonia	Epithelial-like arrangement	No
8 Months *n= 5*	Secondary spermatocytes	Epithelial-like arrangement	No
10 Months *n= 3*	Round spermatids	Epithelial-like arrangement	No
12 Months till 20 months *n= 22*	Elongating spermatids	Epithelial-like arrangement	Yes

Body weight, bi-testis volume, serum testosterone in individual animals from birth till the sacrifice and the bi-testis weight, seminiferous tubule diameter and moset advanced germ cell type on the day of sacrifice

The individual animal's body weight, bi-testis volume and serum testosterone concentration are presented from birth till the day of sacrifice. The bi-testis weight which was measured after the removal of the testis, the serum testosterone concentration on the day of sacrifice along with the histomorphometric data including the seminiferous tubules diameter and the most advanced germ cell type present are also shown. Note the sudden rise in serum testosterone at 7 month of age (Fig 4.10, 4.11, 4.13, 4.14, 4.15, 4.16, 4.18, 4.18, 4.26, 4.28, 4.36, 4.38) while some animals fail to show a substantial rise in serum testosterone at 7 months of age. In these animals the substantial rise in serum testosterone is seen either before 7 months of age (6 months) (Fig 4.17, 4.20, 4.25, 4.32) or after 7 months of age (Fig 4.21, 4.22, 4.23, 4.24, 4.27, 4.30, 4.31, 4.33, 4.35, 4.37, 4.39, 4.40). Interestingly the seminiferous tubule diameter and most advanced germ cell type were found to be similar in the animals from same age group but having different testosterone levels. Animal # 596 (Fig 4.12) did not show a testosterone rise at 7 month of age but the seminiferous diameter is comparable along with the most advanced germ cell type with those animals which show substantial rise at 7 month of age and were in the same age group as animals# 596 at the sacrifice day (Fig 4.10, 4.11, 4.13). Few animals did not show any testosterone rise during the complete sampling period (Fig 4.19, 4.29), but the testis weight and seminiferous tubule diameter of these animals were comparable with the other animals from the same age group. The significant rise in bi-testis weight was observed in 12 month old group (Fig 4.21, 4.22, 4.23) as the testis weight doubled as compared with the 11 month old group (Fig 4.19, 4.20). This sudden increase in testis weight was mainly due to the spermatogonial expansion as the sperms were observed in the epididymis in the 12 month old group (Table. 1). Although the seminiferous tubules diameter did not show a remarkable difference in these two age groups.

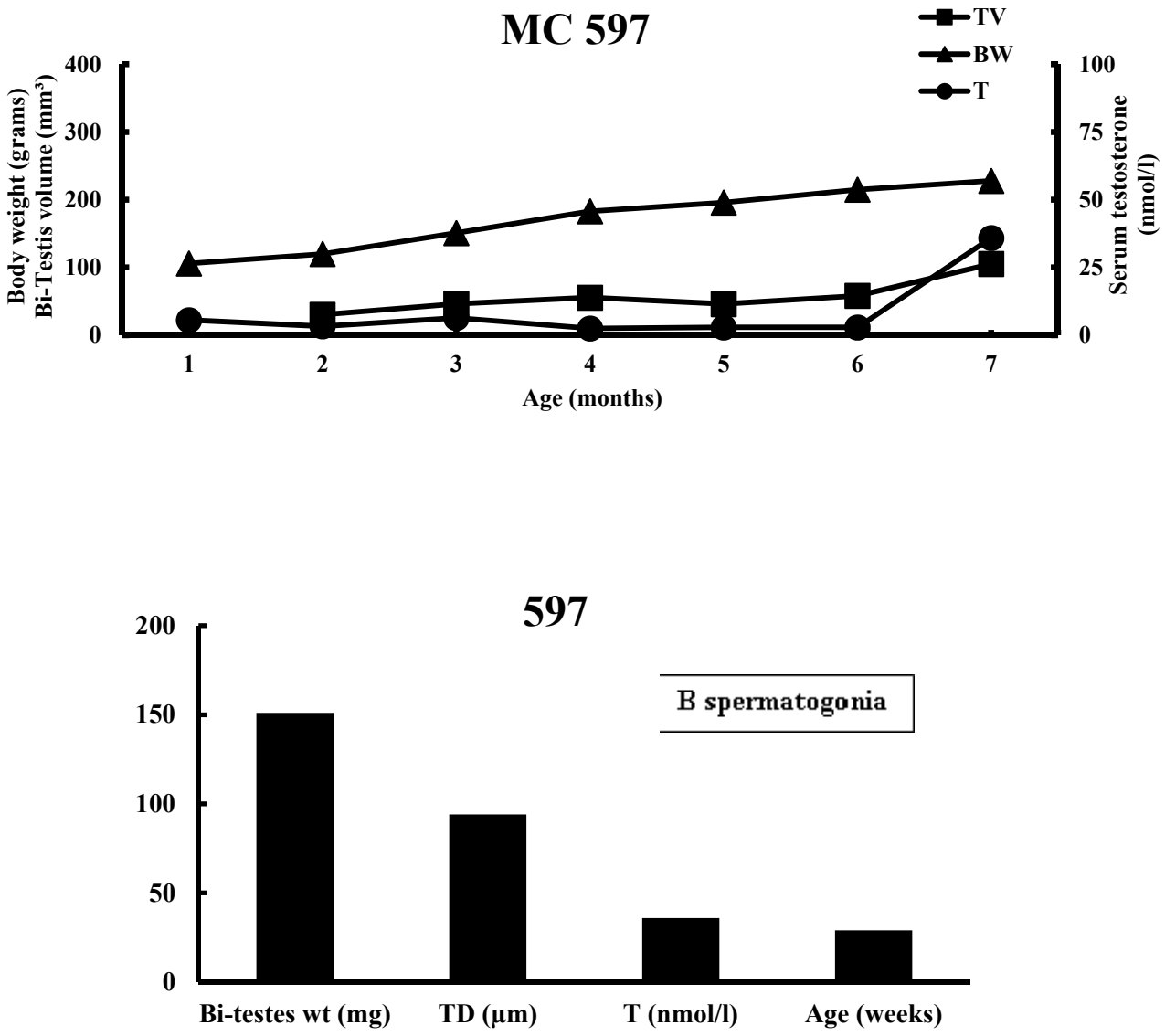


Figure 4.10 Body weight, serum testosterone and bi-testis volume from birth till 7 month of age in common marmoset (#597) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

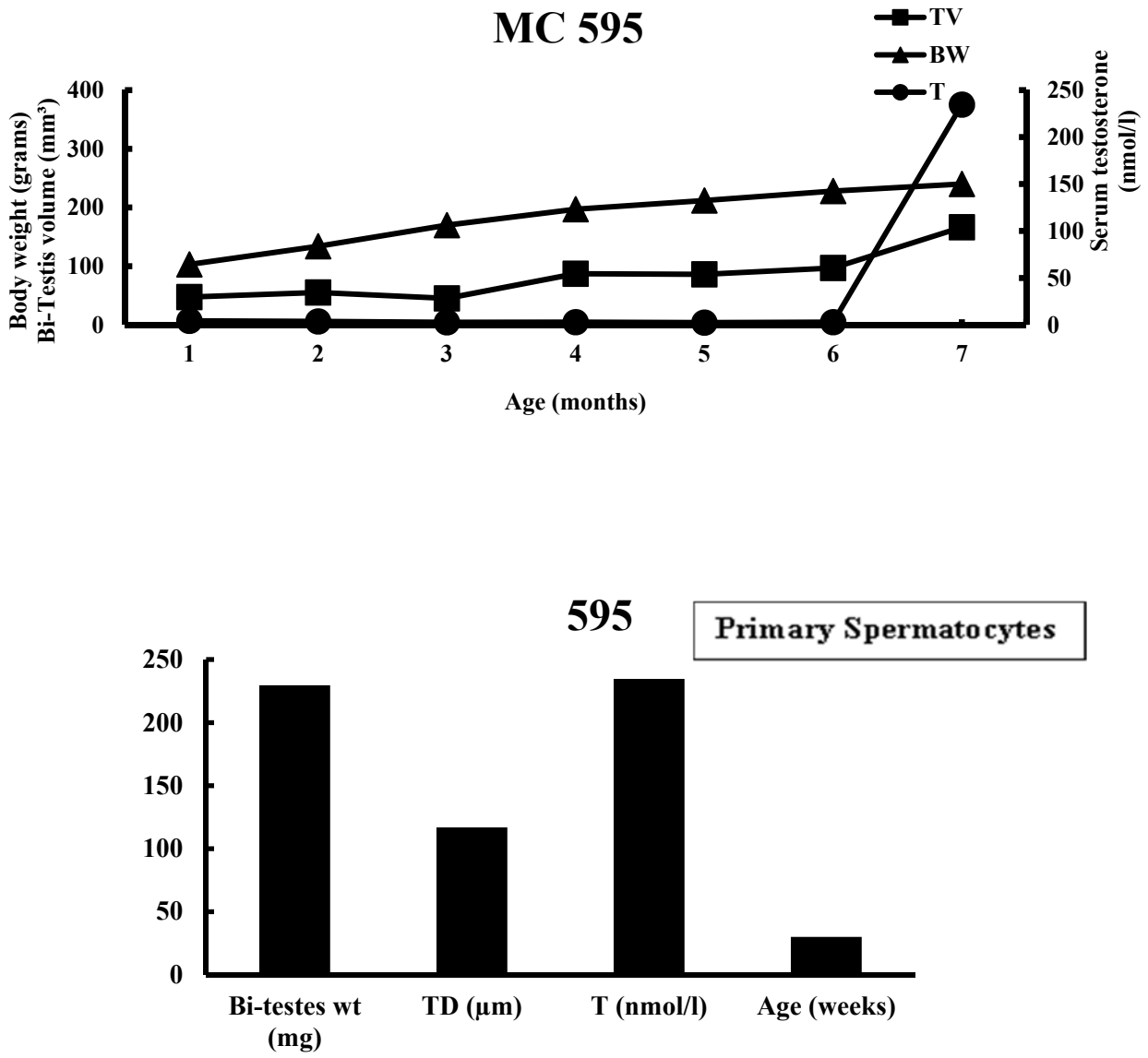
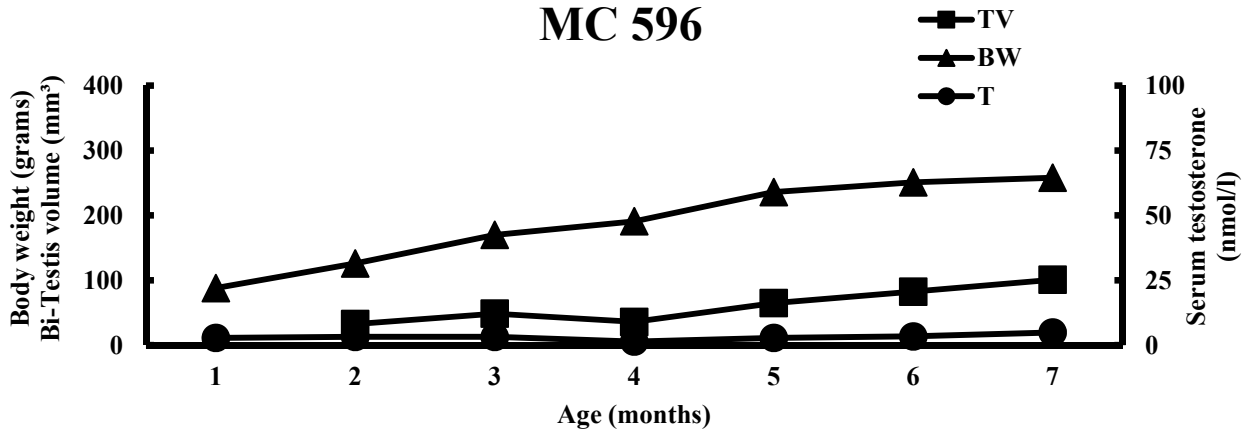


Figure 4.11 Body weight, serum testosterone and bi-testis volume from birth till 7 month of age in common marmoset (#595) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



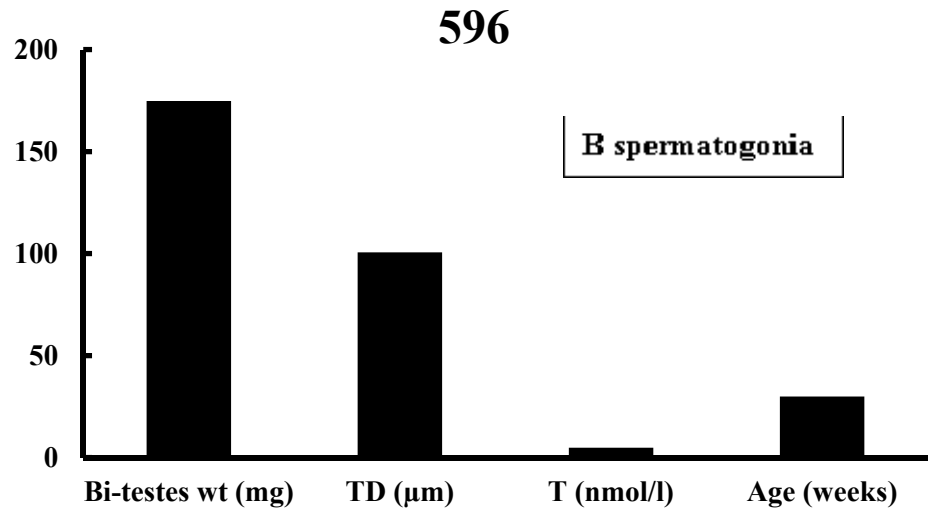


Figure 4.12 Body weight, serum testosterone and bi-testis volume from birth till 7 month of age in common marmoset (#596) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

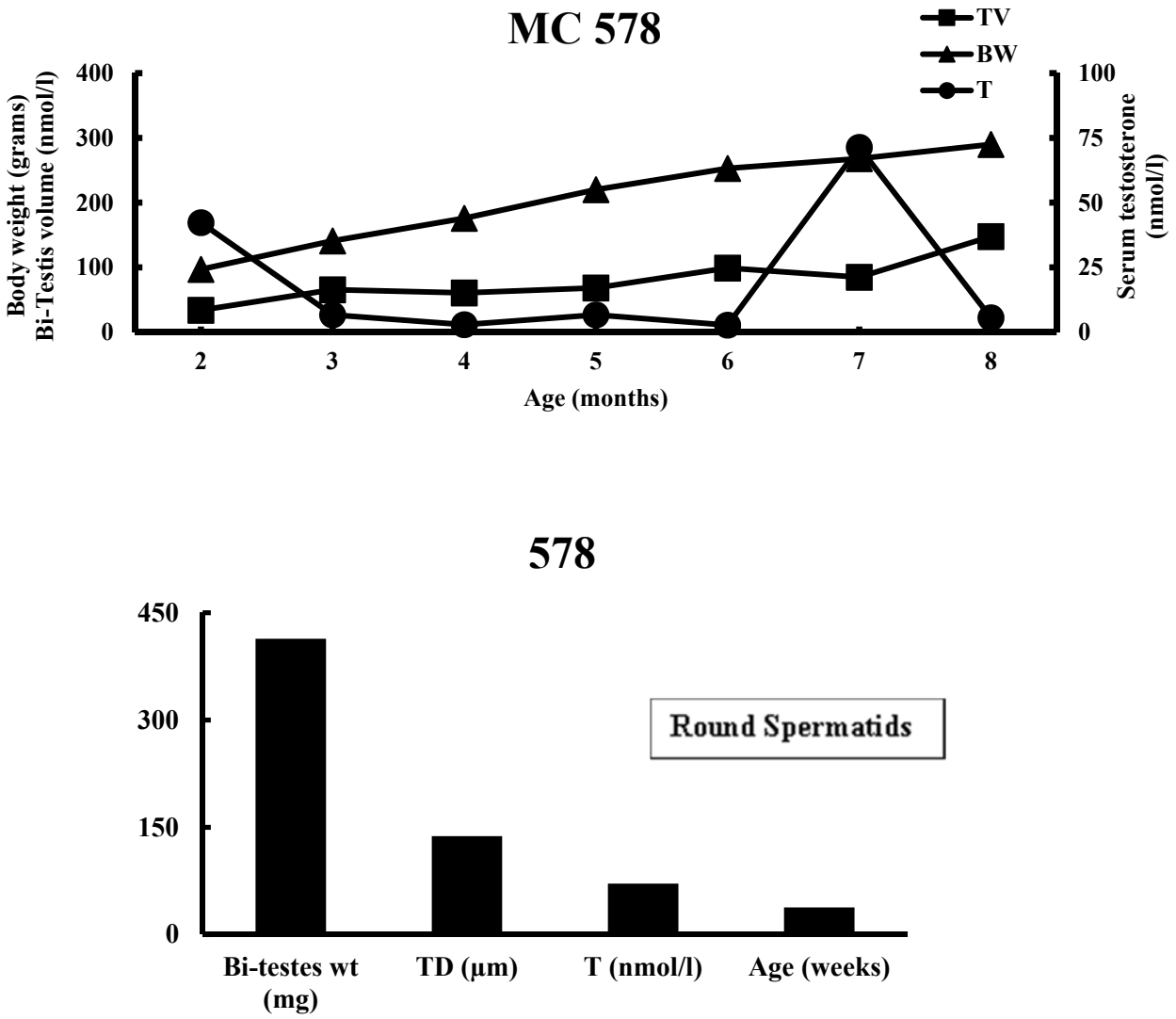


Figure 4.13 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#578) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

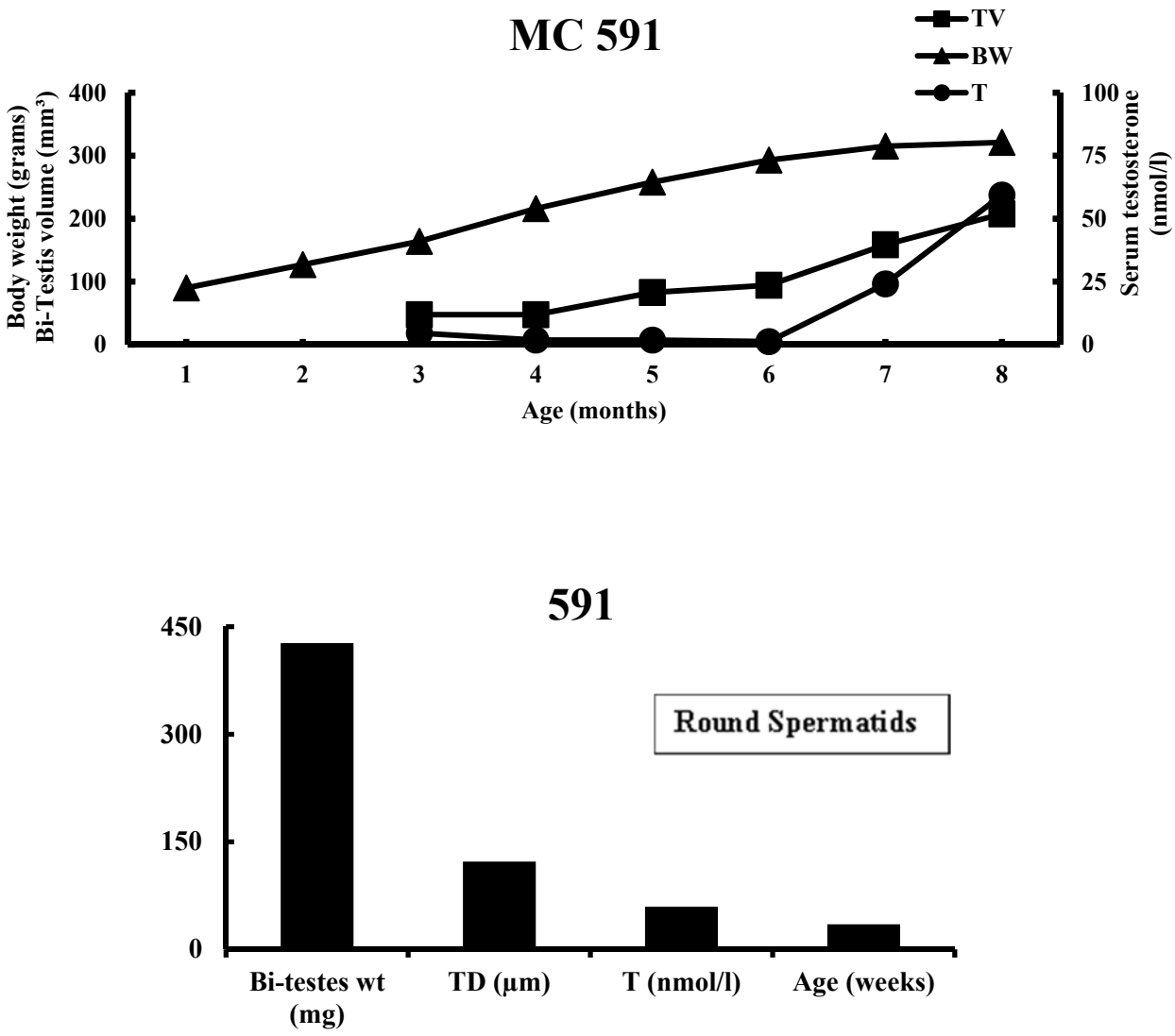


Figure 4.14 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#591) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

