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Departamento de Biología Celular, Fisiología e Inmunología

TESIS DOCTORAL

ANALYSIS OF THE ROLES OF NEUROPEPTIDERGIC SYSTEMS AND MICRORNAS IN THE CONTROL OF PUBERTY

María Manfredi Lozano

Córdoba, 16 de diciembre de 2014

TITULO: *Analysis of the roles of neuropeptidergic system and micrornas in the control of puberty*

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ANALYSIS OF THE ROLES OF NEUROPEPTIDERGIC SYSTEMS AND MICRORNAS IN THE CONTROL OF PUBERTY

Memoria de Tesis Doctoral presentada por **María Manfredi Lozano**, licenciada en biología por la Universidad de Sevilla, para optar al grado de **Doctora** en Ciencias.

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ÍTULO DE LA TESIS: ANALYSIS OF THE ROLES OF NEUROPEPTIDERGIC SYSTEMS AND MICRORNAS IN THE CONTROL OF PUBERTY

DOCTORANDO/A: María Manfredi Lozano

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El trabado de Tesis Doctoral titulado "Analysis of the roles of neuropeptidergic systems and microRNAs in the control of puberty" ha sido realizado satisfactoriamente por Dña. María Manfredi Lozano, licenciada en Biología, entre los años 2009-2014 en la Sección de Fisiología del Departamento de Biología Celular, Fisiología e Inmunología bajo nuestra dirección. El objetivo general de esta Tesis fue fue caracterizar el papel de los sistemas neuropeptidérgios, concretamente α-MSH y kisspeptinas, y del sistema Lin28/let-7 en el control fisiológico de la pubertad, empleando la rata y el ratón como animal modelo, incluyendo modelos genéticamente modificados. El grado de aprovechamiento y la adquisición de conocimientos, por parte de la doctoranda, durante el tiempo de desarrollo de esta Tesis Doctoral fue máximo. Prueba de ello, es la publicación de 11 artículos en revistas de primer orden en el campo de la Endocrinología. A continuación se citan las 5 publicaciones más relevantes:

1. Sangiao-Alvarellos S*, **Manfredi-Lozano M***, Ruiz-Pino F, Navarro VM, Sánchez-Garrido MA, Leon S, Dieguez C, Cordido F, Matagne V, Dissen GA, Ojeda SR, Pinilla L, Tena-Sempere M. Changes in hypothalamic expression of the Lin28/let-7 system and related microRNAs during postnatal maturation and after experimental manipulations of puberty. *Endocrinology.* 2013 Feb; 154(2):942-55. **IF: 4.64**. Journal Ranking: 24/122 in Endocrinology & Metabolism **(Q1**)
   Nota: * Estos autores han contribuido igualmente a este trabajo

2. Gaytan F*, Sangiao-Alvarellos S*, **Manfredi-Lozano M**, García-Galiano D, Ruiz-Pino F, Romero-Ruiz A, León S, Morales C, Cordido F, Pinilla L, Tena-Sempere M. Distinct expression patterns predict differential roles of the miRNA-binding proteins, Lin28 and Lin28b, in the mouse testis: Studies during postnatal development and in a model of hypogonadotropic hypogonadism. *Endocrinology*. 2013 Mar; 154(3):1321-36. **IF: 4.64**. Journal Ranking: 24/122 in Endocrinology & Metabolism (**Q1**)
   Nota: * Estos autores han contribuido igualmente a este trabajo

3. Ruiz-Pino F*, Garcia-Galiano D*, **Manfredi-Lozano M**, Leon S, Sánchez-Garrido MA, Roa J, Pinilla L, Navarro VM, Tena-Sempere M. Dissecting the roles of kisspeptin partners, NKB and dynorphin, in the control of the gonadotropic axis: Shaping the KNDy paradigm. *Endocrinology*. 2014 Dec [Epub ahead of print] **IF: 4.64**. Journal Ranking: 24/122 in Endocrinology & Metabolism (**Q1**)
Nota: * Estos autores han contribuido igualmente a este trabajo

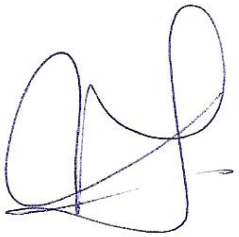
4. Sangiao-Alvarellos S, Pena-Bello L, **Manfredi-Lozano M**, Tena-Sempere M, Cordido F. Perturbation of hypothalamic microRNA expression patterns in male rats following metabolic distress: Impact of obesity and conditions of negative energy balance. *Endocrinology.* 2014 May; 155(5):1838-50. **IF: 4.64**. Journal Ranking: 24/122 in Endocrinology & Metabolism (**Q1**)

5. Navarro VM*, Ruiz-Pino F*, Sánchez-Garrido MA, García-Galiano D, Hobbs SJ, **Manfredi-Lozano M**, León S, Sangiao-Alvarellos S, Castellano JM, Clifton DK, Pinilla L, Steiner RA, Tena-Sempere M. Role of neurokinin B in the control of female puberty and its modulation by metabolic status. *Journal of Neuroscience*. 2012 Feb 15; 32(7):2388-97. **IF: 6.90**. Journal Ranking: 22/251 in Neurosciences **(D1**).

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 16 de diciembre de 2014

Firma del/de los director/es



Fdo.:Dr. Manuel Tena Sempere **Fdo.: Leonor Pinilla Jurado**

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ABBREVIATIONS

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(X)-IR: X –immunoreactive

3´UTR : 3´ untranslated region

3V: Third cerebral ventricle

aa: Amino acid

ACTH: Adrenocorticotropic hormone

ADAR1/ ADAR2: Adenosine deaminase acting on RNA enzyme

Ago: Argonaute

AgRP: Agouti-Related Protein

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AR: Androgen receptor

ARC: Arcuate nucleus

AUC: Area under curve

AVPV: Anterior ventral periventricular area

BPS: Balano-preputial separation

BW: Body weight

cAMP: Cyclic adenosine monophosphate

CART: Cocaine and Amphetamine-regulated transcript

CD: Constant darkness

CLIP: Corticotrophin-like intermediate peptide

CNS: Central nervous system

CR: Caloric restriction

CRH: Corticotropin-release hormone

D-1: Diestrus-1 or metestrus

D-2: Diestrus-2

DAG: Diacylglycerol

db/db: Leptin receptor-deficient mice

DGCR8: DiGeorge syndrome chromosomal region 8

DHT: Dihydrotestosterone

DMN: Dorsomedial nucleus

dsRNA: Double-stranded RNA

Dyn: Dynorphin

E_2: 17β-estradiol

EAA: Excitatory amino acids

EB: Estradiol benzoate

EOPs: Endogenous opioid peptides

ERα: Estrogen receptor α

ERβ: Estrogen receptor β

ESCs: Embryonic stem cells

FSH: Follicle-stimulating hormone

FSH-R: FSH receptor

GABA: γ-aminobutyric acid

GAL: Galanin

GALP: Galanin-like peptide

GH: Growth hormone

GHRH: Growth hormone-releasing hormone

GHS-R: GH secretagogue receptor

Glu: Glutamate

GnIH: *Gonadotropin-inhibiting hormone*

GnRH: Gonadotropin-releasing hormone

GnRH-R: GnRH receptor

GNX: Gonadectomy

GPCR: G protein-coupled receptor

hCG: *Human* chorionic gonadotropins

HH: Hypogonadotropic hypogonadism

hnRNPA1: Heterogeneous nuclear ribonucleoprotein A1

HPG: Hypothalamic-pituitary-gonadal

HPX: Hypophysectomized

icv: Intracerebroventricular

iGluRs: Ionotropic Glu receptors

IHC: Immunohistochemistry

ip: Intraperitoneal