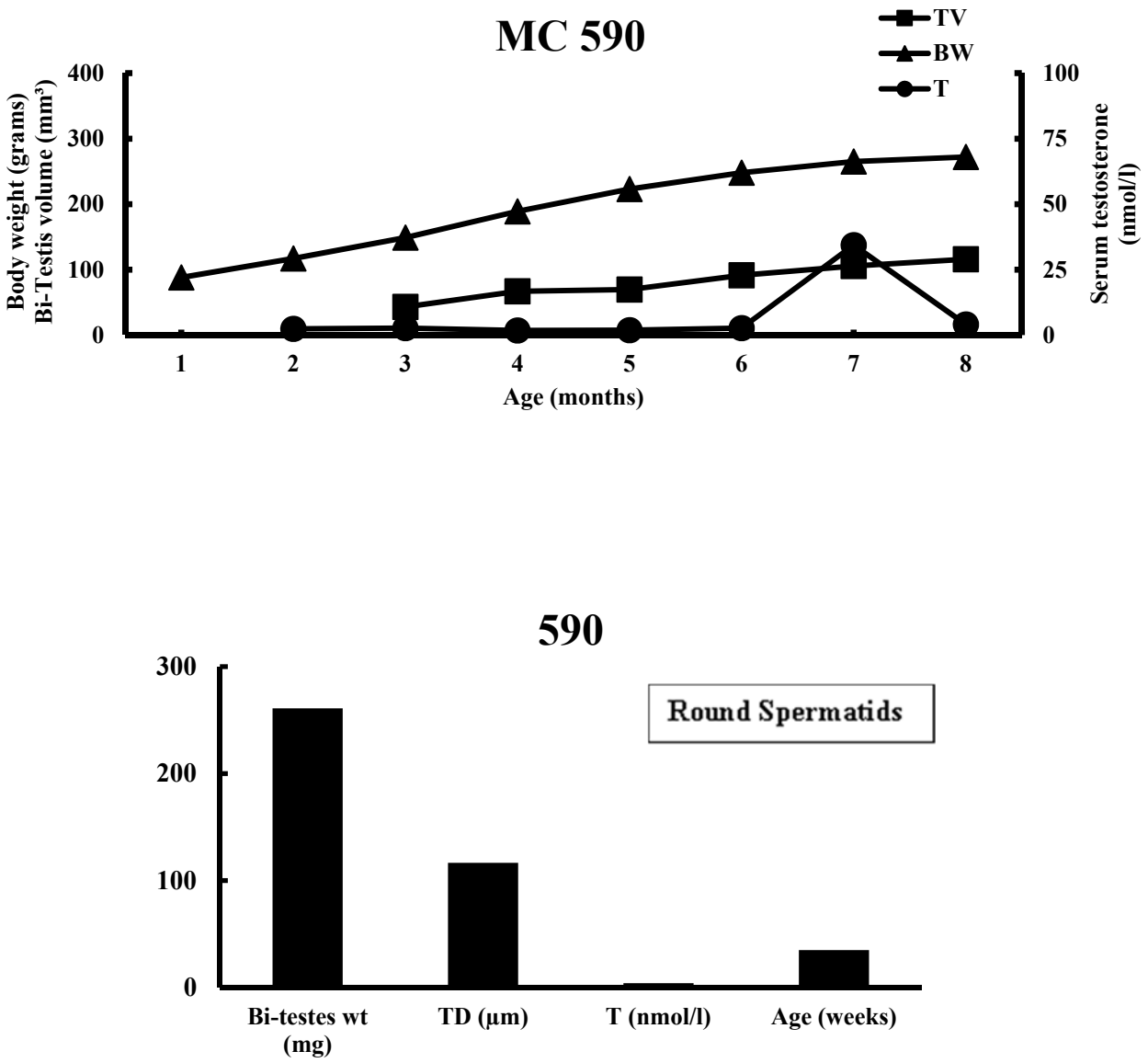


Figure 4.15 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#590) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

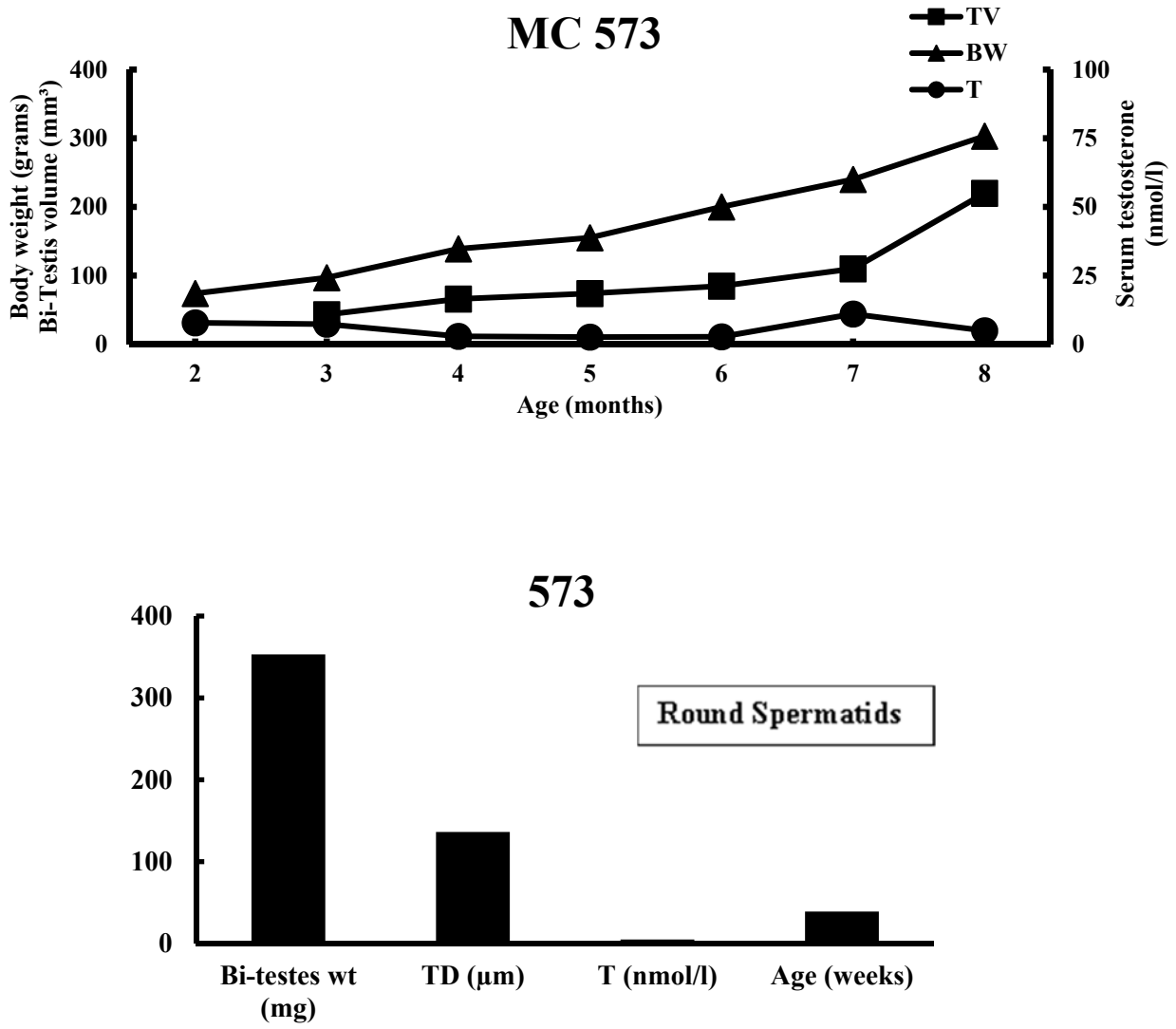


Figure 4.16 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#573) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

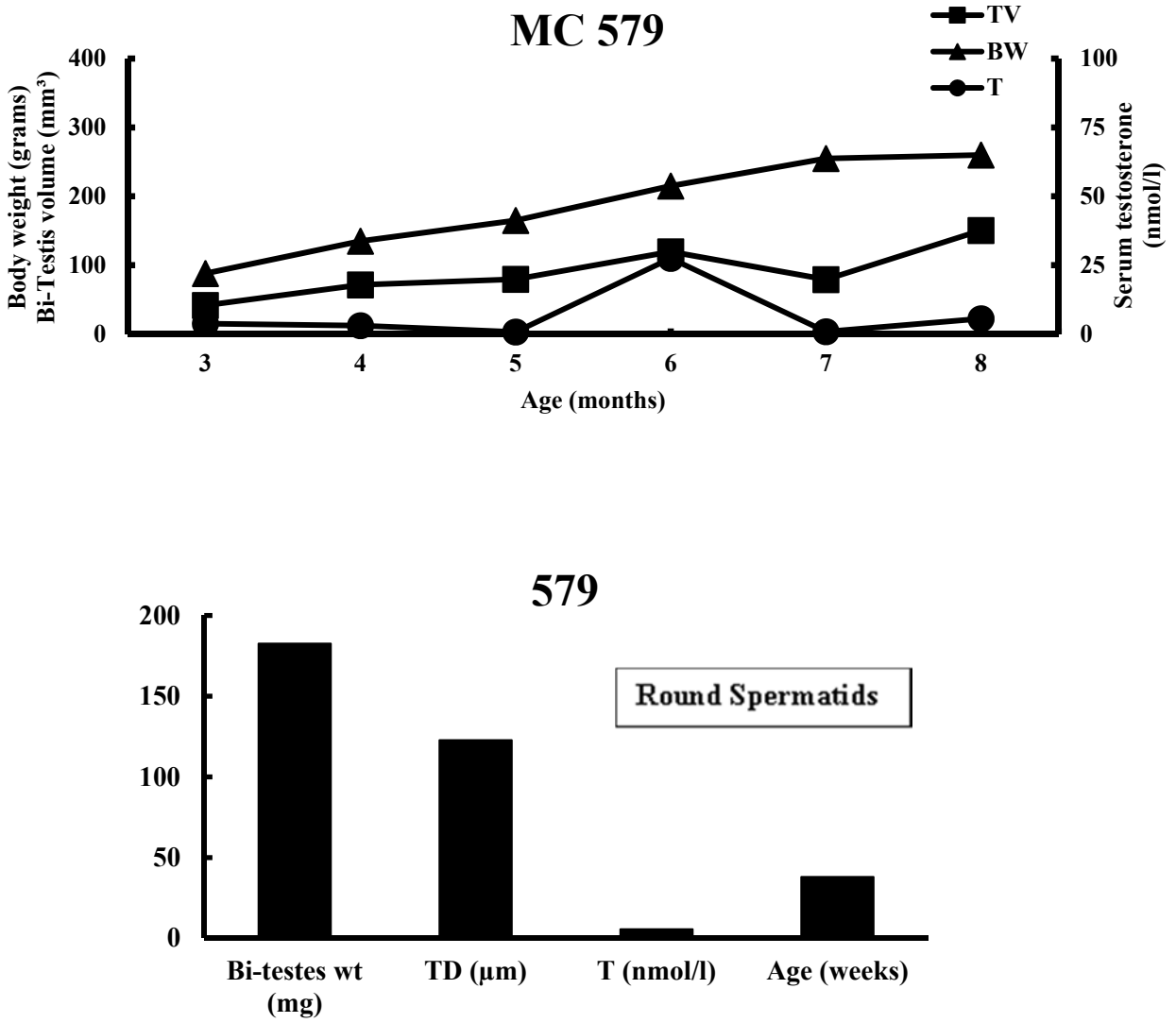


Figure 4.17 Body weight, serum testosterone and bi-testis volume from birth till 8 month of age in common marmoset (#579) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

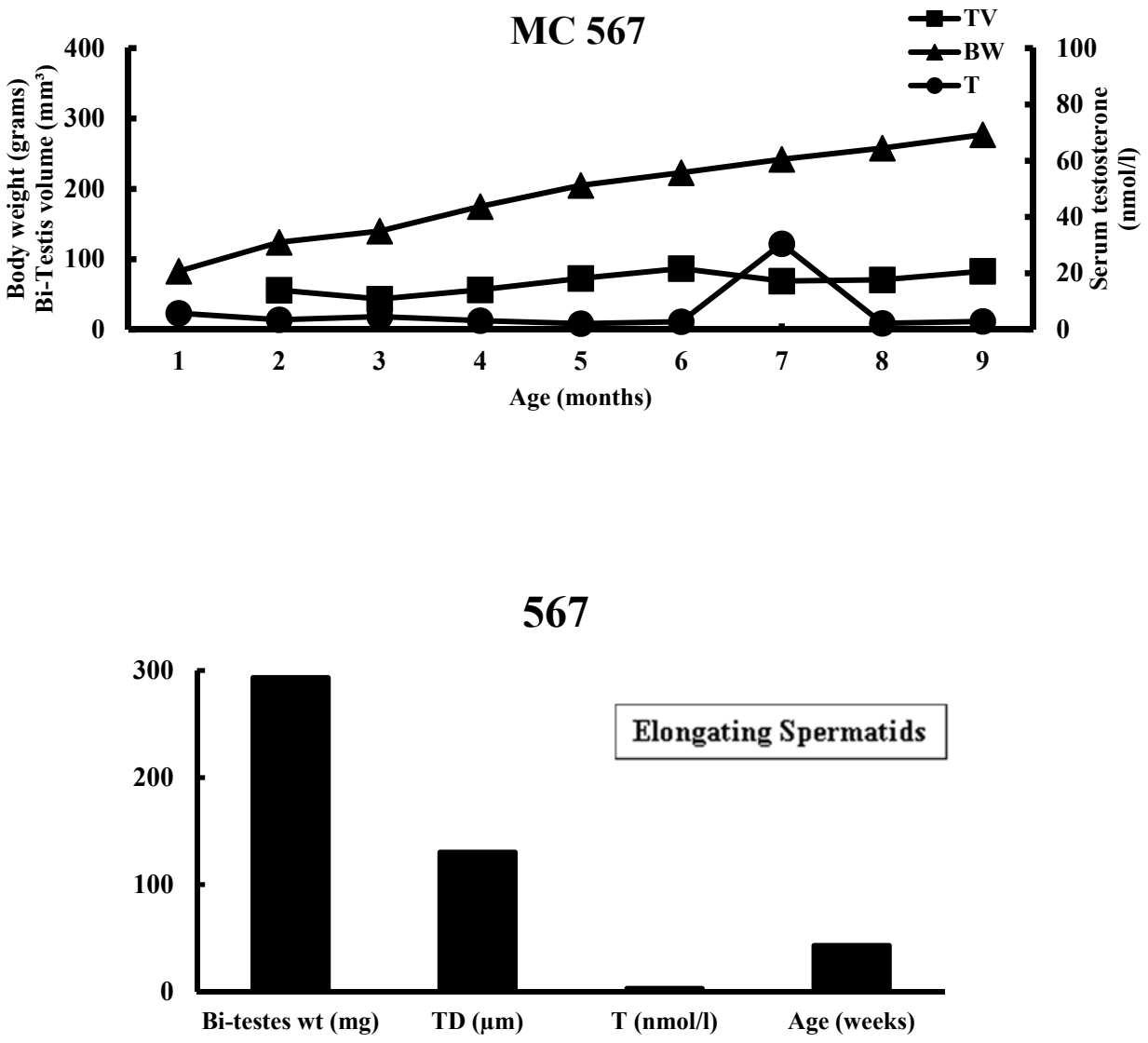


Figure 4.18 Body weight, serum testosterone and bi-testis volume from birth till 9 month of age in common marmoset (#567) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

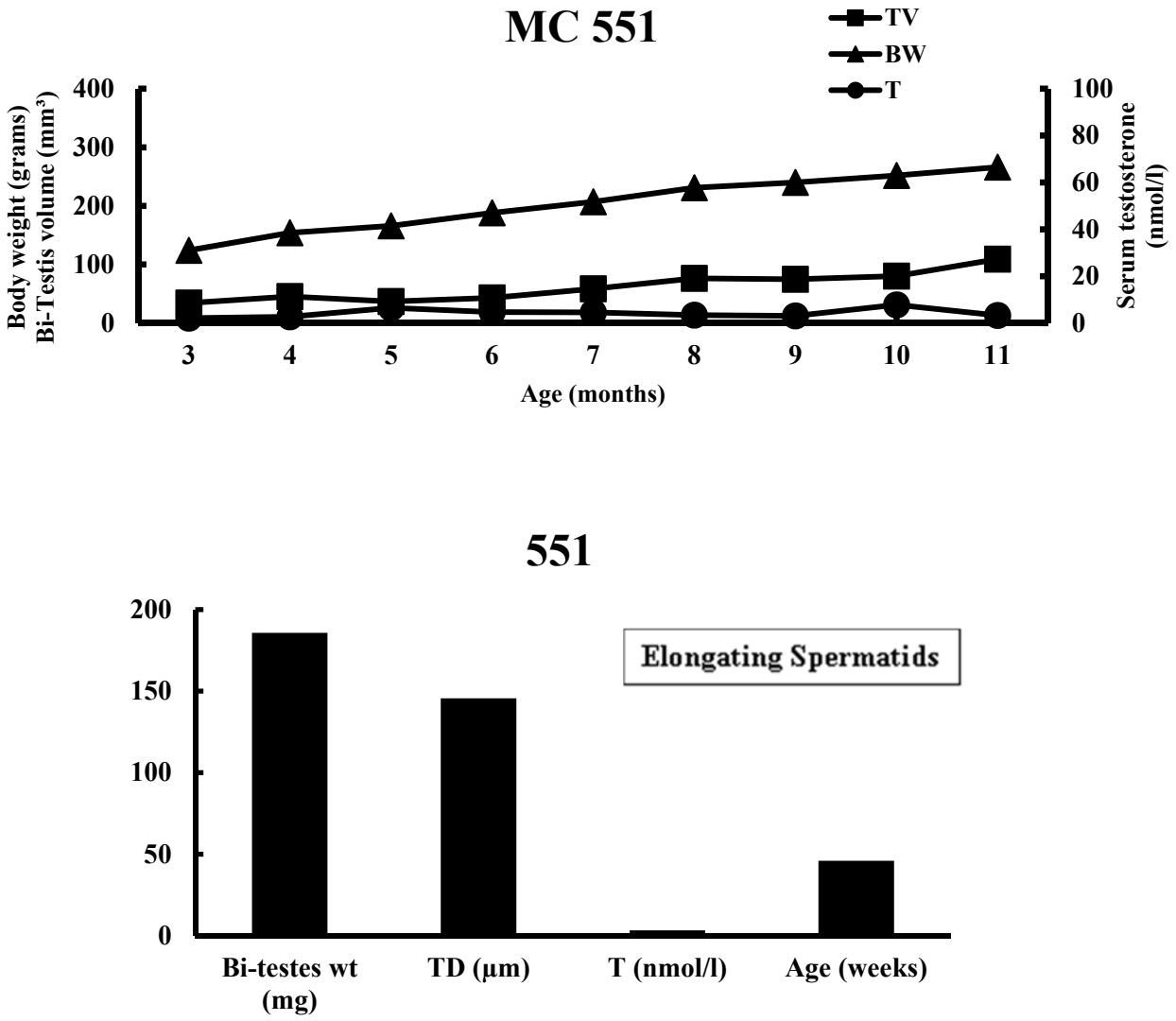


Figure 4.19 Body weight, serum testosterone and bi-testis volume from birth till 11 month of age in common marmoset (#551) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

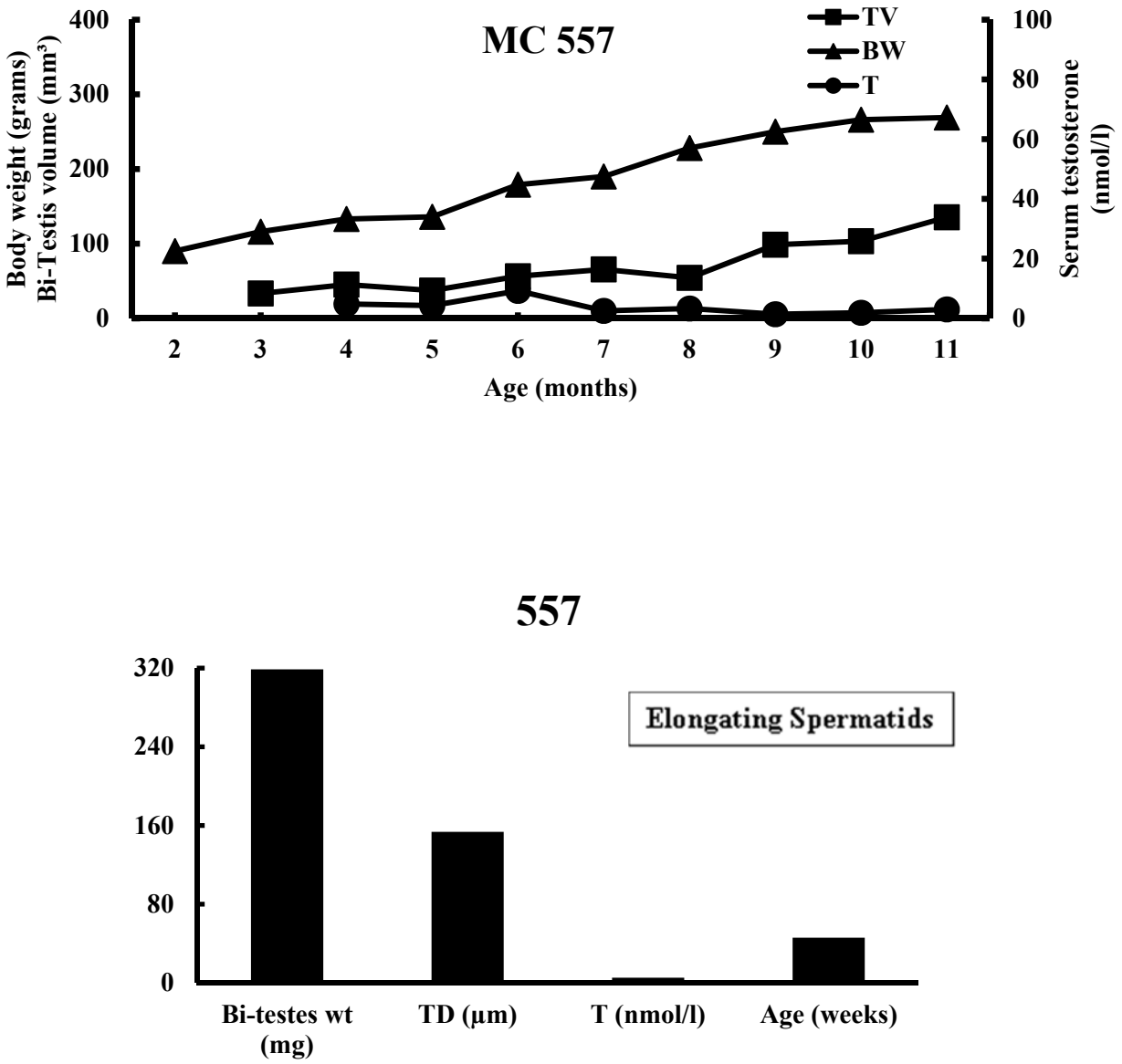


Figure 4.20 Body weight, serum testosterone and bi-testis volume from birth till 11 month of age in common marmoset (#557) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