IP3: Inositol 1,4,5-trisphosphate

iPS: Induced pluripotent stem

IR: Insulin receptor

ISH: *in situ* hybridization

KA: Kainate

KO: Knock-out

Kp-10: Kisspeptin-10

Kp-13: Kisspeptin-13

Kp-54: Kisspeptin-54

KSRP: KH-type splicing regulatory protein

LH: Luteinizing hormone

LHA: Lateral hypothalamic area

LH-R: LH/hCG receptor

LHRH: Luteinizing hormone-releasing hormone

MBH: Medio basal hypothalamus

MC-R: Melanocortin receptor

ME: Median eminence

mGluRs: Metabotropic Glu receptors

min: Minute

miRISC: miRNA-induced silencing complex

miRNA*: Passenger strand in the miRNA/miRNA*

miRNA: MicroRNA

mRNA: Messenger RNA

MSH: Melanocyte stimulating hormone

MT II: Melanotan, MC3-R and MC4-R agonist

NA: Noradrenaline

NGR: Neuroregulin

NK1R: Tachykinin type 1 receptor

NK2R: Tachykinin type 2 receptor

NK3R: NKB receptor

NKB: Neurokinin B

NMDA: N-Methyl-D-Aspartate

NO: Nitric oxide

NOS: Nitric oxide synthase

NPFF-1R: Neuropeptide FF receptor type 1

NPFF-2R: Neuropeptide FF receptor type 2

NPY: Neuropeptide Y

nt: Nucleotides

ob/ob: Leptin-deficient mice

Ob-R: Leptin receptor

ORX: Orchidectomized

OT: Oxytocin

OVX: Ovariectomized

OW: Ovarian weight

P: Progesterone

PCR: Polymerase chain reaction

PeVN: Periventricular nucleus

PIF: Prolactin-release inhibiting factor

PIP2: Phosphatidylinositol 4,5-bisphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

PND: Postnatal day

POA: Preoptic area

Pol II: Polymerase II

POMC: Proopiomelanocortin

PR: Progesterone receptor

pre-miRNA: Precursor miRNA

PRF: Prolactin-releasing factor

pri-miRNA: Primary miRNA

PRL: Prolactin

PRO: Proestrus

PrRP: PRL-releasing peptide

PVN: Paraventricular nucleus

PYY: Peptide YY

RFRP: RF-amide related peptide

RNA: Ribonucleic acid

RP3V: Rostral periventricular area of the 3V

sc: Subcutaneous

SEM: Standard error of the mean

SHBG: Sex hormone-binding-globulin

SHU9119: MC3-R and MC4-R antagonist

SON: Supraoptic nucleus

T: Testosterone

Tg: Transgenic

TNF- α: Tumor necrosis factor- α

TP: Testosterone propionate

TRBP: Tar RNA binding protein

TRH: Thyrotropin-release hormone

TSH: Thyroid stimulating hormone

TW: Testicular weight

UW: Uterus weight

VMN: Ventromedial nucleus

VO: Vaginal opening

WT: Wild type

α-MSH: α- melanocyte stimulating hormone

β-END : β-endorphin

β-MSH: β-melanocyte stimulating hormone

γ-MSH: γ-melanocyte stimulating hormone

INTRODUCTION

1. INTRODUCTION

Reproduction is essential for the perpetuation of the species. In mammals, the acquisition of reproductive ability and its subsequent maintenance in adulthood is subjected to the coordinated participation of numerous central and peripheral signals that regulate the so-called hypothalamic-pituitary-gonadal (HPG), also known as neuroendocrine reproductive, axis. The regulatory signals controlling this axis range from environmental cues, as photoperiod or stress conditions, to endogenous signals, as circulating hormones or metabolic factors.