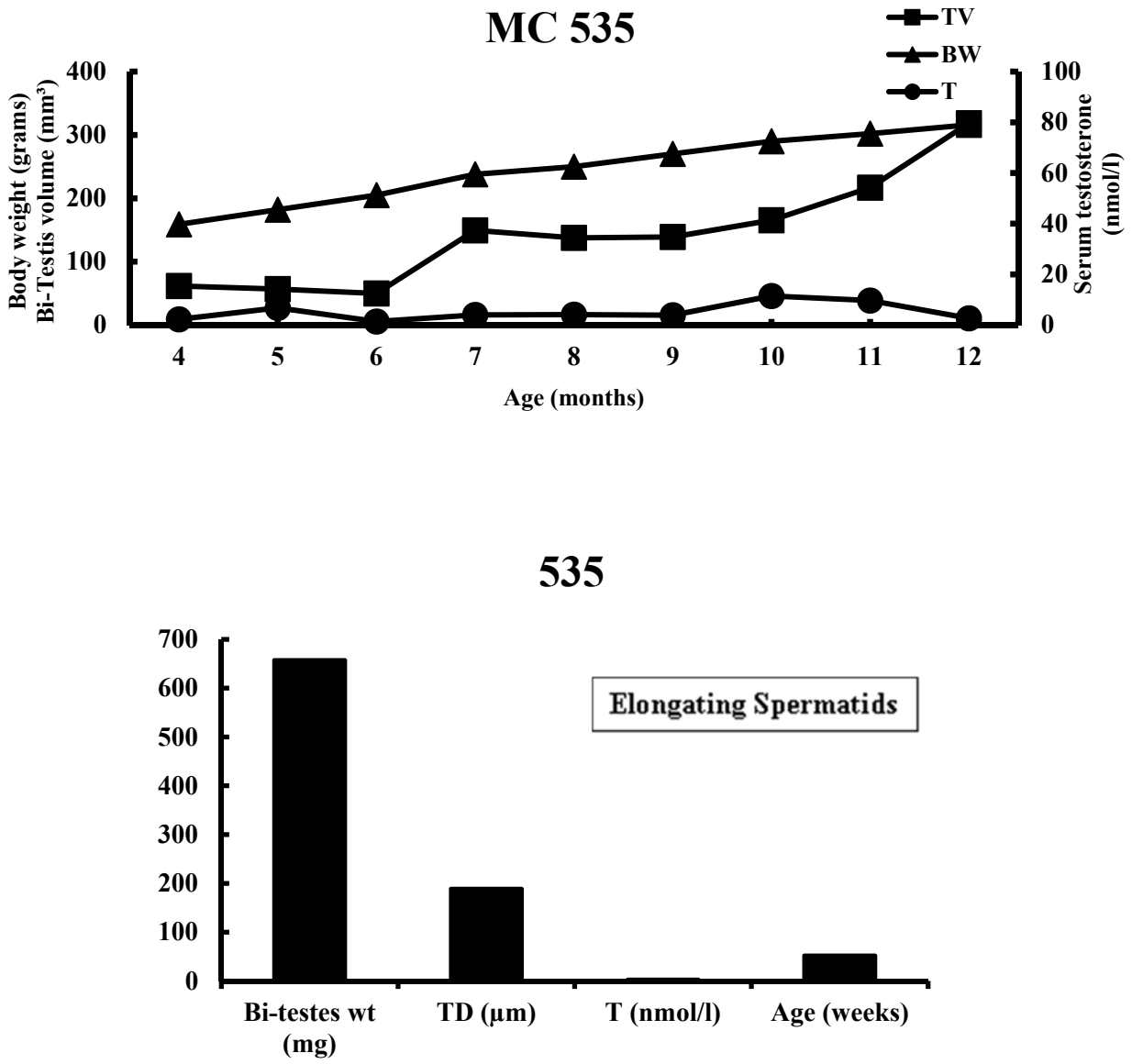


Figure 4.21 Body weight, serum testosterone and bi-testis volume from birth till 12 month of age in common marmoset (#535) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

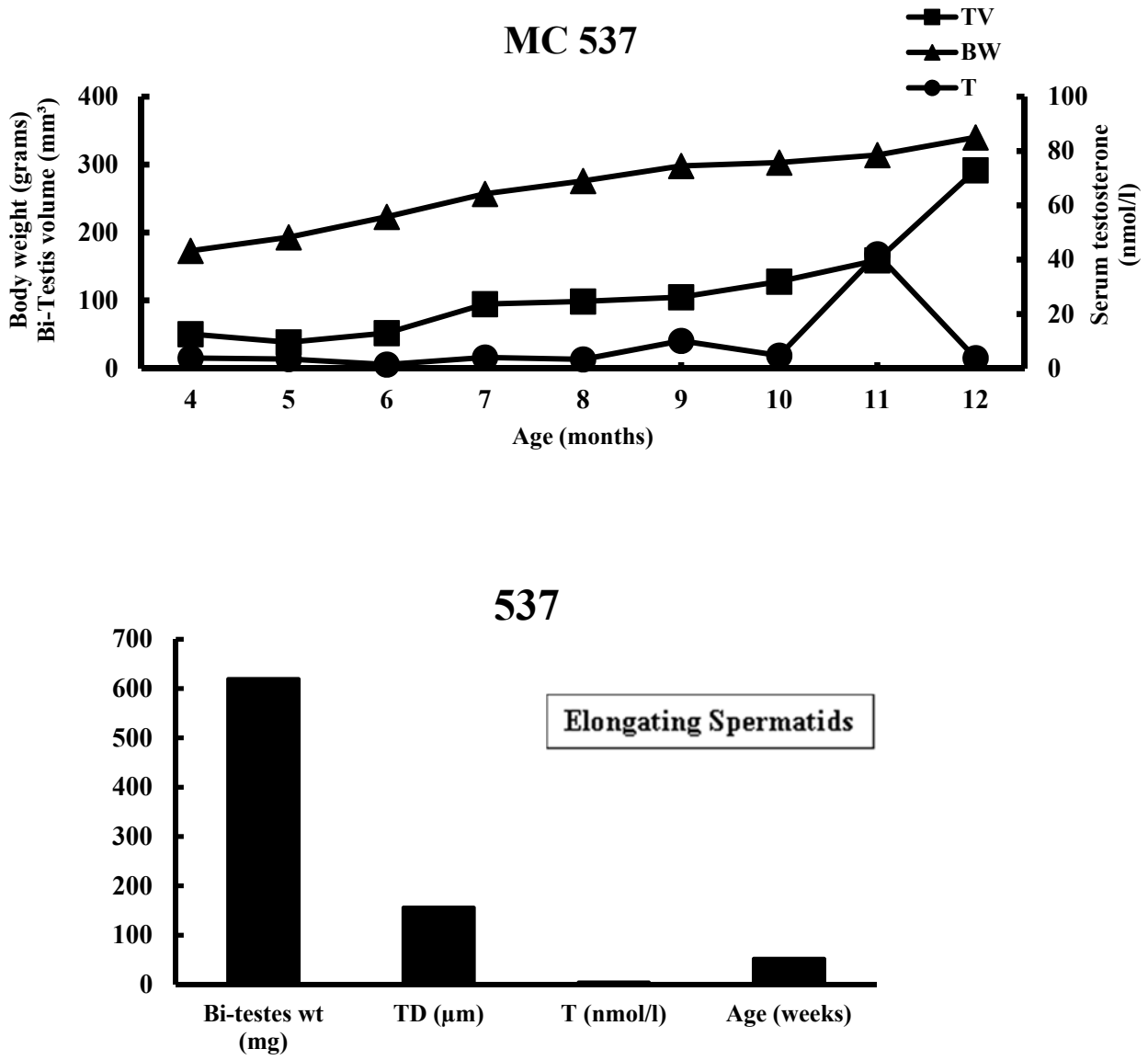
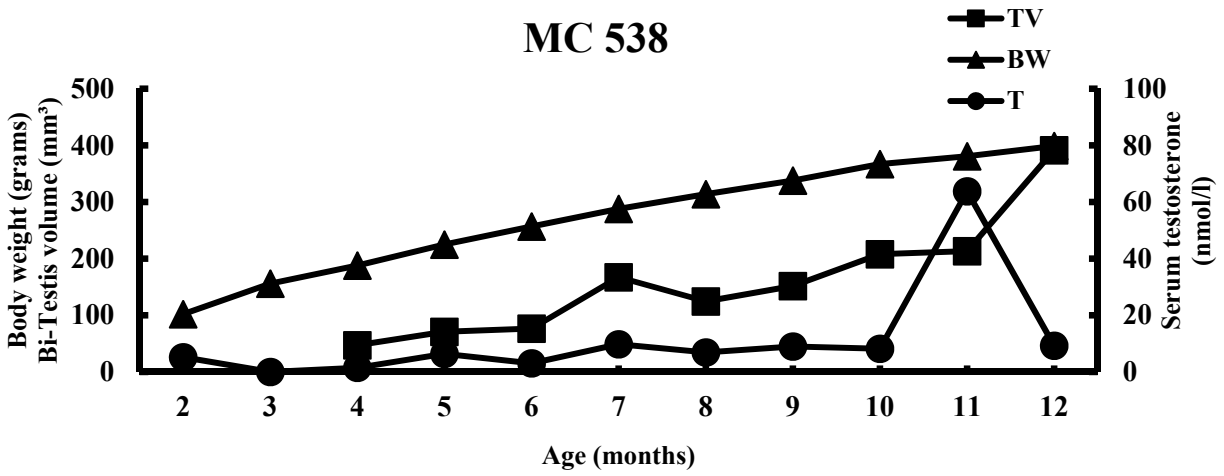


Figure 4.22 Body weight, serum testosterone and bi-testis volume from birth till 12 month of age in common marmoset (#537) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



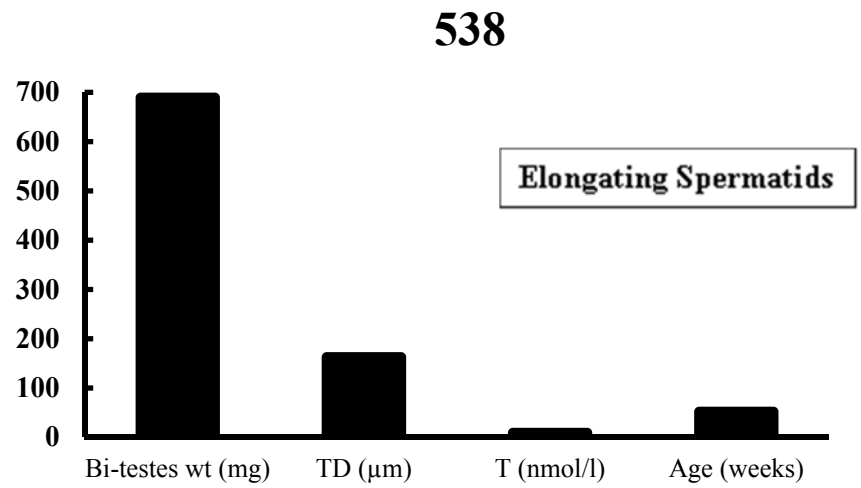
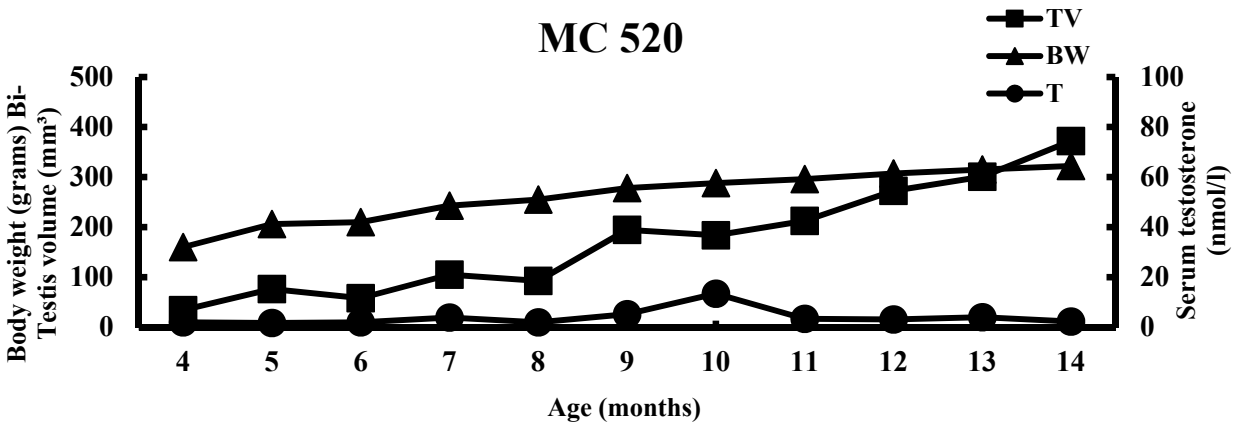


Figure 4.23 Body weight, serum testosterone and bi-testis volume from birth till 12 month of age in common marmoset (#538) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



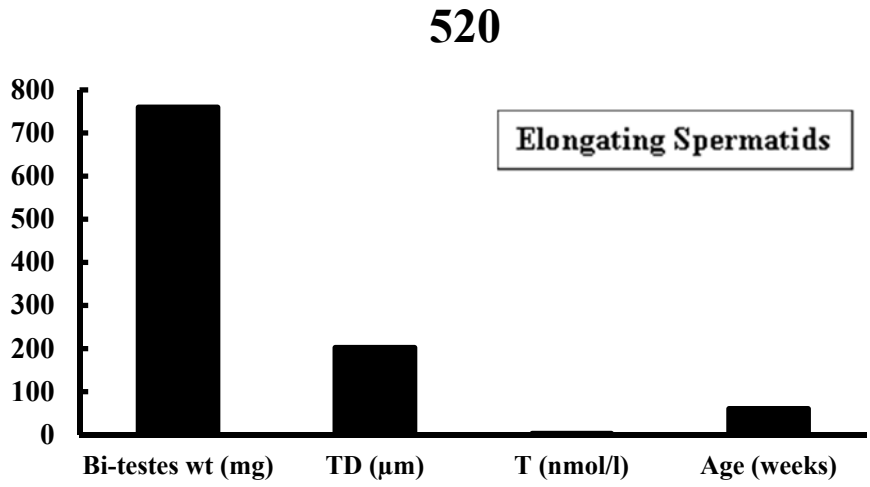
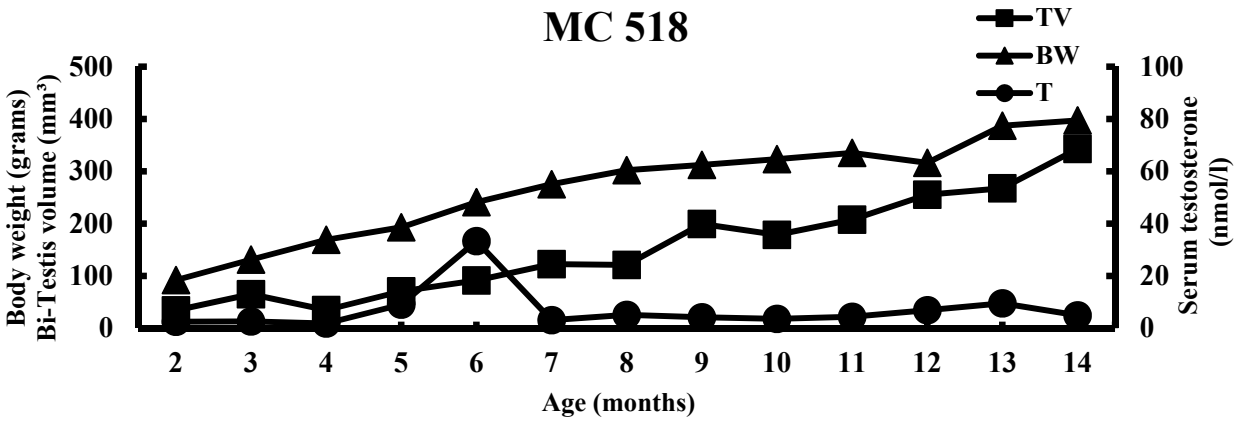


Figure 4.24 Body weight, serum testosterone and bi-testis volume from birth till 14 month of age in common marmoset (#520) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



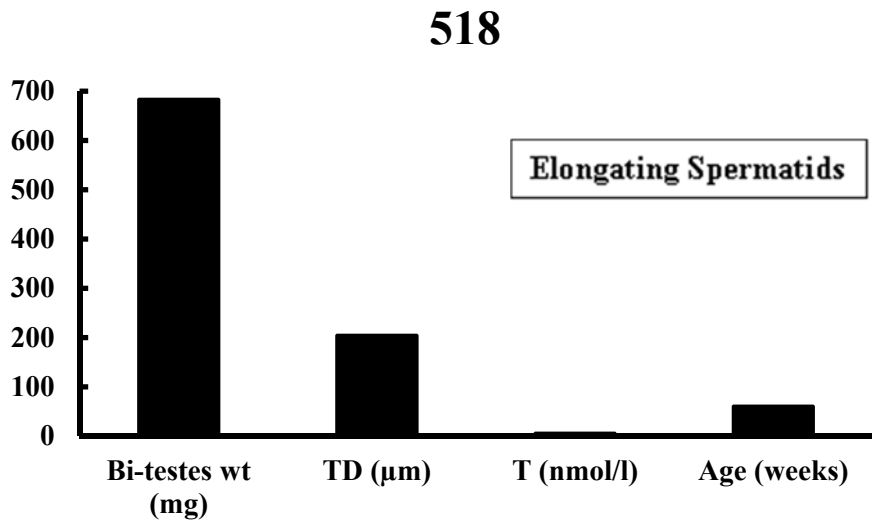


Figure 4.25 Body weight, serum testosterone and bi-testis volume from birth till 14 month of age in common marmoset (#518) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

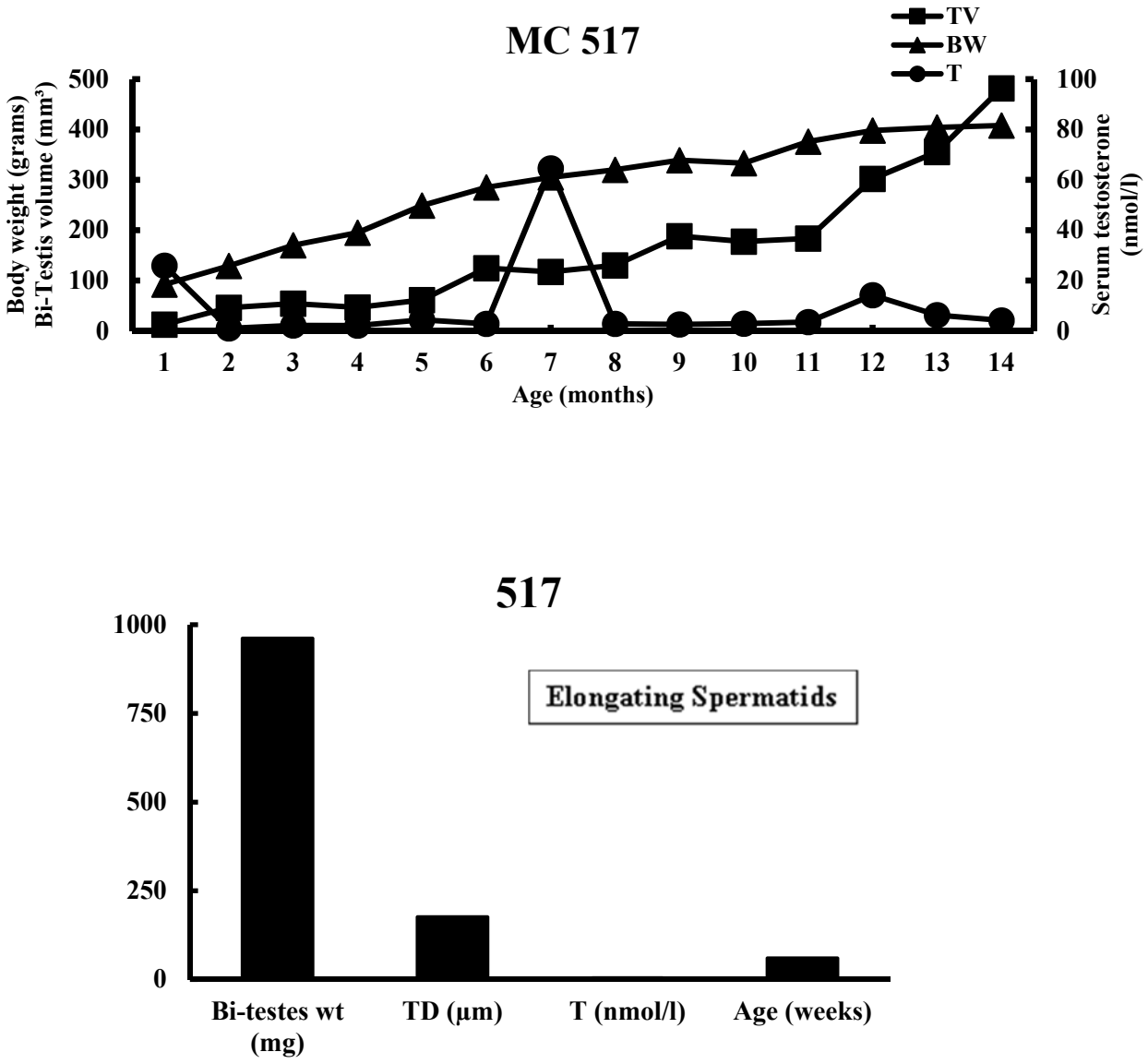


Figure 4.26 Body weight, serum testosterone and bi-testis volume from birth till 14 month of age in common marmoset (#517) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

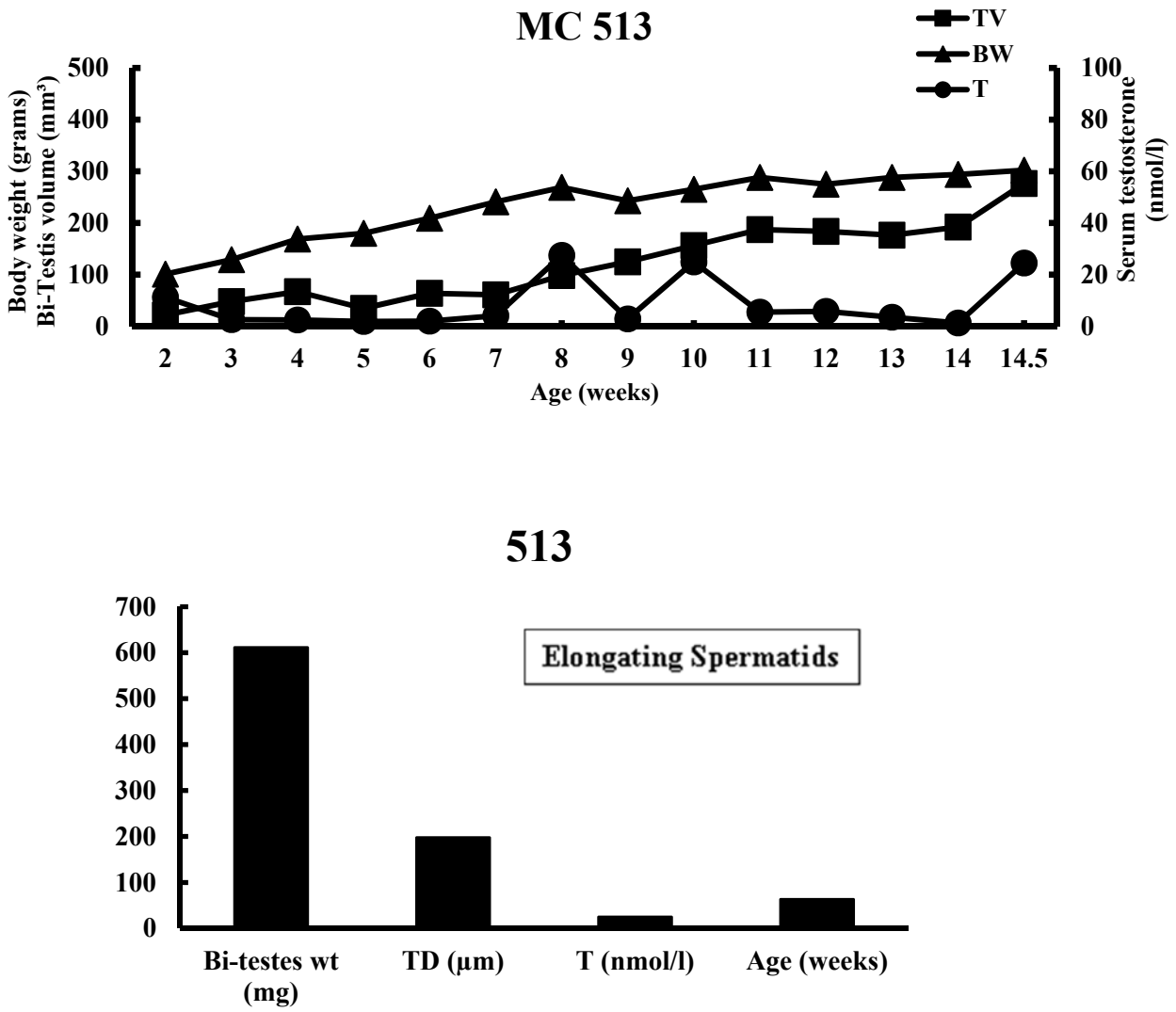


Figure 4.27 Body weight, serum testosterone and bi-testis volume from birth till 14.5 month of age in common marmoset (#513) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