1.1. HYPOTHALAMIC-PITUITARY-GONADAL AXIS

The HPG axis is organized at three major levels: *1)* the hypothalamus, where a small group of neurons synthesize and release, in a pulsatile manner, the decapeptide gonadotropin-releasing hormone (GnRH) [1, 2], the key hierarchical element of the HPG axis; *2)* the anterior pituitary or adenohypophysis, where gonadotrope cells are stimulated by GnRH to synthesize and release the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH); and *3)* the gonads, testes and ovaries, which, in response to gonadotropins, generate gametes from puberty onwards (gametogenesis), and are responsible for the synthesis and release of sex steroids and peptide hormones (hormonogenesis). In turn, gonadal hormones operate via negative and positive feedback loops, the latter exclusive of females, to regulate the GnRH secretion from the hypothalamus and gonadotropin secretion from the pituitary [3-5] (**Figure 1**).

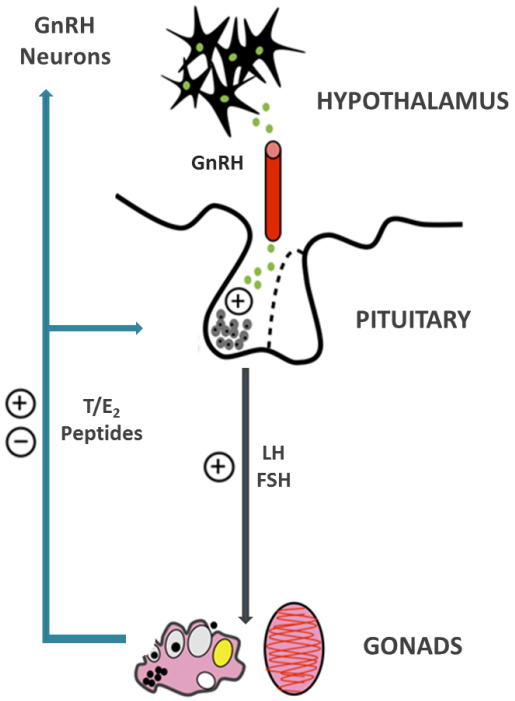

Figure 1. Schematic presentation of the hypothalamic-pituitary-gonadal (HPG) axis.

1.1.1. HYPOTHALAMUS

The hypothalamus acts as the central regulator of endocrine glands through neurotransmitters and neuropeptides secreted by different neuronal populations.

The hypothalamus is located at the base of the diencephalon, between the thalamus and the third cerebral ventricle (3V). The front side is delimited by the *lamina terminalis* and the caudal edge of the mammillary bodies [6]. From a functional point of view, hypothalamic neurons are grouped into different nuclei distributed in three regions: *1)* the rostral or supraoptic region; 2*)* the middle or tuberal region; and 3*)* the caudal or mammillary region [6]. The major hypothalamic nuclei and their location in the different areas are described below (**Figure 2**).

The **rostral area** includes different nuclei as the preoptic area (POA), the anterior hypothalamic area, the supraoptic nucleus (SON) and the paraventricular nucleus (PVN). Neurons of the SON and the OVN mainly project toward to the neurohypophysis [7]. The POA is considered a key area in the control of the reproductive axis, since it contains the most of the GnRH neurons.

The **medial area** contains the lateral hypothalamic area (LHA), the arcuate nucleus (ARC), the ventromedial nucleus (VMN) and the dorsomedial nucleus (DMN). Neurons of the medial area project towards the capillaries of the median eminence (ME) where they release their secretions to the portal system which carries them into the adenohypophysis [7, 8].

The **caudal region** mainly consists of the mammillary bodies whose endocrine function is less known.