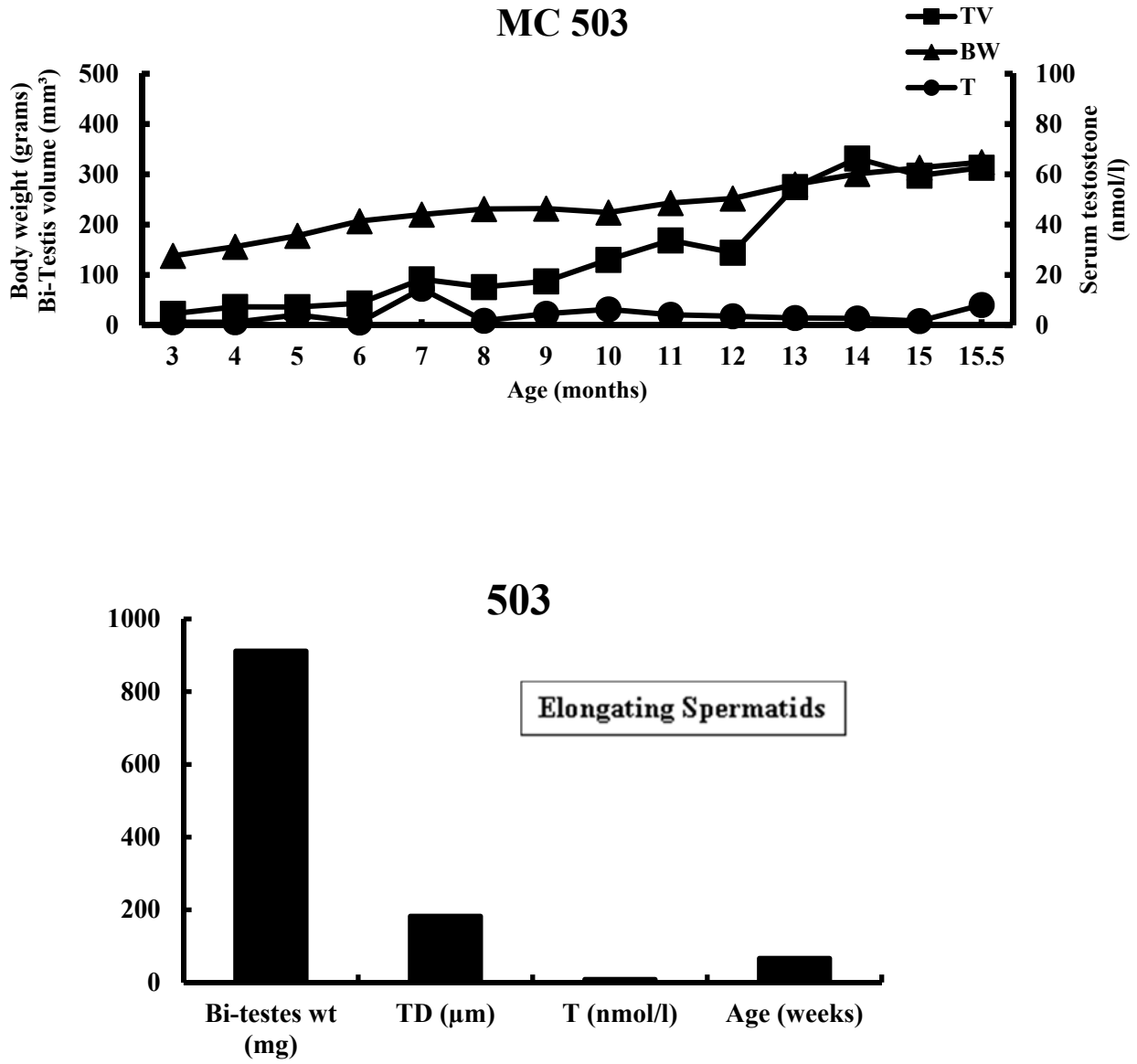


Figure 4.28 Body weight, serum testosterone and bi-testis volume from birth till 15.5 month of age in common marmoset (#503) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

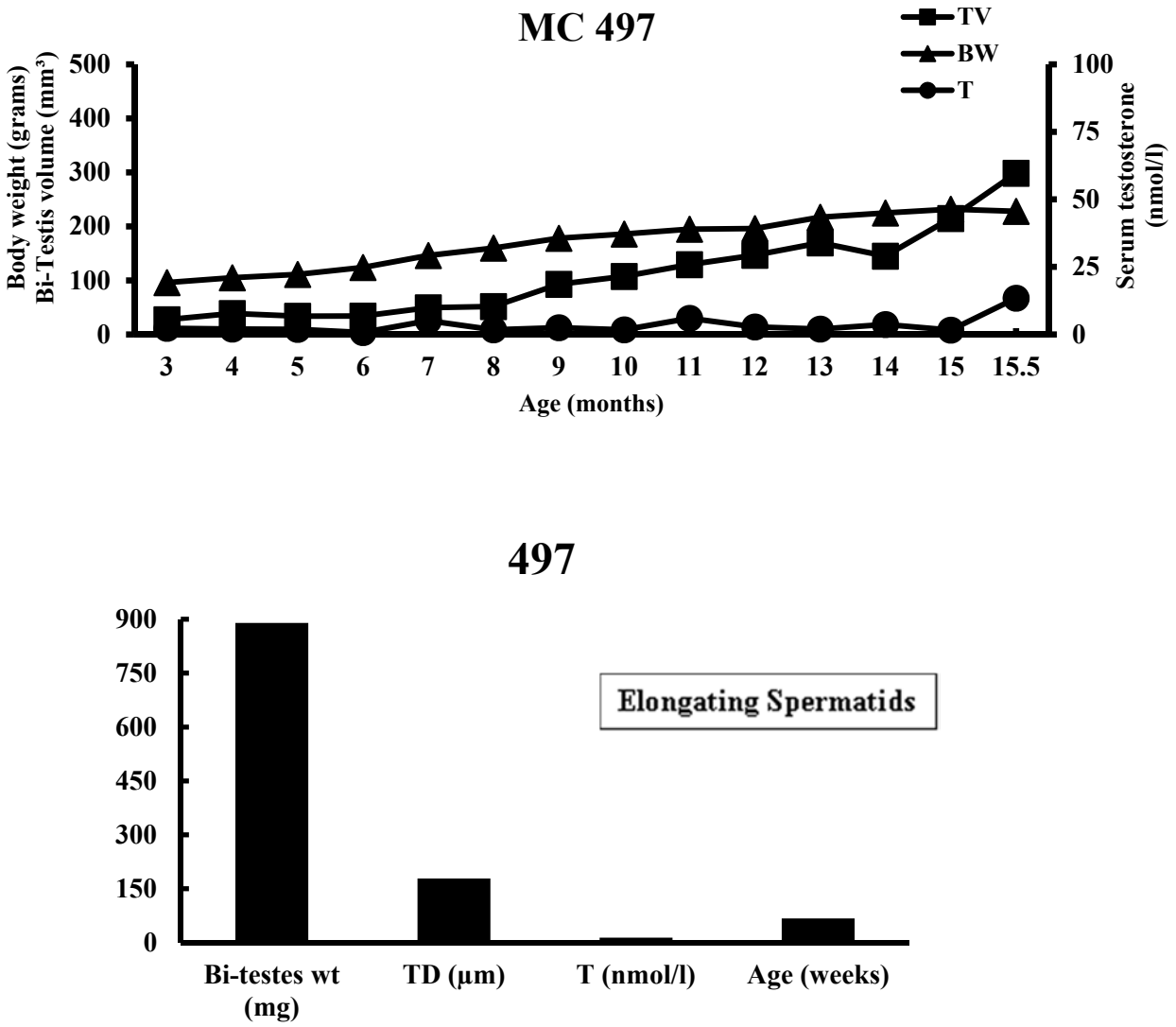


Figure 4.29 Body weight, serum testosterone and bi-testis volume from birth till 15.5 month of age in common marmoset (#497) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

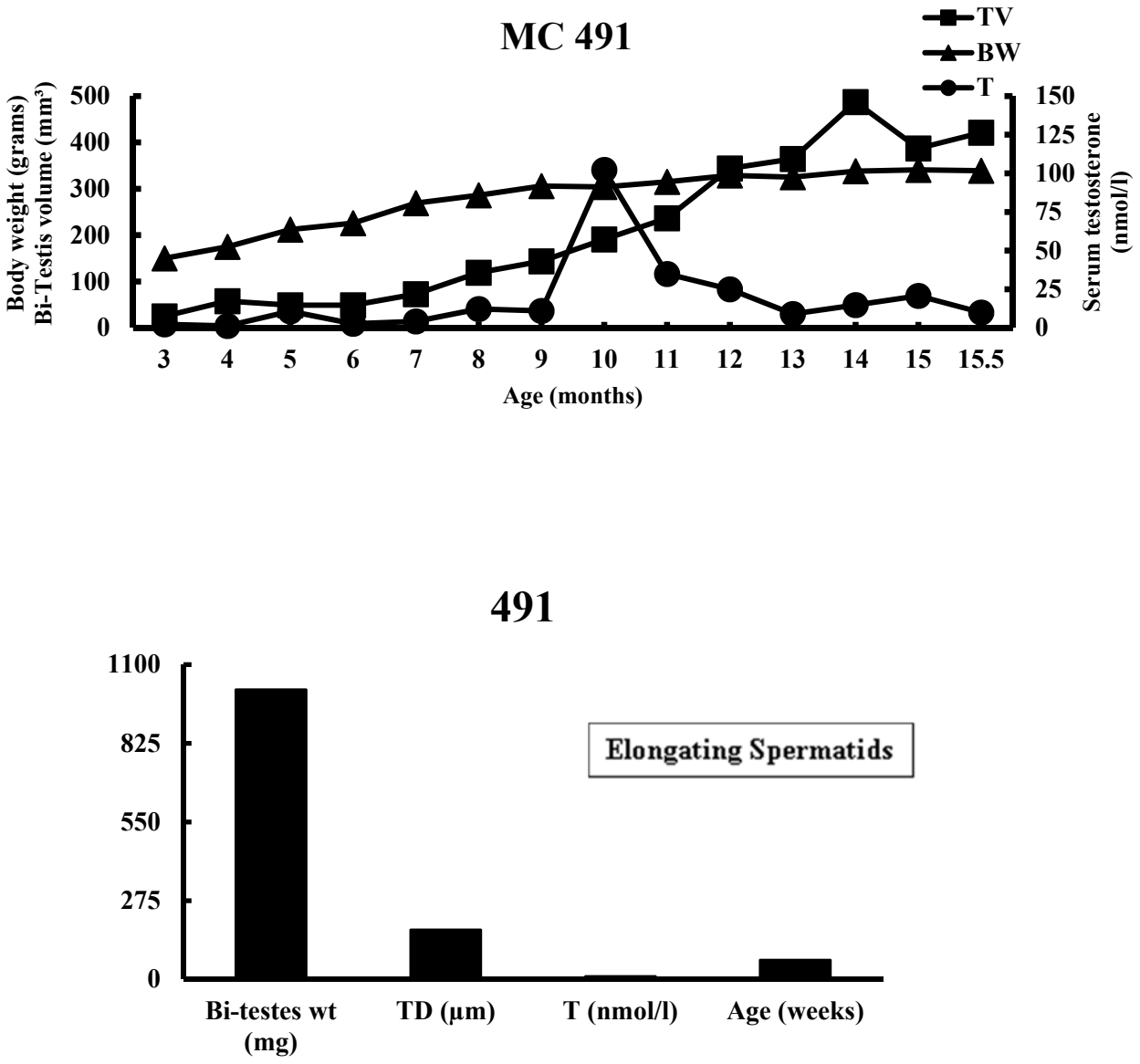


Figure 4.30 Body weight, serum testosterone and bi-testis volume from birth till 15.5 month of age in common marmoset (#491) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

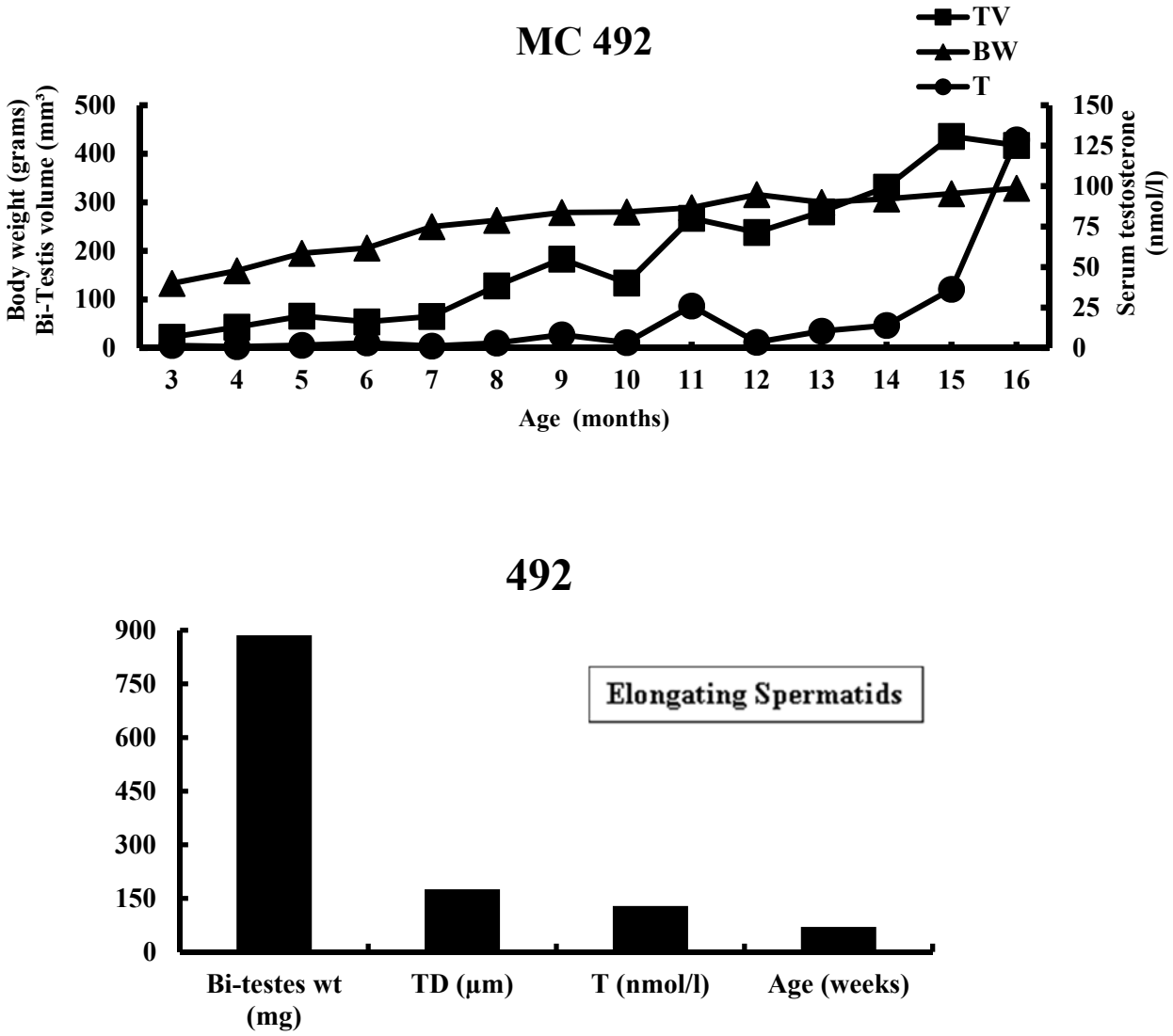
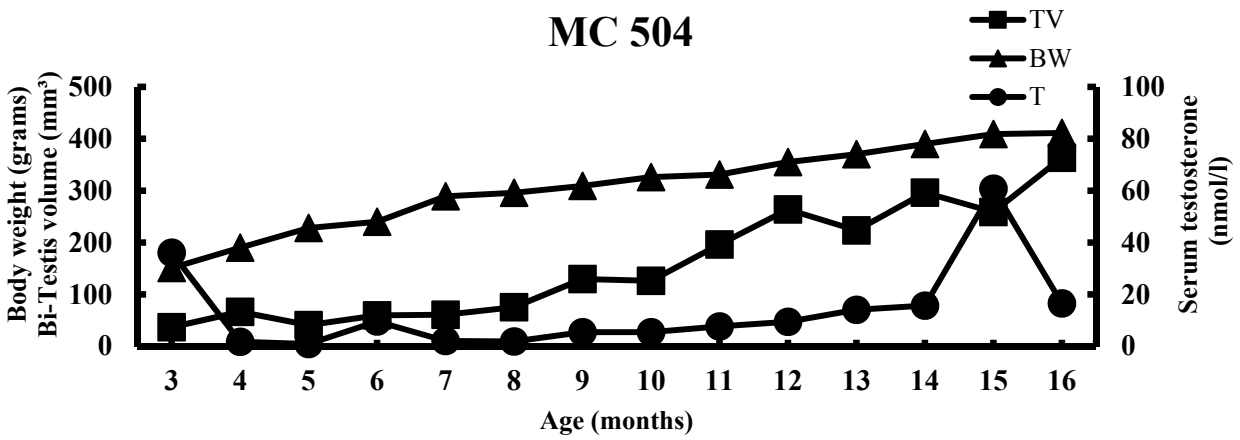


Figure 4.31 Body weight, serum testosterone and bi-testis volume from birth till 16 month of age in common marmoset (#492) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



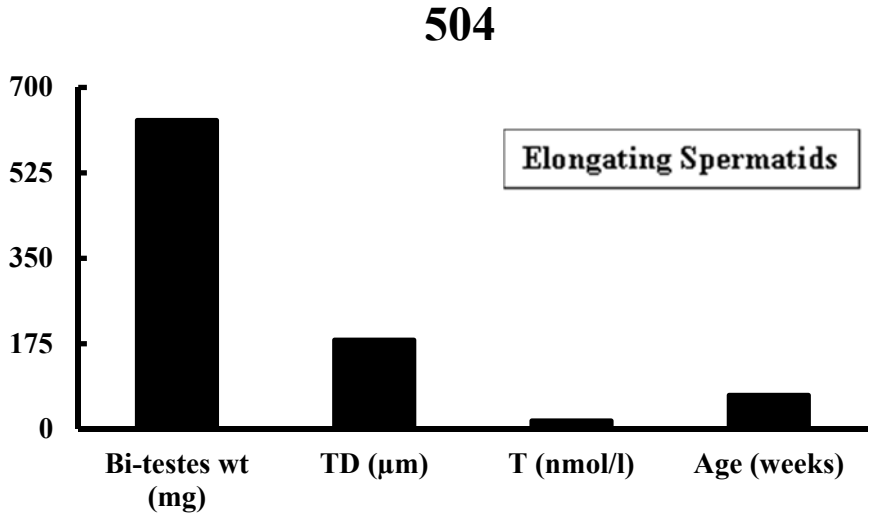


Figure 4.32 Body weight, serum testosterone and bi-testis volume from birth till 16 month of age in common marmoset (#504) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

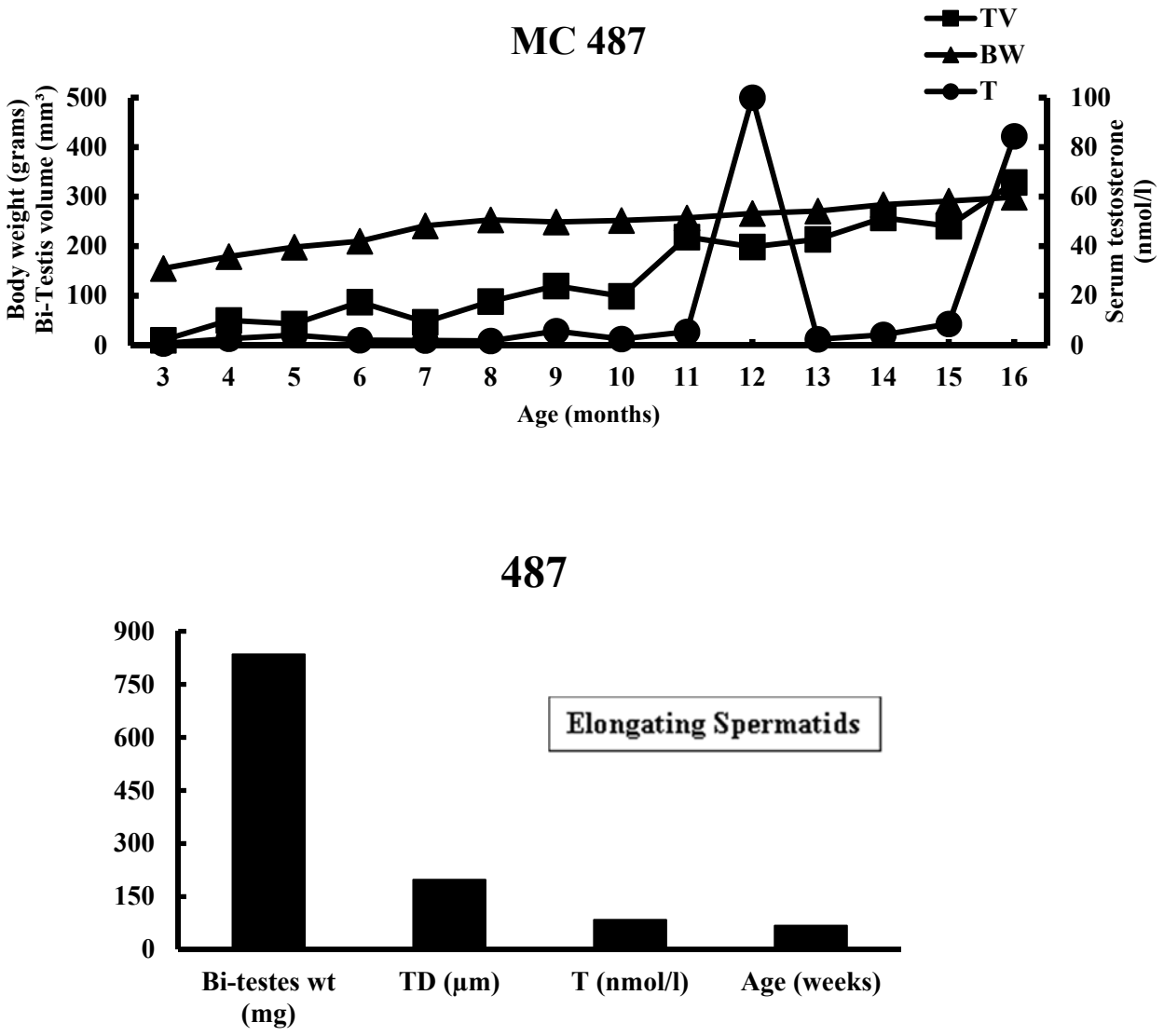


Figure 4.33 Body weight, serum testosterone and bi-testis volume from birth till 16 month of age in common marmoset (#487) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

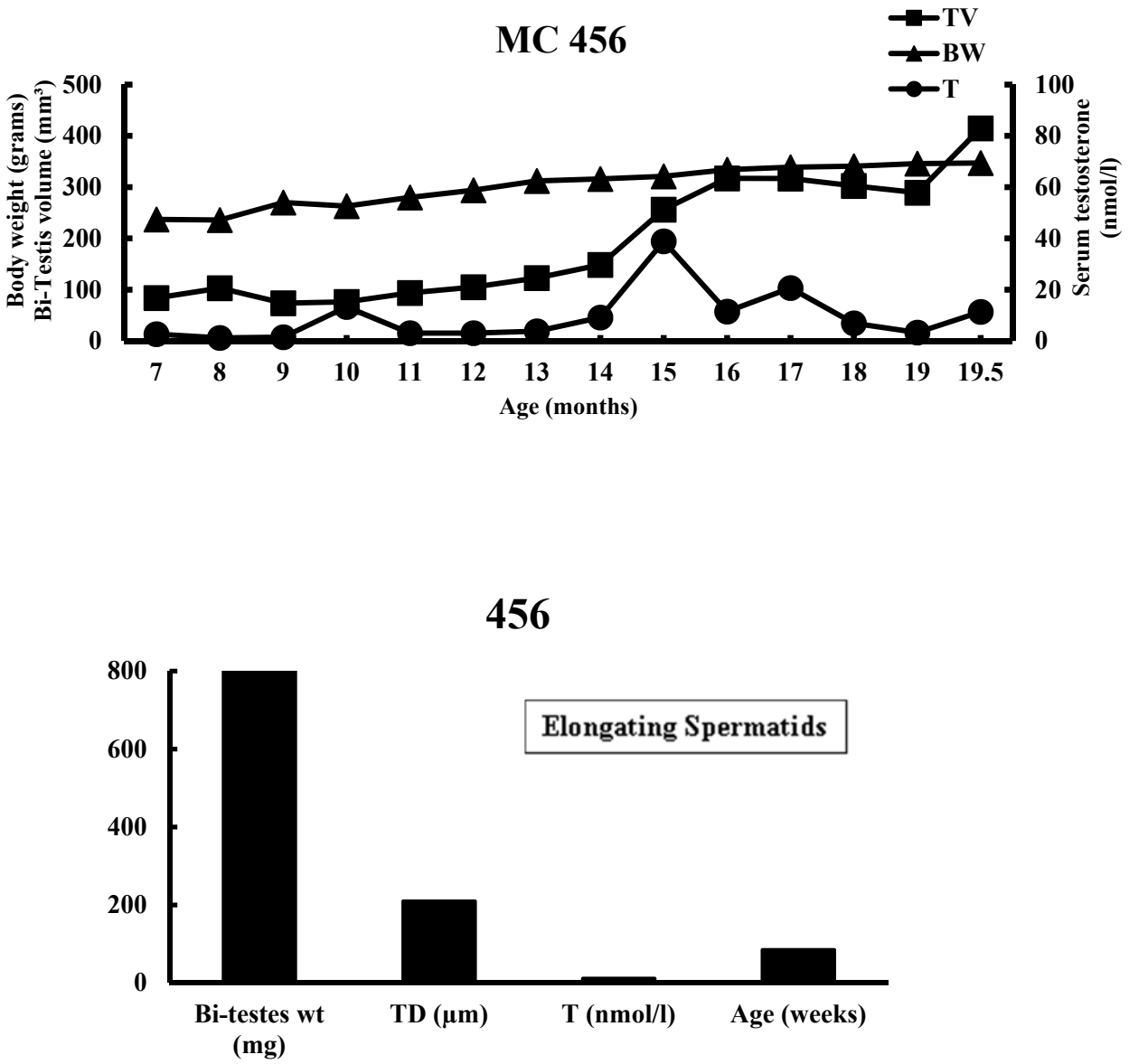


Figure 4.34 Body weight, serum testosterone and bi-testis volume from birth till 19.5 month of age in common marmoset (#456) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

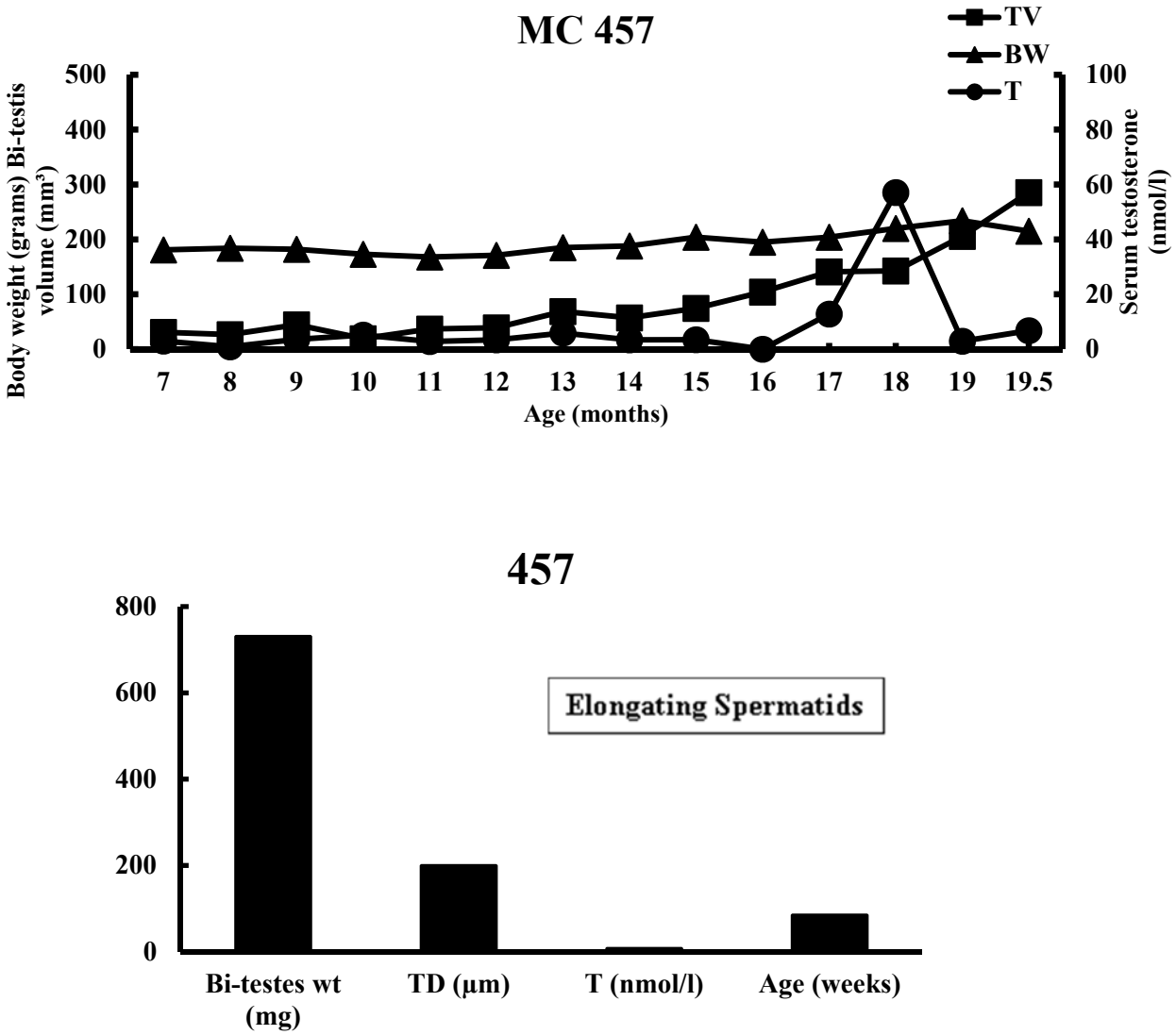


Figure 4.35 Body weight, serum testosterone and bi-testis volume from birth till 19.5 month of age in common marmoset (#457) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

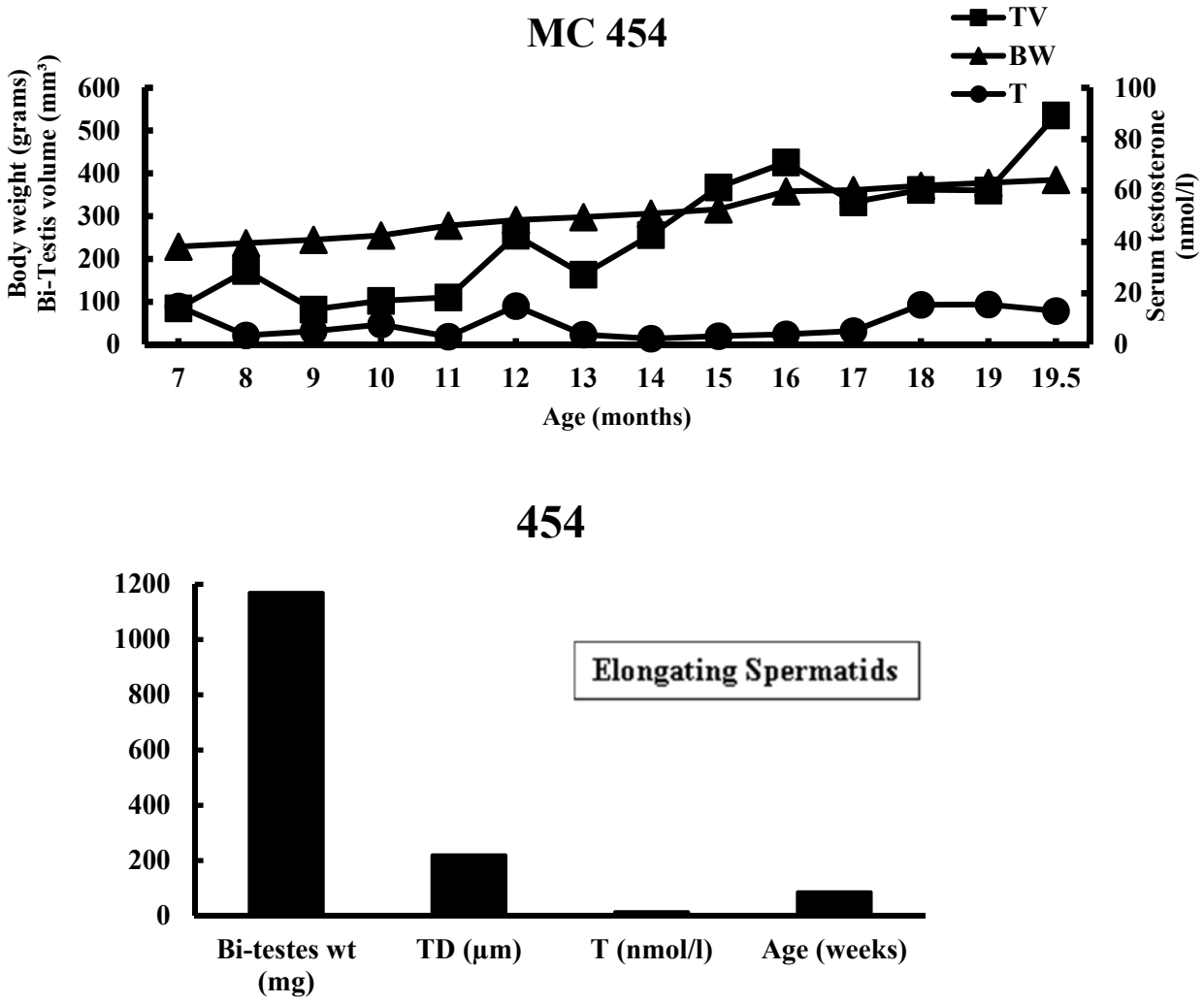
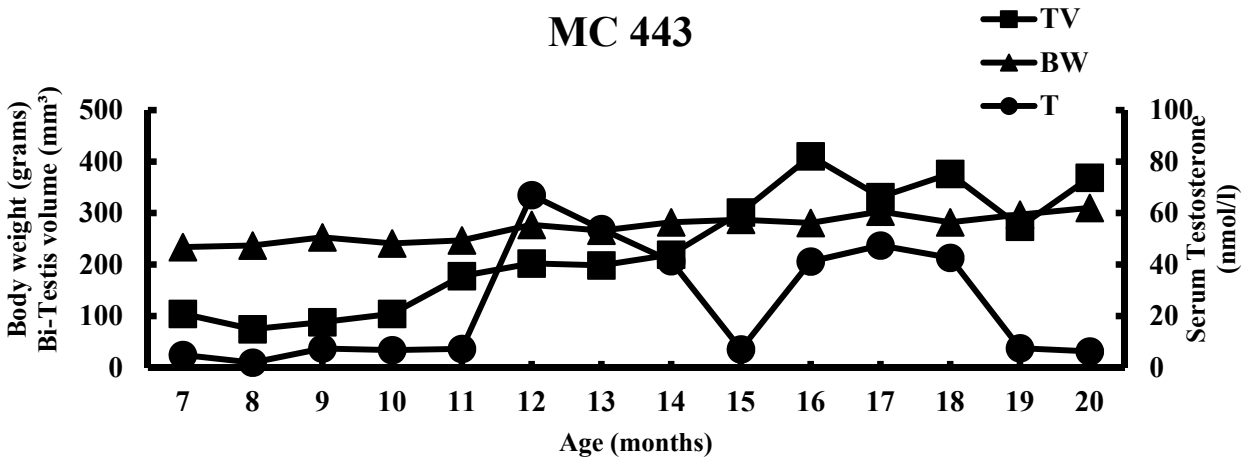


Figure 4.36 Body weight, serum testosterone and bi-testis volume from birth till 19.5 month of age in common marmoset (#454) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



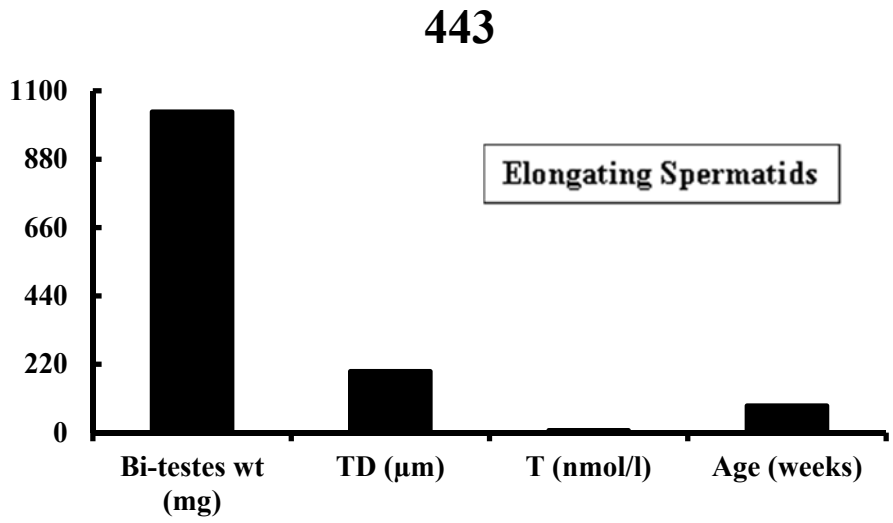


Figure 4.37 Body weight, serum testosterone and bi-testis volume from birth till 20 month of age in common marmoset (#443) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

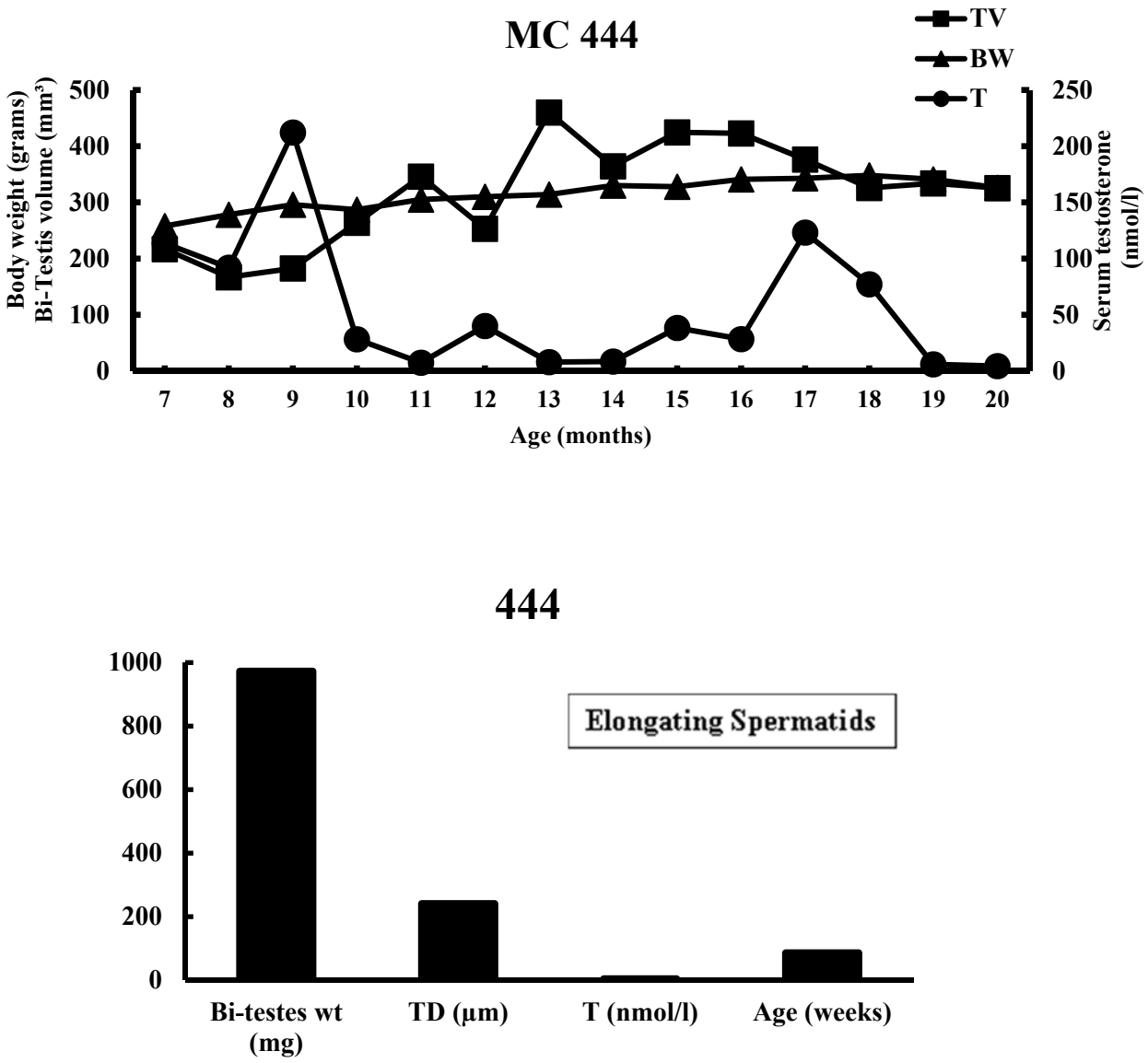


Figure 4.38 Body weight, serum testosterone and bi-testis volume from birth till 20 month of age in common marmoset (#444) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

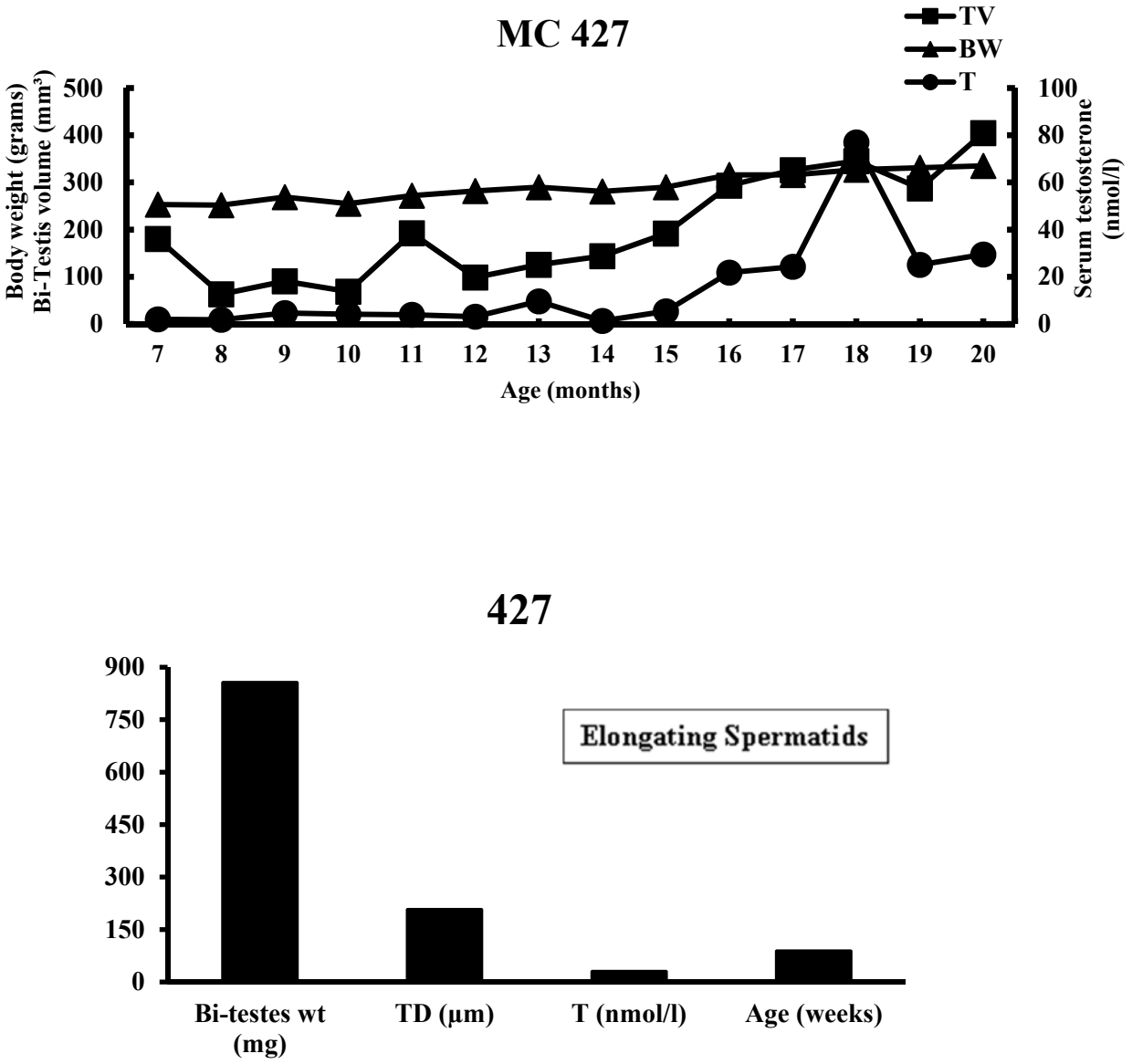
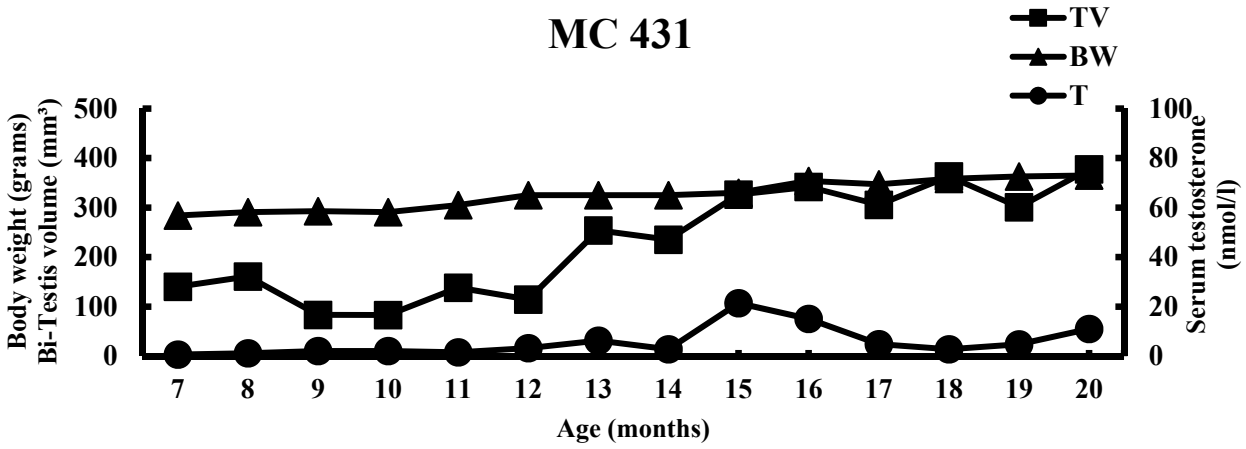