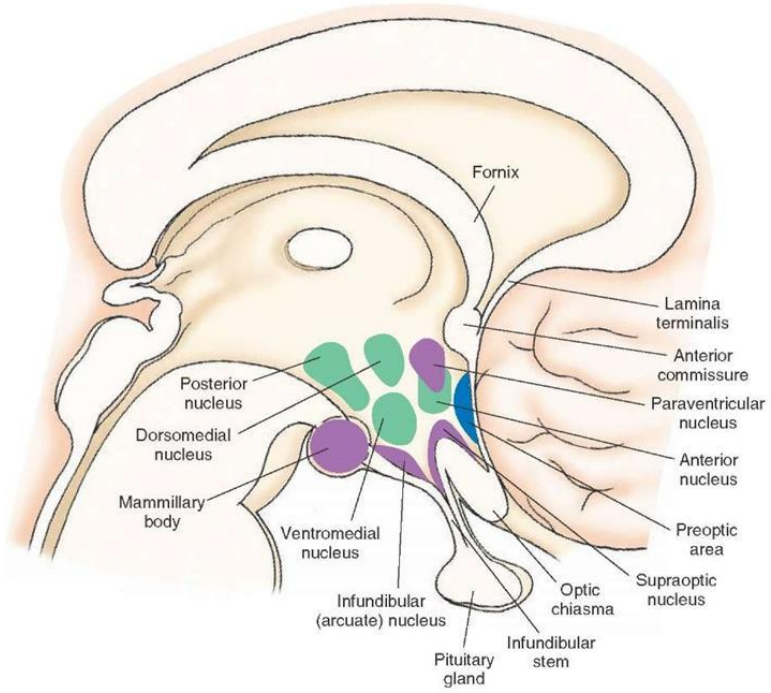

Figure 2. Schematic presentation of the major hypothalamic nuclei.

In the hypothalamic areas and nuclei are synthetized a large number of hormones which are release into the hypophyseal portal system to control the secretion of the different pituitary hormones [9]. Among them, given their importance in this Thesis, we highlight the following: corticotropin-release hormone (CRH), which acts on pituitary corticotrophs to stimulate the secretion of adrenocorticotropic hormone (ACTH); and GnRH, the hypothalamic decapeptide that stimulates the synthesis and release of LH and FSH from the gonadotropes in the adenohypophysis [1]. This hormone will be described in detail in the following section.

1.1.1.1. GONADOTROPIN-RELEASING HORMONE

GnRH (also known as luteinizing hormone-releasing hormone, LHRH) is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) which was first isolated in the 70s of the past century from the hypothalamus of pig and sheep [10, 11]. As remarkable feature, the N- and C-terminal domains are evolutionarily conserved; they are involved in binding and activation of the GnRH receptor. Several structural variants of GnRH have been identified in diverse vertebrates [12]: GnRH-I, GnRH-II and GnRH-III.

GnRH-I, termed as GnRH in the present Thesis, is known as the mammalian hypothalamic variant. It has an essential role in the control of the HPG axis as final effector of the set of central and peripheral regulators involved in the neuroendocrine control of reproduction [13]. GnRH is also present in amphibian and primitive fish species.

GnRH-II, also known as chicken GnRH, is the midbrain variant and is present in most vertebrate species, including human. It appears to be involved in the regulation of sexual behavior, food intake and energy balance [14].

GnRH-III, also known as salmon GnRH, has been identified in olfactory and telencephalic brain regions of several species of fish and reptiles and appears to play a role as a hypophysiotropic factor [15].

GnRH neurons arise in the olfactory placode and migrate to the rostral hypothalamus areas during development, specifically to the middle septum, the diagonal band of Broca and the POA. Most of them extend their axonal projections to the capillary plexus of the ME where GnRH is released as highly synchronized pulses [16].

In humans, GnRH is synthesized from a single gene which encodes the pre-pro-GnRH, a precursor of 92 amino acids (aa) [17, 18] (**Figure 3**). In this precursor protein, the decapeptide is preceded by a signal peptide of 23 aa and followed by a Gly-Lys-Arg sequence, essential for proteolytic processing and carboxyl terminal amidation. The last 56 aa residues are designated as GnRH-associated peptide [19]. The biologically active conformation of GnRH contains a fold in the central region of the molecule (Gly^6) [20].