Figure 4.39 Body weight, serum testosterone and bi-testis volume from birth till 20 month of age in common marmoset (#427) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



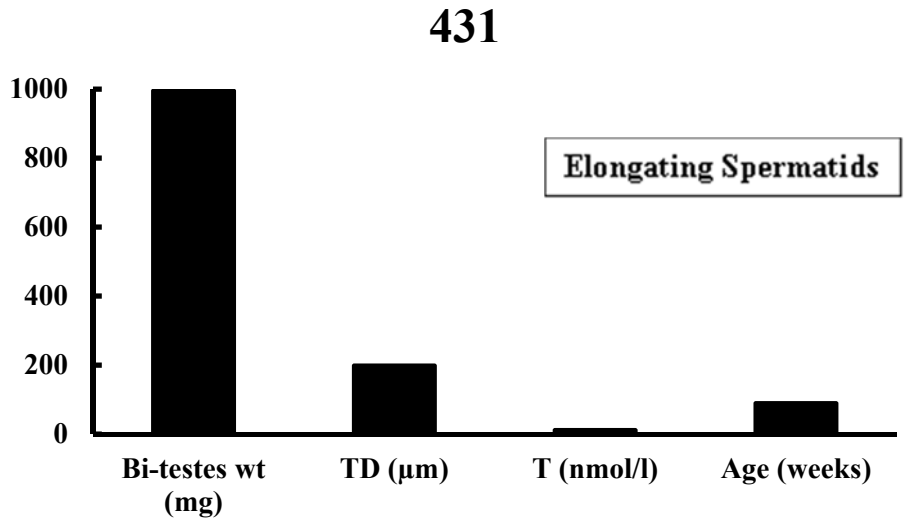
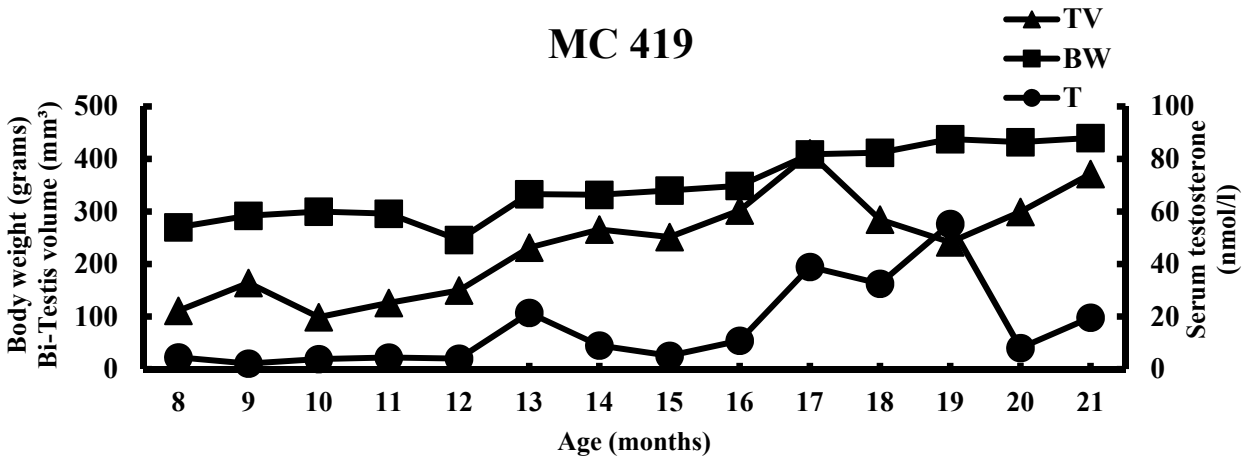


Figure 4.40 Body weight, serum testosterone and bi-testis volume from birth till 20 month of age in common marmoset (#431) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



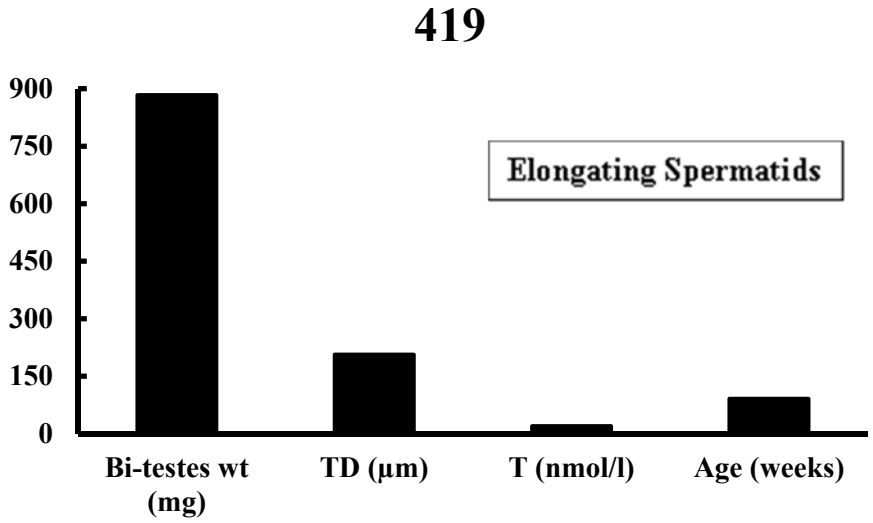
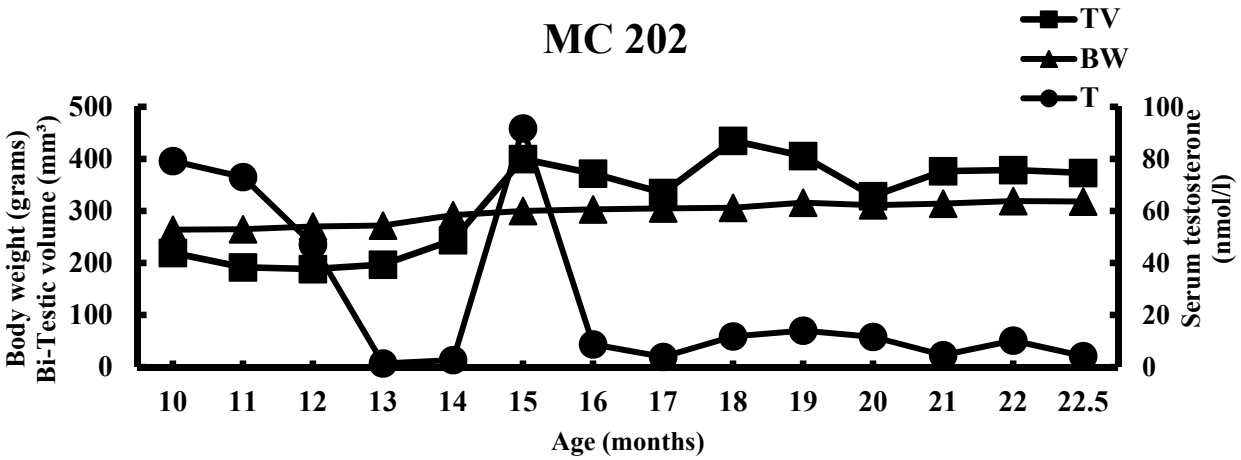


Figure 4.41 Body weight, serum testosterone and bi-testis volume from birth till 21 month of age in common marmoset (#419) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



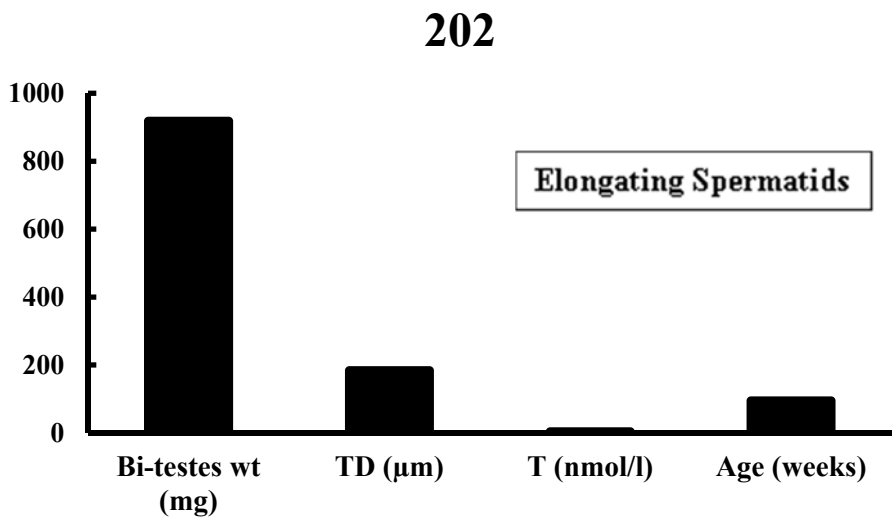
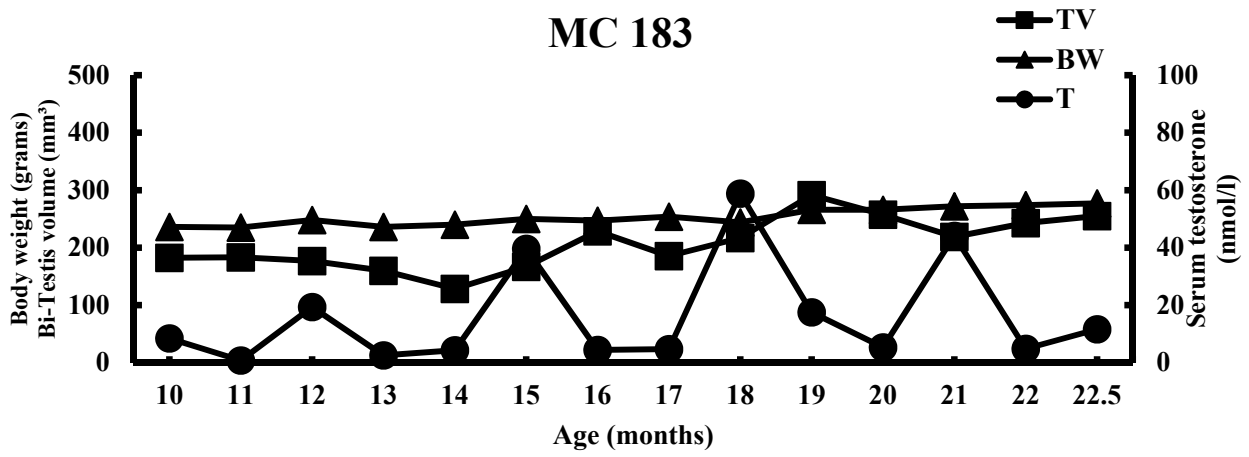


Figure 4.42 Body weight, serum testosterone and bi-testis volume from birth till 22.5 month of age in common marmoset (#202) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).



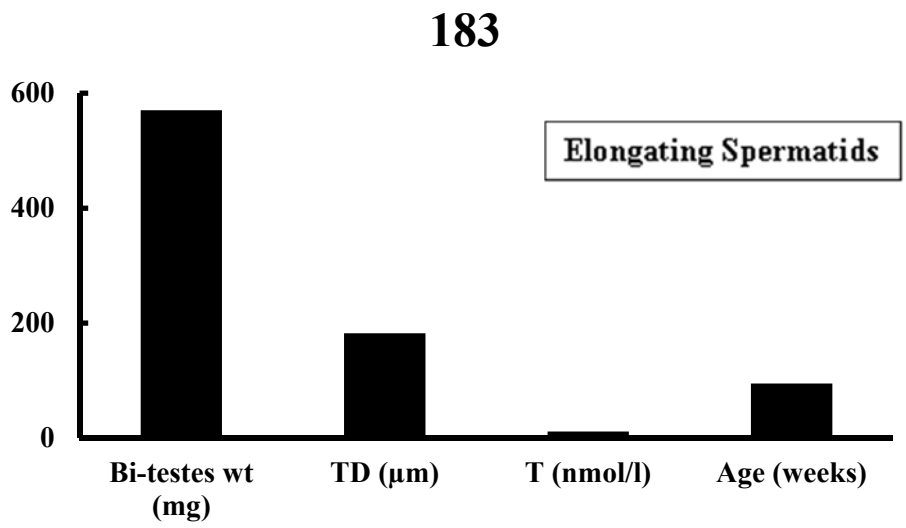


Figure 4.43 Body weight, serum testosterone and bi-testis volume from birth till 22.5 month of age in common marmoset (#183) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

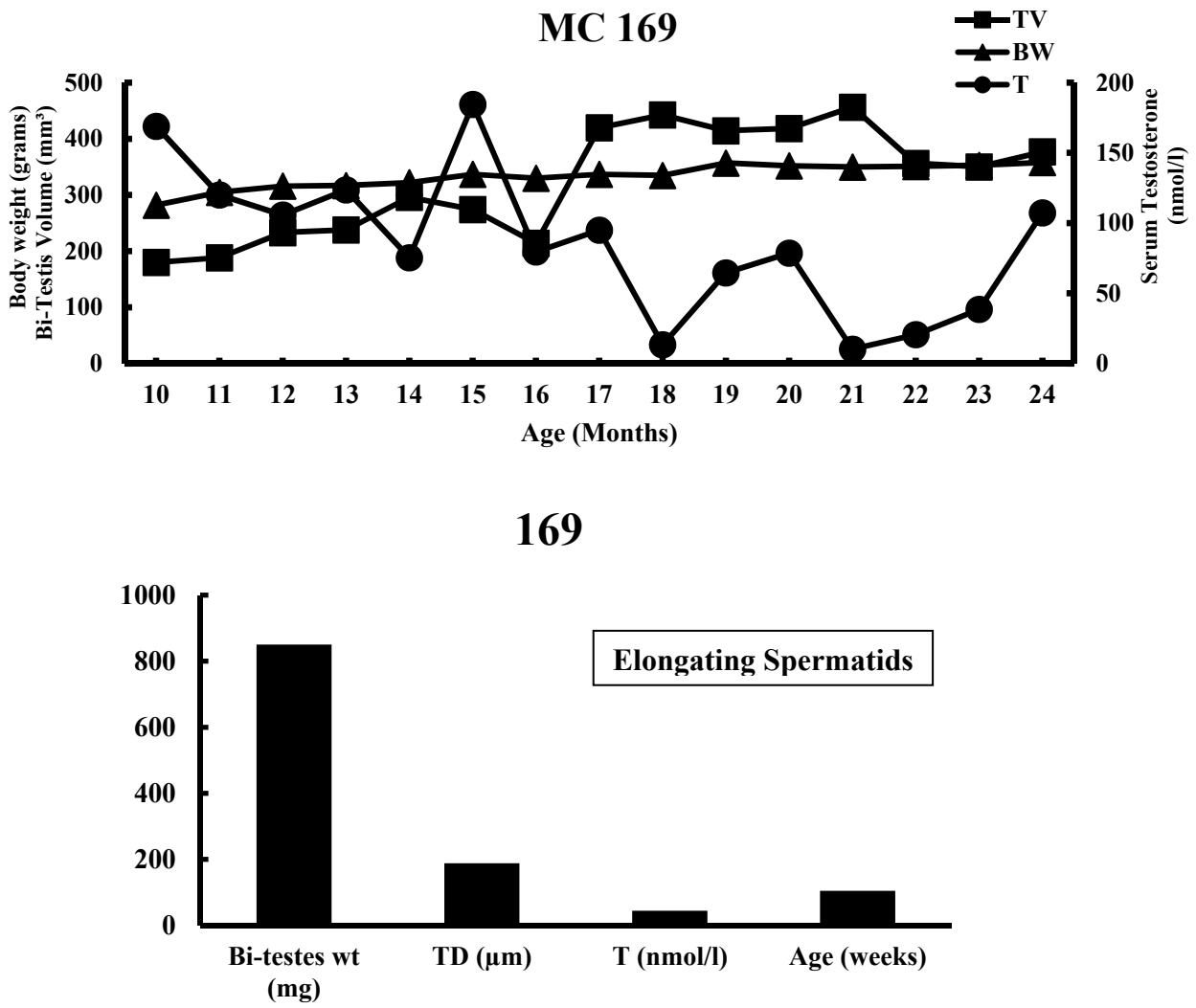


Figure 4.44 Body weight, serum testosterone and bi-testis volume from birth till 24 month of age in common marmoset (#169) (Line graph) and the bi-testis weight, serum testosterone on the day of sacrifice along with tubule diameter (Column graph) and most advanced germ cell type (Text box).

Discussion

The pubertal and testicular development in common marmoset was highlighted almost 30 years ago (Abbot and Hearn, 1978; Jackson and Edmonds, 1984). Since these classical reports the testicular characterization during development in common marmoset has been attempted regarding Sertoli cells and Leydig cell morphology (Rune et al., 1991; Rune et al., 1992). The observational attempts to unravel the ontogeny of pubertal onset in common marmoset has been scarce (Dixon, 1986; Chandolia et al 2006). Apart from these above mentioned studies, it is interesting to note here that nearly all of the other studies conducted on common marmoset to unravel the postnatal aspect of sexual development at the testis level were manipulative instead of observatory (Lunn et al., 1990, 1992, 1994, 1997; Sharpe et al., 2000, 2003).

The scarcity of the available observational data creates poor understanding of the pubertal and testicular development and poised a question on the validity of the data generated by studies involving hormonal manipulation. For example, Abbot and Hearn reported that testis volume and plasma testosterone began to rise on 250th day or 7th month of age (Abbott and Hearn, 1978). This postnatal time is considered as start of puberty and the end of the juvenile phase and is charatectrized by night time increase in LH pulses followed by an sudden increase in testis volumen in higher primates (Plant, 2005). Jackson and Edmonds pointed 60th week (14 months) of age to be considered as testicular maturity interms of accomplishement of spermatogenesis where all stages of spermatogonia were present (Jackson and Edmonds, 1984). On the other hand Kholkute et al (1983) considered 12-16 month old marmosets as prepubertal animals.

As a clear understanding of the process of primate puberty and the precise time period required for different post natal developmental stages in common marmoset is still under debate and vividly argued. We attempted to unravel the pubertal ontogeny interms of testicular growth in common marmoset. We selected an observatory approach where

the animals were not manipulated in any way. Thus a normal postnatal growth pattern was observed by collecting monthly samples for body weight, testis size and serum testosterone. We found out that the activation of hypothalamic pituitary gonadal axis in terms of the presence of high serum levels of testosterone in common marmoset occurs around (28 weeks ±2). Few animals did not show any testosterone rise till 10 or 12 months, although their testis size was comparable with animals which have shown testosterone rise at 7 month of age. As in common marmoset the predominant trigger for testosterone is a CG-like molecule having a short serum half life, unlike the LH in higher primates. We assumed that the animals, which failed to show any testosterone rise up till 10^{th} or 12^{th} of age, have actually already gone through the pubertal activation of testosterone but the testosterone peaks were missed in once a month blood sampling regime, as the CG-like molecule is active for a very short time in serum as compared with LH. Our assumption is also supported by the histomorphometric data where the animals with low serum testosterone depicted ongoing spermatogenesis at 10 and 12 month of age. We propose that for the correct analysis of the testosterone status of the 6-12 months old marmoset monkeys, weekly blood sampling regime should be employed because normally it is not possible to histologically analyze the testis tissue if the respective animals is to be employed for further experimentation. Our testosterone data also mimicked the classical findings documented by Abbot and Hearn (Abbott and Hearn, 1978). Although it was shown previously that in common marmoset the quiescent juvenile period lasts till 35 weeks (8.5 months) of age (Lunn et al., 1997; Kelnar et al., 2002). Our data concerning the body weight gain pattern depicted an important aspect of postnatal development in common marmoset. The maximum body weight gain had been observed well within the first 6 months of age, or in other words before the pubertal activation. After 6 months the animals showed a slow rise in the body weight. Either this initial weight is a prerequisite for the pubertal activation is unknown. It seems that growth and reproductive axis are controlled independently in common marmoset where the time courses for somatic and reproductive development do not overlap.