GnRH secretion profile is not constant over development. In rodents and humans, GnRH secretion is increased during late fetal and early neonatal period and subsequently decreases, remaining at low levels until puberty [21]. The onset of puberty is characterized by a gradual increase in the frequency and amplitude of pulses of GnRH secretion into the hypophyseal portal system [12]. Thus, an adequate pulsatile secretion of GnRH is obligatory for proper attainment and maintenance of reproductive function.

The biological actions of GnRH are conducted after its binding with GnRH-R, a G protein-coupled receptor (GPCR). The NH_2- and COOH-terminal domains, on the extracellular and intracellular side respectively, contribute to receptor binding and activation, whereas the central region appears to be involved in spatial configuration and conformational changes after ligand binding. The number of GnRH-R in the pituitary are regulated by GnRH itself or gonadal steroids, rising before the preovulatory surge of gonadotropins and during pregnancy and lactation [22].

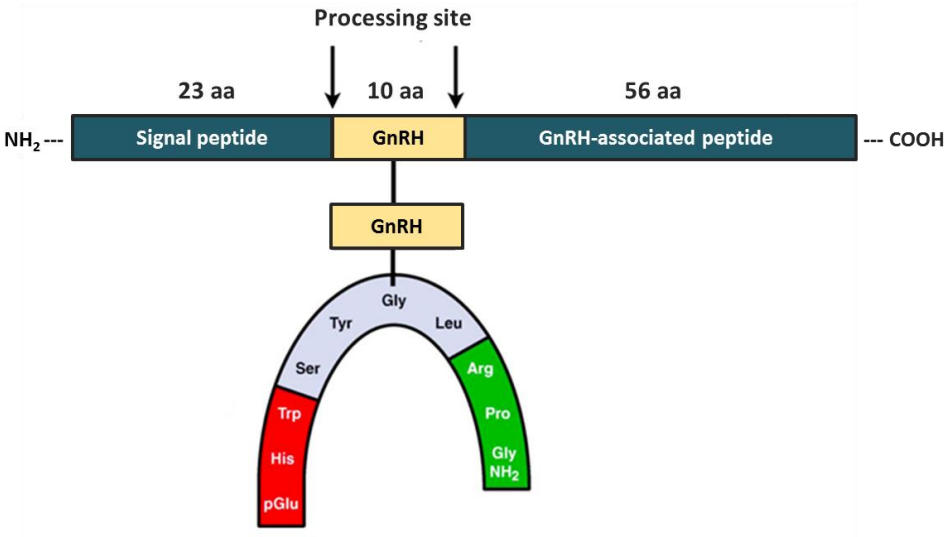

Figure 3. Schematic representation of mammalian GnRH. NH_2-terminal [10] and COOH-terminal [19] domains are involved in the receptor binding and activation, whereas the central region, Gly^6, is involved in spatial configuration and conformation of GnRH molecule. Taken from [23].

1.1.2. PITUITARY

The pituitary or hypophysis, is a pea-sized endocrine gland located in a cavity of the sphenoid bone called *sella turcica*, and is functionally connected to the hypothalamus by the ME via a small tube called the pituitary stalk (**Figure 4**). It is

composed of two functionally distinct structures, namely adenohypophysis and neurohypophysis, which differ in their embryological origin and their capacity to synthesize hormones.

The **adenohypophysis** arises from ectoderm of the pharyngeal roof (oral cavity) where there is a pocket called Rathke's pouch [3]. It is subdivided into three parts: *pars distalis*, *pars tuberalis*, these two parts constitute the anterior lobe of pituitary, and *pars intermedia*, located between anterior and posterior lobes.

The anterior lobe contains two basic types of cell, non-hormonal-secreting cells and hormonal-secreting cells. These last ones are responsible to synthesize and release different hormones whose secretion is controlled by hypothalamic hormones. Among them we highlight the following: gonadotropes, which synthesize and release LH and FSH [24]; and corticotropes, which synthesize a major precursor protein called proopiomelanocortin (POMC) which generates, through proteolytic cleavage, several biologically active peptides including ACTH, endorphins and melanocyte stimulating hormone (MSH). The physiological role of POMC and POMC-derived peptides will be further detailed in section 1.6.