At the testicular level the major germ cell migration towards the periphery and the arrangement of Sertoli cell nuclei in an epithelial like fashion seems to be completed well within the first 6 months of development. After 6 month of age the seminiferous tubules

in common marmoset showed a rapid response towards the high serum levels of testosterone by the formation of a lumen around 28 weeks (7 month) of age. The formation of the lumen is indicative of the fluid secretion from Sertoli cells which attain functional maturity characterized by an increase in Sertoli cell cytoplasm (Sharp et al., 2003). As depicted by the point counting, due to the increase in size the of the Sertoli cells, the percentage of the epithelial area also increased sharply after 7 month of age. The formation of lumen and the increase in the Sertoli cell size collectively increase the seminiferous tubule diameter at the start of the puberty. The increase in tubule diameter as well as in the testis volume observed after 44 week (10 months) was mainly due to the proliferation of germ cell as the B spermatogonia, primary spermatocytes and secondary spermatocytes were observed during this time. So the initial increase in testis size observed at 7 month of age is caused by somatic activation while the increase in testis size observed after 8-10 months of age is due to the increase in the germ cell compliment. Sperm were first observed in the epididymis at 12 months of age and the testis weight and testis volume increased almost two fold as compared with the 10 month old age group. This sudden increase in testis volume at 12 month of age is not the start of puberty in common marmoset. Thus a one year old marmoset should be considered a pubertal animal, although the testis volume continues to increase after this age but the spermatogenesis is qualitatively achieved at this time. So the time from the normal onset of puberty till the presence of epididymal sperm in common marmoset seems to be around 5 months. This time period is very short as compared with higher primates and also shows that testicular cells in common marmosets are normally capable of a swift response towards pituitary gonadotropins. Our endocrine, orchidectomy and histological data in common marmoset suggest a similar pattern of pubertal testicular development towards higher primates but requiring a significantly less time to achieve these developmental features as their higher primate counterparts.

In conclusion the puberty in common marmoset is not initiated before 6 months of age. Pubertal onset is preceded by a period of accelerated somatic growth. The hypothalamic control of the somatic and reproductive axis seems to be in a chronological order where the pubertal growth comes later. Either this early weight gain is a prerequisite for the normal pubertal initiation remains to be answered. The Sertoli

arrangement and germ cell migration seems to be completed before the onset of puberty in common marmoset. Further studies on the involvement of kisspeptin in pubertal activation, dynamics of GnRH and CG interplay at the pituitary level, the timing of the androgen expression by developing Sertoli cells and the hormonal regulation of germ cell development are needed to fully characterize hypothalamic and gonadal ontogeny of pubertal development in common marmoset.

Chapter 5

General Discussion

General Discussion

The current thesis focuses on functional and developmental aspects of the primate testis. The specific aims and objectives of the present study were firstly to sort out a possible functional role of kisspeptin-kisspeptin receptor signaling in adult primate testis, secondly to sort out the cytological localization of the kisspeptin receptor in the adult primate testis and thirdly to characterize the pubertal testicular development in common marmoset: a new world monkey. To achieve these objectives, three separate experiments were conducted using an in vivo approach. For the first two objectives adult animals were employed whereas for the third objective immature animals were used. The animal models selected in the current study included Old World monkeys and New World monkeys. Rhesus monkey (*Macaca mulatta*): a representative higher primate was employed for the first and second objective whereas common marmoset (*Callithrix jacchus*) was employed in the second and third objective.

The primate testis performs two main functions namely spermatogenesis and steroidogenesis where successful spermatogenesis is dependent on the availability of testosterone through the process of steroidogenesis. The first objective which is the functional aspect of kisspeptin-kisspeptin receptor signaling in the adult primate testis was sorted out in terms of the ability of the kisspeptin to affect the testosterone secretion (steroidogenesis). It is interesting to note that the kisspeptin has the ability to enhance the serum levels of testosterone but this increase in the testosterone levels is due an increase in the LH levels. Increased LH secretion is in turn occasioned by action of kisspeptin at the hypothalamus resulting in an enhanced GnRH release. In general, to observe an effect of a given iv bolus on the testosterone secretion which is solely at the testis level, it is a prerequisite that the testis should not be exposed to pituitary gonadotropins.

In the first experiment, to observe an effect of kisspeptin which is solely at the testis level and to prevent the kisspeptin to influence pituitary LH secretion, pituitary gonadotropin clamped monkey model was employed. The chemical hypophysectomy was employed and achieved by pretreating the animals with acyline, a GnRH receptor antagonist. In the pituitary clamped monkey model, the kisspeptin (50μg) given as an iv bolus significantly enhances stimulated testosterone levels while having no effect on the basal testosterone levels. This result demonstrates that kisspeptin does not affect Leydig cells directly, as the direct action of kisspeptin at the Leydig cells would result in an effect on the basal testosterone levels. Although the observation of an effect on the stimulated testosterone levels indicates the presence of a functional kisspeptin-kisspeptin receptor signaling cascade which can affect the Leydig cell responsiveness towards LH/hCG. However, it seems that kisspeptin-kisspeptin receptor signaling can only influence the Leydig cell steroidogenesis in the presence of LH/hCG. These results also hint towards an indirect action of kisspeptin on Leydig cells via another testicular cell type. As the paracrine interaction exist between different testicular cell types and this local communication is necessary for the proper functioning of the testis (Schlatt et al., 1997). So it is plausible that kisspeptin-kisspeptin receptor signaling affect Leydig cell steroidogenesis indirectly through another testicular cell type. Sertoli cell, germ cell and peritubular myoid cell are the other cell types present in the testis where Sertoli cells has been shown to secrete factors which have the ability to modify Leydig cell steroidogenesis (Saez et al., 1989). It has been demonstrated in vitro that Sertoli cell factors increased Leydig cell steroidogenesis and this effect was augmented in the presence of LH or hCG (Papadopoulus et al., 1987; Papadopolus, 1991; Sharpe, 1985).

The second objective was to find out the testicular cell type positive for kisspeptin receptor. To localize the specific cell type immunocytochemistry was employed on the testis tissue obtained from two adult rhesus monkeys as well as two adult common marmoset monkeys. The testicular immunocytochemistry revealed that the kisspeptin receptor is localized inside the seminiferous tubules. The area found positive for the kisspeptin receptor was the outer periphery of the seminiferous tubules. This area contains the undifferentiated A spermatogonia along with the basal surface of the Sertoli cells. The detailed analysis revealed

that Sertoli cell membrane around the A spermatogonia exhibited strong staining for kisspeptin receptor. The same staining pattern was also found in the new world monkey testis. The kisspeptin like immunoreactivity was found to be present in the interstitial area. It has been shown previously that interstitial area was positive for kisspeptin like immunoreactivity in mouse and vertebrate testis (Anjum et al., 2012; Chianese et al., 2013: Hua et al., 2013). In particular Leydig cell were found positive for kisspeptin in these above mentioned studies. These immunocytochemical findings not only support our previous finding of an indirect action of kisspeptin on Leydig cell steroidogenesis but might also reveal a much unexpected and important aspect of kisspeptin-kisspeptin receptor signaling at the testis level. As kisspeptin was initially discovered as an anti-metastatic peptide, initially called metastin found in the metastatic cancerous tissue (Lee et al., 1996; Lee and Welch, 1997). Kisspeptin has been shown to influence metastasis through its antimetastatic effects by inhibiting chemotaxis and cell migration (Hori et al., 2001; Yan et al., 2001; Harms et al., 2003; Nash and Welch, 2006; Navenot et al., 2009). During the testicular development the germ cell migrate from the center of the tubules towards the periphery. During migration, these cells are called gonocytes (Culty, 2009) and once they reach the periphery and establish contact with the basement membrane they are known as undifferentiated A spermatogonia (Plant, 2010). It is not clear that whether these cells inherently lost the migratory ability once they establish the contact with basement membrane or this migratory ability is somehow compromised through the action of another cell i.e. Sertoli cell. As the peripheral area of seminiferous tubules which exhibited strong signal for kisspeptin receptor has also been implicated as spermatogonial stem cell niche (de Rooij, 2009). It seems that kisspeptin receptor is the new candidate for the spermatogonial stem cell niche region. Further studies are needed to delineate the precise role of kisspeptin receptor as a niche player in primate testis. Keeping in view the finding of an efficacious intratesticular action of kisspeptin on stimulated testosterone and the immunocytochemical finding of a tubular localization for kisspeptin receptor where Sertoli cells were found to be the main cell type positive for kisspeptin

receptor signal, it is probable that kisspeptin might act on the Sertoli cells via kisspeptin receptor and the receptor activation initiates a secretory response from the Sertoli cells which in turn enhances the responsiveness of the Leydig cells towards LH/hCG. This notion is supported by previous studies where Sertoli cell produced factors have been shown to influence testosterone secretion from the Leydig cell or affect Leydig cell response towards gonadotropins (reviewed in Lejuene et al., 1992).

The third objective of the study was to characterize the pubertal and subsequent testicular development in common marmoset (*Callithrix jacchus*): a representative New World monkey model. Common marmoset exhibits unique reproductive endocrinology in terms of the presence of a chorionic gonadotropin (CG) like molecule instead of LH in HPG axis interplay. This CG-like molecule has a much shorter serum half life as compared with LH (Muller at al., 2004). Combined with the classical report about the different steroidogenic pathway ($\Delta 4$) used by marmoset leydig cells compared with higher primates including man (Preslock and steinberger, 1977). And the lack of the availability of functional data on the dynamics of hypothalamic-pituitary-gonadal axis during puberty in the presence of CG instead of LH not only obscure the recent efforts to associate pituitary and testicular interplay during pubertal development in common marmoset (Chandolia et al., 2006) but also demands different approach towards understanding the pubertal ontogeny in common marmoset. Our results demonstrated that the earliest pubertal onset characterized by high serum testosterone levels in common marmoset was found to occur around 7 months of age. The major body weight gain was observed in the first 6 months employing that the somatic growth and pubertal growth do not coincide in this monkey. The puberty in higher primates is preceded by a juvenile phase of development characterized by stable testis volume, low serum testosterone and a rapid body weight gain (Plant, 2005, 2006). Common marmoset shows a somewhat higher primate like pattern of postnatal development although higher primates require much longer time to reach puberty whereas the common marmoset require significantly less time, depicting a highly responsive testicular tissue towards pituitary gonadotropins. At the testis level the time period from the first

testosterone peak to the presence of epididymal sperm was around 5 months. These 5 months are characterized by rigorous somatic and germ cell activity at the testis. While the major cellular activity shown by a prepubertal and juvenile testis is in terms of gonocyte migration from the center of the seminiferous tubules towards the periphery. This migratory activity was completed before the onset of puberty indicating that the germ cell migration is an innate ability of gonocytes to reach the peripheral areas of the seminiferous tubules and is also independent of HPG axis interplay. This feature had been documented before and found to be highly similar with gonocytes migration in human testis (Mitchell et al., 2008). In conclusion common marmoset has a swift progression from an immature state to a mature stage and one year old marmoset testis show qualitatively established spermatogenesis although the quantitative spermatogenic out continue to increase after one year of age.

The overall conclusion of the current thesis is that the non human primates offer an excellent model to study the primate testis biology as the testicular morphology and functionally is preserved across non-human primate models. The findings of the current thesis emphasize that further studies are needed to delineate the involvement of kisspeptin-kisspeptin receptor signaling in the Sertoli cell functionality by using pharmacological approaches in *in vitro* and organ culture model. Also the possible involvement of kisspeptin receptor in spermatogonial stem cell niche in the context of germ cell migratory ability in the immature testis as well as its role as an anti migratory signal for the A spermatogonia in the adult primate testis needs to be examined. In common marmoset, studies are needed to characterize the endocrine requirements for the pubertal onset in terms of kisspeptin signaling at hypothalamic level as well as the kinetics of the gonadotropic secretory activity from the pituitary gland during pubertal activation and the downstream requirement by LH/CG receptor present on Leydig cells for activation and producing an adequate androgenic response during pubertal activation.

Chapter 6

References

REFRENCES

Abbott DH, Barnett DK, Colman RJ, Yamamoto ME, Schultz-Darken NJ 2003 Aspects of common marmoset basic biology and life history important for biomedical research. Comp Med 53(4):339-350

Abbott DH and Hearn JP 1978 Physical, hormonal and behavioural aspects of sexual development in the marmoset monkey, *Callithrix jacchus*. J Reprod Fertil 53:155–166

Abbott DH, Hodges JK, George LM 1988 Social status controls LH secretion and ovulation in female marmoset monkeys (*Callithrix jacchus*). J Endocrinol 117(3):329-339

Albert S, Ehmcke J, Wistuba J, Eildermann K, Behr R, Schlatt S, Gromoll J 2010 Germ cell dynamics in the testis of the postnatal common marmoset monkey (*Callithrix jacchus*). Reproduction 140(5): 733-742

Anjum S, Krishna A, Sridaran R, Tsutsui K 2012 Localization of gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin and GnRH receptor and their possible roles in testicular activities from birth to senescence in mice. J Exp Zool A Ecol Genet Physiol 317(10):630-644

Arslan M and Qazi MH 1976 Effect of hCG on testicular function in juvenile rhesus monkey (*Macaca mulatta*). J Reprod Fertil 48: 181-183

Arslan M, Weinbauer GF, Schlatt S, Shahab M, Nieschlag E 1993 FSH and testosterone, alone or in combination, initiate testicular growth and increase the number of spermatogonia and Sertoli cells in a juvenile non-human primate (*Macaca mulatta*). J Endocrinol 136(2): 235-243

Arslan M, Zaidi P, Akhtar FB, Amin S, Rana T, Qazi MH 1981 Effects of gonadotrophin treatment in vivo on testicular function in immature rhesus monkeys (*Macaca mulatta*). Int J Androl 4(4): 462-474

Ascoli M, Fanelli F, Segaloff DL 2002 The lutropin/choriogonadotropin receptor, a 2002 perspective. Endocr Rev 23, 141–174.

Austad SN, Fischer KE 2011 The development of small primate models for aging research. ILAR J 52(1):78-88

Bambino TH, Schreiber JR, Hsueh AJ 1980 Gonadotropin-releasing hormone and its agonist inhibit testicular luteinizing hormone receptor and steroidogenesis in immature and adult hypophysectomized rats. Endocrinology. 107(4):908-917

Baker JV, Abbott DH, Saltzman W 1999 Social determinants of reproductive failure in male common marmosets housed with their natal family. Anim Behav 58(3):501-513

Bercu BB, Lee BC, Pineda JL, Spiliotis BE, Denman DW, Hoffman HJ, Brown TJ, Sachs HC 1983 Male sexual development in the monkey. I. Cross-sectional analysis of pulsatile hypothalamic-pituitary-testicular function. J Clin Endocrinol Metab 56(6): 1214-1226

Bernatein IR, Ruehlmann TE, Judge PG, Lindquist T, Weed JL 1991 Testosterone changes during the period of adolescence in male rhesus monkeys (*Macaca mulatta*). Amer J Primatol 24: 29-38

Bourne GA, Regiani S, Payne AH, Marshall JC 1980 Testicular GnRH receptors--characterization and localization on interstitial tissue. J Clin Endocrinol Metab 51(2):407-409

Carrion R Jr, Patterson JL 2012 An animal model that reflects human disease: the common marmoset (*Callithrix jacchus*). Curr Opin Virol 2(3):357-362

Cavicchia JC and Dym M 1978 Ultrastructural characteristics of monkey spermatogonia and preleptotene spermatocytes. Biol Reprod 18(2):219-228

Chandolia RK, Luetjens CM, Wistuba J, Yeung CH, Nieschlag E, Simoni M 2005 Changes in endocrine profile and reproductive organs during puberty in the male marmoset monkey (*Callithrix jacchus*). Reproduction 132(2):355-363

Chemes HE 2001 Infancy is not a quiescent period of testicular development. Int J Androl 24(1): 2-7

Chianese R, Ciaramella V, Fasano S, Pierantoni R, Meccariello R 2013 Kisspeptin receptor, GPR54, as a candidate for the regulation of testicular activity in the frog *Rana esculenta*. Biol Reprod 88(3):73-77.

Culty M 2009 Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res (C) 87(1):1-26.

Dang DC and Meusy-Dessolle N 1985 Quantitative study of testis histology and plasma androgens at onset of spermatogenesis in the prepuberal laboratory-born macaque (*Macaca fascicularis*). Arch Androl 12 Suppl: 43-51

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

de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci USA 100(19):10972-10976

de Rooij DG 2009 The spermatogonial stem cell niche. Microsc Res Tech 72(8):580-585

Dockray GJ 2004 The expanding family of -RFamide peptides and their effects on feeding behaviour. Exp Physiol 89(3):229-235

Dixson AF 1986 Plasma testosterone concentrations during postnatal development in the male common marmoset. Folia Primatologica (Basel) 47: 166–170

Dym M 1974 The fine structure of the monkey (*Macaca mulatta*) Sertoli cell and its role in maintaining the blood-testis barrier. Anat Rec 175: 639-656

Dym M and Fawcett DW 1970 The blood–testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol Reprod 3, 308–326.

Eik-Nes KB, Hall PF 1965 Secretion of steroid hormones in vivo. Vitam Horm 23, 153–208

Ehmcke J and Schlatt S 2006 A revised model for spermatogonial expansion in man: lessons from non-human primates. Reproduction 132(5): 673-80

Ehmcke J, Luetjens CM, Schlatt S 2005 Clonal organization of proliferating spermatogonial stem cells in adult males of two species of non-human primates, *Macaca mulatta* and *Callithrix jacchus*. Biol Reprod 72(2):293-300

Eslamboli A 2005 Marmoset monkey models of Parkinson's disease: which model, when and why? Brain Res Bull 68(3):140-149

Fawcett DW 1975 Ultrastructure and function of the Sertli cell. In "Handbook of Physiology Male Reproductive System" Vol 5, Section 7 Endocrinology, Eds D. Hamilton and R. O. Greep, Waverly Press, Baltimore, USA

Fawcett D W and Burgos M 1956 The fine structure of the Sertoli cells in the human testis. Anat Rec 124: 401–402

Fraser MO, Pohl CR, Plant TM 1989 The hypogonadotropic state of the prepubertal male rhesus monkey (*Macaca mulatta*) is not associated with a decrease in hypothalamic gonadotropin-releasing hormone content. Biol Reprod 40, 972-980

Frawley LS and Neill JD 1979 Age related changes in serum levels of gonadotropins and testosterone in infantile male rhesus monkeys. Biol Reprod 20(5):1147-1151

Fouquet JP, Meusy-Dessolle N, Dang DC 1984 Relationship between Leydig cell morphometry and plasma testosterone during postnatal development of the monkey, *Macaca fuscicularis*. Reprod Nut Develop 24(3): 281-196

Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, Yang S, Monsma FJ, Gustafson EL 2003 The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem Biophys Res Commun 312(4):1357-1363

Galdieri M, Monaco L, Stefanini M. 1984 Secretion of androgen binding protein by Sertoli cells is influenced by contact with germ cells. J Androl 5(6):409-15

Gassei K and Schlatt S 2007 Testicular morphogenesis: comparison of in vivo and in vitro models to study male gonadal development. Ann NY Acad Sci 1120:152-167

Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA 2004 A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145(9):4073-4077

Griswold MD 1998 The central role of Sertoli cells in spermatogenesis. Semin Cell Dev Biol 9(4), 411–416

Griswold MD 1995 Interactions between germ cells and Sertoli cells in the testis. Biol Reprod 52(2), 211–216

Griswold MD1988 Protein secretions of Sertoli cells. Int Rev Cytol 110, 133–156

Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE 2005 Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 25(49):11349-11356

Harms JF, Welch DR, Miele ME 2003 KISS1 metastasis suppression and emergent pathways. Clin Exp Metastasis 20(1):11-8

Haas GG Jr, D'Cruz OJ, De Bault LE 1988 Distribution of human leukocyte antigen-ABC and -D/DR antigens in the unfixed human testis. Am J Reprod Immunol Microbiol 18(2):47-51

Heckert LL, Griswold MD 2002 The expression of the follicle-stimulating hormone receptor in spermatogenesis. Recent Prog Horm Res 57, 129–148

Hess RA and Franca LR 2005 Structure of Sertoli cell. In “Sertoli cell biology” Eds, Skinner MK and Griswold MD, Elsevier Science, USA, pp 19-40.

Herbst KL, Anawalt BD, Amory JK, Bremner WJ 2002 Acyline: the first study in humans of a potent, new gonadotropin-releasing hormone antagonist. J Clin Endocrinol Metab 87(7):3215-3220

Higashi Y, Takahashi J, Yoshida K, Winters SJ, Oshima H, Troen P 1984 Seasonal changes in steroidogenesis in the testis of the rhesus monkey (*Macaca mulatta*). J Androl 5(2):70-79

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

Hori A, Honda S, Asada M, Ohtaki T, Oda K, Watanabe T, Shintani Y, Yamada T, Suenaga M, Kitada C, Onda H, Kurokawa T, Nishimura O, Fujino M 2001 Metastin suppresses the motility and growth of CHO cells transfected with its receptor. Biochem Biophys Res Commun 286(5):958-963

Hua M, Doran J, Kyle V, Yeo S and Colledge WH 2013 Does kisspeptin signalling have a role in the testes?. 4:198. doi:10.3389/fendo.2013.00198

Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA 2004 Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80(4):264-72

Jackson MR and Edmunds JG 1984 Morphological assessment of testicular maturity in marmosets (*Callithrix jacchus*). Laboratory Animals 18: 173–178

Johnson BH and Ewing LL 1971 Follicle stimulating hormone and the regulation of testosterone secretion in rabbit testis. Science 173: 635-637

Kamberi IA, Mical RS, and Porter JC 1971 Pituitary portal infusion of hypothalamic extract and release of LH, FSH and prolactin. Endocrinology 88, 1294–1299.

Keen KL, Wegner FH, Bloom SR, Ghatei MA, Terasawa E 2008 An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. Endocrinology 149 (8): 4151-4157

Kerr JB and Sharpe RM 1985 Follicle stimulating hormone induction of Leydig cell maturation. Endocrinology 116: 2592-2604

Kelnar CJH, McKinnell C, Walker M, Morris KD, Wallace WHB, Saunders PTK, Fraser HM, Sharpe RM 2002 Testicular changes during infantile `quiescence' in the marmoset and their gonadotrophin dependence: a model for investigating susceptibility of the prepubertal human testis to cancer therapy? Hum Reprod 17: 1367-1378

Kholkute SD, Aitken RJ, Lunn SF 1983 Plasma testosterone response to hCG stimulation in the male marmoset monkey (*Callithrix jacchus*). J Reprod Fertil 67(2): 457-463

Kirby HR, Maguire JJ, Colledge WH, Davenport AP 2010 International Union of Basic and Clinical Pharmacology. LXXVII. Kisspeptin receptor nomenclature, distribution, and function. Pharmacol Rev 62(4):565-578

Kirilov M, Clarkson J, Liu X, Roa J, Campos P, Porteous R, Schütz G, Herbison AE 2013 Dependence of fertility on kisspeptin-GPR54 signaling at the GnRH neuron. Nat Commun 4:2492-3492

Kluin PM, Kramer MF, de Rooij DG 1983 Testicular development in *Macaca irus* after birth. Int J Androl 6: 25-43

Knobil, E, Plant TM, Wildt L, Belchetz PE, and Marshall, G 1980 Control of the rhesus monkey menstrual cycle: permissive role of hypothalamic gonadotropinreleasing hormone. Science 207, 1371–1373.

Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SW, Vassart G and Parmentier M 2001 The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligand of the orphan G protein coupled receptor GPR54. J Biol Chem 276: 34631-34536

Ku WW, Pagliusi F, Foley G, Roesler A, Zimmerman T 2010 A simple orchidometric method for the preliminary assessment of maturity status in male cynomolgus monkeys (*Macaca fascicularis*) used for nonclinical safety studies. J Pharm Toxicol Meth 61:32–37

Lanfranco F, Gromoll J, von Eckardstein S, Herding EM, Nieschlag E, Simoni M 2005 Role of sequence variations of the GnRH receptor and G protein-coupled receptor 54 gene in male idiopathic hypogonadotropic hypogonadism. Eur J Endocrinol 153(6):845-852.

Lee DK, Nguyen T, O'Neill GP, Cheng R, Liu Y, Howard AD, Coulombe N, Tan CP, Tang-Nguyen AT, George SR, O'Dowd BF 1999 Discovery of a receptor related to the galanin receptors. FEBS Lett 446(1):103-107

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

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

Lehman MN, Ladha Z, Coolen LM, Hileman SM, Connors JM, Goodman RL. 2010 Neuronal plasticity and seasonal reproduction in sheep. Eur J Neurosci 32(12):2152-2164

Lejeune H, Skalli M, Chatelain PG, Avallet O, Saez JM 1992 The paracrine role of Sertoli cells on Leydig cell function. In "Cell Biology and Toxicology, Vol. 8, No 3, Princeton Scientific Publishing Co., Inc. pp. 73-83

Li LH, Donald JM & Golub MS 2005 Review on testicular development, structure, function, and regulation in common marmoset. Birth Defects Research Part B: Dev Reprod Toxicol 74: 450–469

Lui WY, Mruk D, Lee, WM, Cheng CY 2003 Sertoli cell tight junction dynamics: their regulation during spermatogenesis. Biol Reprod 68, 1087–1097.

Lunn SF, Recio R, Morris K & Fraser HM 1994 Blockade of the neonatal rise in testosterone by a gonadotrophin-releasing hormone antagonist: effects on timing of puberty and sexual behaviour in the male marmoset monkey. J Endocrinol 141: 439–447

Lunn SF, Cowen GM & Fraser HM 1997 Blockade of the neonatal increase in testosterone by a GnRH antagonist: the free androgen index, reproductive capacity and postmortem findings in the male marmoset monkey. J Endocrinol 154: 125–131

McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, de Kretser DM, Pratis K, Robertson DM 2002 Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Prog. Horm Res 57, 149–179.

Mansfield K 2003 Marmoset models commonly used in biomedical research. Comp Med 53(4):383-392

Majumdar SS, Sarda K, Bhattacharya I, Plant TM 2012 Insufficient androgen and FSH signaling may be responsible for the azoospermia of the infantile primate testes despite exposure to an adult-like hormonal milieu. Hum Reprod 27(8):2515-2525

Mann DR, Akinbami MA, Gould KG, Paul K, Wallen K 1998 Sexual maturation in male rhesus monkeys: importance of neonatal testosterone exposure and social rank. J Endocrinol 156: 493–501

Mann DR, Akinbami MA, Gould KG, Tanner JM, Wallen K 1993 Neonatal treatment of male monkeys with a gonadotropin-releasing hormone agonist alters differentiation of central nervous system centers that regulate sexual and skeletal development. J Clin Endocrinol Metab 76: 1319–1324

Mann DR, Davis-DaSilva M, Wallen K, Coan P, Evans DE, Collins DC 1984 Blockade of neonatal activation of the pituitary–testicular axis with continuous administration of a gonadotropin-releasing hormone agonist in male rhesus monkeys. J Clin Endocrinol Metab 58: 262–267

Mann DR, Gould KG, Collins DC, Wallen K 1989 Blockade of neonatal activation of the pituitary-testicular axis: effect on peripubertal luteinizing hormone and testosterone secretion and on testicular development in male monkeys. J Clin Endocrinol Metab 68: 600–607

Marshall GR, Ramaswamy S, Plant TM 2005 Gonadotropin independent proliferation of the pale type A spermatogonia in the adult rhesus monkey (*Macaca mulatta*). Biol Reprod 73: 222-229

Marshall GR and Plant TM 1996 Puberty occurring either spontaneously or induced precociously in rhesus monkey (*Macaca mulatta*) is associated with a marked proliferation of Sertoli cells. Biol Reprod 54(6):1192-1199

Marshall GR, Wickings EJ, Nieschlag E 1984 Testosterone can initiate spermatogenesis in an immature nonhuman primate, (*Macaca fascicularis*). Endocrinology 114: 2228–2233

Marshall GR, Jockenhovel F, Ludecke D, Nieschlag E 1986 Maintenance of complete but quantitatively reduced spermatogenesis in hypophysectomized monkeys by testosterone alone. Acta Endocrinol (Copenh) 113:424–431

Matsui H, Takatsu Y, Kumano S, Matsumoto H, Ohtaki T 2004 Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. Biochem Biophys Res Commun 320(2):383-388

McKinnell C, Saunders PT, Fraser HM, Kelnar CJ, Kivlin C, Morris KD, Sharpe RM 2001 Comparison of androgen receptor and oestrogen receptor beta immunoexpression in the testes of the common marmoset (*Callithrix jacchus*) from birth to adulthood: low androgen receptor immunoexpression in Sertoli cells during the neonatal increase in testosterone concentrations. Reproduction 122(3): 419-429

Mead EJ, Maguire JJ, Kuc RE, Davenport AP 2007 Kisspeptins: a multifunctional peptide system with a role in reproduction, cancer and the cardiovascular system. Br J Pharmacol 151(8):1143-1153

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

Meyer JK, Fitzsimmons D, Hastings TF, Chellman GJ 2006 Methods for the prediction of breeding success in male cynomolgus monkeys (*Macaca fascicularis*) used for reproductive toxicology studies. J Amer Assoc Lab Anim Sci 45: 31−36

Meusy-Dessolle N and Dang DC 1985 Plasma concentration of testosterone, dihydrotestosterone, Δ4-androstenedione, dehydroepiandrosterone and oestradiol-17ß in the crab-earing monkey (*Macaca fascicularis*) from birth to adulthood. J Reprod Fertil 74: 347-359

Meinhardt A and Hedger MP 2011 Immunological, paracrine and endocrine aspects of testicular immune privilege. Mol Cell Endocrinol 335(1):60-68

Millar MR, Sharpe RM, Weinbauer GF, Fraser HM, Saunders PT 2000 Marmoset spermatogenesis: organizational similarities to the human. Int J Androl 23(5):266-277

Mitchell RT, Cowan G, Morris KD, Anderson RA, Fraser HM, Mckenzie KJ, Wallace WH, Kelnar CJ, Saunders PT, Sharpe RM 2008 Germ cell differentiation in the marmoset (*Callithrix jacchus*) during fetal and neonatal life closely parallels that in the human. Hum Reprod 23(12): 2755-2765

Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, Szekers PG, Sarav HM, Chambers JK, Murdock P, Steplewski K, Shabon U, Miller JE, Middleton SE, Darker JG, Larminie CG, Wilson S, Bergsma DJ, Einson P, Faull R, Philpott KL and Hanison DC 2001 AXOR12, a novel human G protein- coupled receptor, activated by the peptide KiSS-1. J Biol Chem 276: 28969-28975

Muller J and Skakkebaek N E 1983 Quantification of germ cells and seminiferous tubules by stereological examination of the testicles from 50 boys who suffered from sudden death. Int J Androl 6: 143-156

Muller T, Simoni M, Pekel E, Luetjens CM, Chandolia R, Amato F, Norman RJ & Gromoll J 2004 Chorionic gonadotrophin beta subunit mRNA but not luteinising hormone beta subunit mRNA is expressed in the pituitary of the common marmoset (*Callithrix jacchus*). J Mol Endocrinol 32: 115–128

Murphy KG 2005 Kisspeptins: regulators of metastasis and the hypothalamic-pituitary-gonadal axis. J Neuroendocrinol 17(8):519-525

Mullaney BP, Skinner MK 1991 Growth factors as mediators of testicular cell-cell interactions. Baillieres Clin Endocrinol Metab 5(4):771-790

Nash KT and Welch DR 2006 The KISS1 metastasis suppressor: mechanistic insights and clinical utility. Front Biosci 11:647-659

Navenot JM, Fujii N, Peiper SC. 2009 KiSS1 metastasis suppressor gene product induces suppression of tyrosine kinase receptor signaling to Akt, tumor necrosis factor family ligand expression, and apoptosis. Mol Pharmacol 75(5):1074-1083

Neill JD, Patton JM, Dailey RA, Tsou RC, and Tindall GT 1977 Luteinizing hormone releasing hormone (LHRH) in pituitary stalk blood of rhesus monkeys: relationship to level of LH release. Endocrinology 101: 430–434.

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

Okano H, Hikishima K, Iriki A, Sasaki E 2012 The common marmoset as a novel animal model system for biomedical and neuroscience research applications. Semin Fetal Neonatal Med 17(6):336-340

Orth JM 1984 The role of follicle stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. Endocrinology 115, 1248–1255.

Orth JM, Gunsalus GL, Lamperti AA 1988 Evidence from Sertoli cell depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cell produced during perinatal development. Endocrinology 122: 787-794

Pallais JC, Bo-Abbas Y, Pitteloud N, Crowley WF Jr, Seminara SB 2006 Neuroendocrine, gonadal, placental, and obstetric phenotypes in patients with IHH and mutations in the G-protein coupled receptor, GPR54. Mol Cell Endocrinol 25:70-77

Papadopoulos V, Kamtchouing P, Drosdowsky MA, Hochereau de Reviers MT, Carreau S 1987 Adult rat Sertoli cells secrete a factor or factors which modulate Leydig cell function. J Endocrinol 114(3):459-467

Papadopoulos V 1991 Identification and purification of a human Sertoli cell-secreted protein (hSCSP-80) stimulating Leydig cell steroid biosynthesis. J Clin Endocrinol Metab 72(6):1332-1339

Pelletier RM, Byers SW 1992 The blood–testis barrier and Sertoli cell junctions: structural considerations. Microsc Res Tech 20, 3–33.

Petersen F and Yu X 2010 A novel preclinical model for rheumatoid arthritis research. Arthritis Res Ther 12(6):148

Petersson F, Emons G, Hammar M 1989 Testicular GnRH-receptors and direct effects of a GnRH-agonist on human testicular steroidogenesis. Scand J Urol Nephrol 23(3):161-4.

Pinto FM, Cejudo-Román A, Ravina CG, Fernández-Sánchez M, Martín-Lozano D, Illanes M, Tena-Sempere M, Candenas ML 2012 Characterization of the kisspeptin system in human spermatozoa. Int J Androl 35(1):63-73.

Plant TM 1980 The effects of neonatal orchidectomy on the developmental pattern of gonadotropin secretion in the male rhesus monkey (*Macaca mulatta*). Endocrinology 106, 1451–1454.

Plant TM 1985 A study of the role of the postnatal testes in determining the ontogeny of gonadotropin secretion in the male rhesus monkey (*Macaca mulatta*). Endocrinology 116, 1341–1350.

Plant TM 1996 Environmental factors and puberty in non-human primates. Acta Paediatr Suppl 417: 89-91

Plant TM 2001 Neurobiological bases underlying the control of the onset of puberty in the rhesus monkey: a representative higher primate. Front. Neuroendocrinol. 22, 107–139.

Plant TM 2006 The male monkey as a model for the study of the neurobiology of puberty onset in man. Mol Cell Endocrinol 254-255:97-102

Plant TM 2008 Hypothalamic control of the pituitary-gonadal axis in higher primates: key advances over the last two decades. J Neuroendocrinol 20(6):719-726

Plant TM 2010 Undifferentiated primate spermatogonia and their endocrine control. Trends Endocrinol Metab 21(8):488-495

Plant TM 2012 A comparison of the neuroendocrine mechanisms underlying the initiation of the preovulatory LH surge in the human, Old World monkey and rodent. Front Neuroendocrinol 33(2):160-168

Plant TM, and Barker-Gibb, ML 2004 Neurobiological mechanisms of puberty in higher primates. Hum. Reprod. Update 10, 1–11.

Plant TM, Gay VL, Marshall GR, Arslan M 1989 Puberty in monkeys is triggered by chemical stimulation of the hypothalamus. Proc Natl Acad Sci U S A 86, 2506–2510.

Plant TM and Marshall GR 2001 The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. Endocr Rev 22 (6): 764-786

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

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

Plant TM and Witchel S 2006 Puberty in non-human primates and humans. In "Knobil and Neill's Physiology of Reproduction", 3rd edn, Vol. 2, Eds. E Knobil and JD Neill, Elsevier. San Diego, USA. pp 2177–2230

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

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

Ramaswamy S, Seminara SB, Pohl CR, DiPietro MJ, Crowley Jr WF, Plant TM 2007 Effect of continuous intravenous administration of human metastin 45–54 on the neuroendocrine activity of the hypothalamic-pituitary-testicular axis in the adult male rhesus monkey (Macaca mulatta). Endocrinology 148 (7) 3364-3370

Resko JA 1967 Plasma androgen levels of the rhesus monkey: Effects of age and season. Endocrinology 81: 1203-1212

Rey RA, Campo SM, Bedecarras P, Nagle C, Chemes HE 1993 Is infancy a quiescent period of testicular development? Histologic, morphometric and functional study of the seminiferous tubules of the cebus monkey from birth to the end of puberty. J Clin Endocrinol Metabol 76: 1325-1331

Ritzen EM, Boitani C, Parvinen M, French FC, Feldman M 1982 Stage-dependent secretion of ABP by rat seminiferous tubules. Mol Cell Endocrinol 25 (1): 25-33

Rune GM, de Souza P, Merker HJ 1991 Ultrastructural and histochemical characterization of marmoset (*Callithrix jacchus*) Leydig cells during postnatal development. Anat Embryol (Berl) 183 (2): 179-191

Rune GM, Pretzer D, De Souza P, Bollmann U, Merker HJ 1992 Ultrastructure of adult and juvenile marmoset (*Callithrix jacchus*) Sertoli cells in vivo and in vitro. J Androl 13(6):560-570

Russell L D 1980 Sertoli–germ cell interactions: A review. Gamete Res 3: 179–202

Russell LD, Gardner RJ, Weber JE 1986 Reconstruction of a type-B configuration monkey Sertoli cell: size, shape, and configurational and specialized cell-to-cell relationships. Am J Anat 175(1):73-90

Saez JM 1994 Leydig cells: endocrine, paracrine, and autocrine regulation. Endocr Rev 15 (5):574-626

Saez JM, Avallet O, Naville D, Perrard-Sapori MH, Chatelain PG 1989 Sertoli-Leydig cell communications. Ann NY Acad Sci 56: 210-231

Sameshima H and Hamana K 1996 Testicular response to exogenous gonadotropins in infant cynomolgus monkeys. Int J Toxicol 15 (5): 371-380

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

Schlatt S, Meinhardt A, Nieschlag E 1997 Paracrine regulation of cellular interactions in the testis: factors in search of a function. Eur J Endocrinol 137 (2): 107-117

Schlatt S, Pohl CR, Ehmcke J and Ramaswamy R 2008 Age related changes in diurnal rhythms and levels of gonadotropins, testosterone, and inhibin b in male rhesus monkeys (*Macaca mulatta*). Biol Reprod 79: 93-99

Seminara SB, Dipietro MJ, Ramaswamy S, Crowley WF Jr, Plant TM 2006 Continuous human metastin 45-54 infusion desensitizes G protein-coupled receptor 54-induced gonadotropin-releasing hormone release monitored indirectly in the juvenile male Rhesus monkey (*Macaca mulatta*): a finding with therapeutic implications. Endocrinology 147 (5): 2122-2126

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

Semple RK, Achermann JC, Ellery J, Farooqi IS, Karet FE, Stanhope RG, O'rahilly S, Aparicio SA 2005 Two novel missense mutations in g protein-coupled receptor 54 in a patient with hypogonadotropic hypogonadism. J Clin Endocrinol Metab 90(3):1849-1855

Setchell BP 2008 Blood-testis barrier, junctional and transport proteins and spermatogenesis. Adv Exp Med Biol 636: 212-233

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

Sharpe RM 1985 Intratesticular regulation of testosterone secretion: comparison of the effects and interactions of hCG, and LHRH agonist and testicular interstitial fluid on Leydig cell testosterone secretion *in vitro*. Mol Cell Endocrinol 41: 247-255

Sharpe RM, McKinnell C, Kivlin C, Fisher JS 2003 Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 125 (6): 769-784

Sharpe RM, Walker M, Millar MR, Atanassova N, Morris K, McKinnell C, Saunders PT, Fraser HM 2000 Effect of neonatal gonadotropin-releasing hormone antagonist administration on Sertoli cell number and testicular development in the marmoset: comparison with the rat. Biol Reprod 62 (6): 1685-1693

Sharpe RM, Fraser HM, Brougham MF, McKinnell C, Morris KD, Kelnar CJ, Wallace WH, Walker M 2003 Role of the neonatal period of pituitary-testicular activity in germ cell proliferation and differentiation in the primate testis. Hum Reprod 18 (10): 2110-2117

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

Silverman AJ, Antunes J, Ferin M, and Zimmerman EA 1977 The distribution of luteinizing hormone-releasing hormone (LHRH) in the hypothalamus of the rhesus monkey. light microscopic studies using immunoperoxidase technique. Endocrinology 101, 134–142

Silverman AJ, Antunes JL, Abrams GM, Nilaver G, Thau R, Robinson JA, Ferin M, Krey LC 1982 The luteinizing hormone-releasing hormone pathways in rhesus (*Macaca mulatta*) and pigtailed (*Macaca nemestrina*) monkeys: new observation on thick, unembedded sections. J Comp Neurol 211:309-17

Simorangkir DR, Marshall GR, Ehmcke J, Schlatt S, Plant TM 2005 Prepubertal expansion of dark and pale type A spermatogonia in the rhesus monkey (*Macaca mulatta*) results from proliferation during infantile and juvenile development in a relatively gonadotropin independent manner. Biol Reprod 73 (6): 1109-1115

Simorangkir DR, Ramaswamy S, Marshall GR, Roslund R, Plant TM 2012 Sertoli cell differentiation in rhesus monkey (*Macaca mulatta*) is an early event in puberty and precedes attainment of the adult complement of undifferentiated spermatogonia. Reproduction 143(4): 513-522

Simorangkir DR, Marshall GR, Plant TM 2003 Sertoli cell proliferation during prepubertal development in the rhesus monkey (*Macaca mulatta*) is maximal during

infancy when gonadotropin secretion is robust. J Clin Endocrinol Metab 88 (10): 4984-4989

Skinner MK 1991 Cell-cell interactions in the testis Endocr Rev 12(1):45-77

Skinner MK, Norton JN, Mullaney BP, Rosselli M, Whaley PD, Anthony CT 1991 Cell-cell interactions and the regulation of testis function. Ann NY Acad Sci 637:354-363

Smedley JV, Bailey SA, Perry RW, O'Rourke CM 2002 Methods for predicting sexual maturity in male cynomolgus macaques on the basis of age, body weight, and histologic evaluation of the testes. J Amer Assoc Lab Anim Sci 41: 18−20

Steiner RA and Bremner WJ 1981 Endocrine correlates of sexual development in the male monkey, Macaca fascicularis. Endocrinology 109: 914-919

Suarez-Quian CA, Martinez-Garcia F, Nistal M, Regadera J 1999 Androgen receptor distribution in adult human testis. J Clin Endocrinol Metab 84, 350–358.

't Hart BA, Abbott DH, Nakamura K, Fuchs E 2012 The marmoset monkey: a multi-purpose preclinical and translational model of human biology and disease. Drug Discov Today 17(21-22):1160-1165

Tardif SD, Smucny DA, Abbott DH, Mansfield K, Schultz-Darken N, Yamamoto ME 2003 Reproduction in captive common marmosets (*Callithrix jacchus*). Comp Med 53(4):364-368

Tarulli GA, Stanton PG, Meachem SJ 2012 Is the adult Sertoli cell terminally differentiated? Biol Reprod 87(1):13, 1-11

Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, Seminara SB, Mendonca BB, Kaiser UB, Latronico AC 2008 A GPR54-activating mutation in a patient with central precocious puberty. N Engl J Med 358(7):709-715

Terasawa E, Fernandez DL 2001 Neurobiological mechanisms of the onset of puberty in primates. Endocr Rev 22(1):111-51

Terasawa E, Guerriero KA, Plant TM 2013 Kisspeptin and puberty in mammals. Adv Exp Med Biol 784: 253-273

Terao Y, Kumano S, Takatsu Y, Hattori M, Nishimura A, Ohtaki T, Shintani Y 2004 Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. Biochim Biophys Acta 1678(2-3):102-110

Waites GM, Speight AC, Jenkins N 1985 The functional maturation of the Sertoli cell and Leydig cell in the mammalian testis. J Reprod Fertil 75(1), 317–326

Weinbauer GF, Göckeler E, Nieschlag E 1988 Testosterone prevents complete suppression of spermatogenesis in the gonadotropin-releasing hormone antagonist-treated nonhuman primate (*Macaca fascicularis*). J Clin Endocrinol Metab 67(2):284-290

Weinbauer GF and Wessels J 1999 'Paracrine' control of spermatogenesis. Andrologia 31(5):249-262.

Wickings, E. J. and Nieschlag, E 1980 Seasonality in endocrine and exocrine testicular function of the adult rhesus monkey (*Macaca mulatta*) maintained in a controlled laboratory Environment. Int J Androl 3:87–104

Wickings EJ, Zaidi P, Brabant G, Nieschlag E 1981 Stimulation of pituitary and testicular functions with LH-RH agonist or pulsatile LH-RH treatment in the rhesus monkey during the non-breeding season. J Reprod Fertil 63(1):129-136

Willems A, Batlouni SR, Esnal A, Swinnen JV, Saunders PT, Sharpe RM, França LR, De Gendt K, Verhoeven G 2010 Selective ablation of the androgen receptor in mouse Sertoli cells affects Sertoli cell maturation, barrier formation and cytoskeletal development. PLoS One 5(11):e14168

Wistuba J, Schrod A, Greve B, Hodges JK, Aslam H, Weinbauer GF, Luetjens CM 2003 Organization of seminiferous epithelium in primates: relationship to spermatogenic efficiency, phylogeny, and mating system. Biol Reprod 69 (2):582-591

Yan C, Wang H, Boyd DD 2001 KiSS-1 represses 92-kDa type IV collagenase expression by down-regulating NF-kappa B binding to the promoter as a consequence of Ikappa Balpha-induced block of p65/p50 nuclear translocation. J Biol Chem 276:1164–1172

Zühlke U and Weinbauer G 2003 The common marmoset (*Callithrix jacchus*) as a model in toxicology. Toxicol Pathol Suppl: 123-127