The intermediate lobe contains the melanotroph cells which synthesize MSH from POMC. In human fetal life, MSH is secreted and causes the release of melanin pigment in skin melanocytes. However, this area is normally very small or entirely absent in adulthood.

The **neurohypophysis** develops from neural ectoderm from the floor of the diencephalon [3]. It is subdivided into: *pars nervosa, pars infundibularis* and the ME. The first two parts comprise the posterior lobe of pituitary. Two hormones are stored and released in the neurohypophysis, OT and ADH [25]. Both of them are synthesized by hypothalamic neurons whose cell bodies are located in the SON and in the PVN.

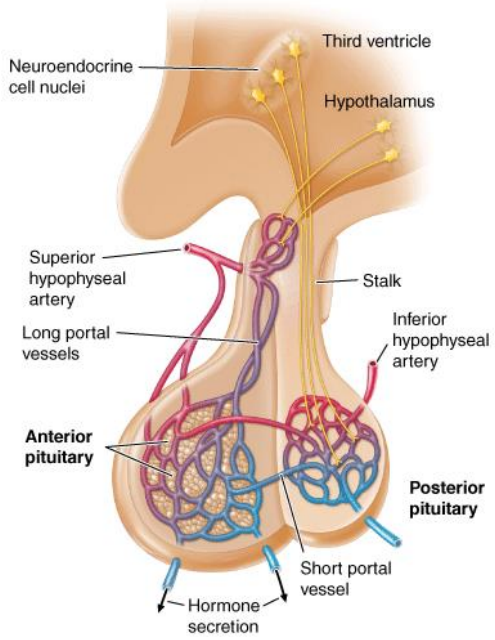

Figure 4. Diagram of hypothalamic-pituitary vasculature.

1.1.2.1. GONADOTROPINS: LH AND FSH

LH, FSH, human chorionic gonadotropins (hCG) and thyroid stimulating hormone (TSH), are structurally related peptides and they belong to the family of glycoprotein hormones [26]. In adenohypophysis, LH and FSH are produced by gonadotropes and TSH by tyrotropes, while hCG is synthesized in the placenta.

These hormones are composed of two noncovalently bound subunits: a common α subunit, an identical polypeptide structure of 92 aa and encoded by a single gene located on human chromosome 6; and an unique β subunit, which confers biological specificity to each hormone. The β subunit molecule is encoded by genes present on different chromosomes, except LH-β and hCG-β subunits that are arranged in a gene cluster structure (one LH-β gene and six hCG-β genes) present on chromosome 19 [27]. The different β subunits contains between 111-145 aa in humans [28]. In rodents, gonadotropins exhibit human-like structural features, while there is no production of placental chorionic gonadotropins.

Gonadotropins exert their biological actions by binding to GPCRs [29]. Both LH and hCG share the same receptor (LH/hCG receptor or LH-R), while FSH binds to a specific receptor (FSH-R) [26]. Gonadotropin receptors, via activation of adenylate

cyclase, induce the rise of intracellular cyclic adenosine monophosphate (cAMP) levels, and as result, the activation of protein kinase A (PKA) [30]. Additionally, it was found that these receptors can also activate other signaling pathways, such as phospholipase C (PLC) which induces an increase of intracellular Ca^{2+} [31]. Have been reported that the prolonged stimulation of LH-R and FSH-R produces a receptor desensitization, via downregulation and functional uncoupling of the receptor from G proteins [32].

Although LH-R expression was observed in extragonadal tissues [33, 34], gonadotropins receptors are mainly expressed in gonads. In ovaries, LH-R are expressed in theca cells and lutein cells [2], and mediate LH-induced steroid hormone synthesis, ovulation and *corpus luteum* formation. In testes, this receptor is expressed in Leydig cells where LH stimulates testosterone (T) production. As regards the FSH-R, they are expressed in the ovarian granulose cells and in the testicular Sertoli cells, suggesting a role in gametogenesis and follicular development.

1.1.3. GONADS

The gonads include the testes in males and the ovaries in females and play a dual role in both sexes: *1)* generating and releasing of mature gametes from germ cells (gametogenesis), and *2)* synthesis and secretion of steroid and peptidergic hormones (hormonogenesis) that regulate, among other actions, the production of these gametes.

1.1.3.1. TESTES

The mammalian testes are paired oval structures whose main functions are the production of sperm and the androgen synthesis. Testes have a heterogeneous cell structure and are organized in two compartments: the interstitial or intertubular compartment and the tubular compartment, formed by the seminiferous tubules [35] (**Figure 5**). Interactions between the different cells populations are required for the proper development of gonadal functions.

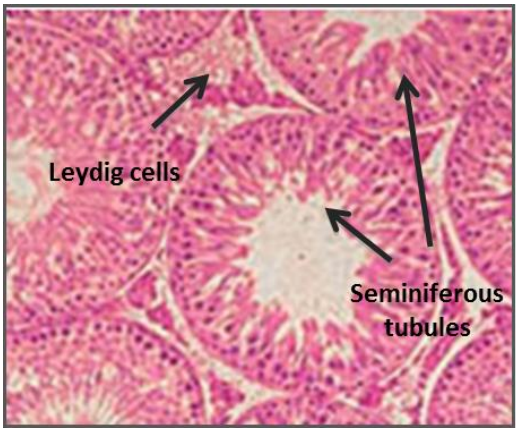

Figure 5. Illustration of testis organization which consists of the Leydig cells which are found in clusters within the interstitial tissue between seminiferous tubules.

In the **testicular interstitial** compartment, the following cell types can be identified: Leydig cells, macrophages, lymphocytes, stromal fibroblast cell and endothelial cells [36]. The main interstitial cell type are Leydig cells and their major function is the synthesis of T from cholesterol in response to LH [37]. Intratesticular T is required to stimulate spermatogenesis, while a substantial fraction is secreted into the systemic circulation where a majority of T binds to plasma proteins such as albumin and globulins, mainly to the sex hormone-binding-globulin (SHBG).

The androgenic effects of T in peripheral tissues require its metabolization, via the enzyme 5α-reductase, into dihydrotestosterone (DHT). By contrast, the central actions of T are carried out as such or after its aromatization to estradiol. The most important functions of androgens are: regulation of LH and FSH secretion from the gonadotropes by negative feedback mechanisms at hypothalamic and pituitary level; sexual differentiation of central nervous system (CNS) before birth and during neonatal period; stimulation of the spermatogenesis; and acquisition and maintenance of secondary sex characteristics after puberty [38].

In the **seminiferous tubules** germ cell and Sertoli cells are located [36] (**Figure 5**). Germ cells, under FSH and T influence, will develop into mature sperm. Sertoli cells support germ cell nutrition and their development [39], and also they respond to FSH stimulation inducing the synthesis of proteins such as activins and inhibins, which regulate FSH release at pituitary level, or the testicular androgen-binding protein (ABP). In the seminiferous tubules, FSH and T regulate the interaction between the Sertoli cells and germ cells [40-42].

1.1.3.2. OVARIES

The main functions of the ovaries are the production of fertilizable gametes (oogenesis) and the synthesis of sex steroids such as estrogens, progesterone (P), and to a lesser degree, 17α-hydroxyprogesterone, androstenedione, estrone and T. In addition, ovaries also contribute to maintaining optimal hormonal conditions during early stages of gestation.

The first level of follicular organization is the primordial follicle, which is formed by a primary oocyte surrounded by a monolayer of folicullar cells called granulosa cells. This primordial follicle undergoes a series of successive transformations leading to the formation of primary follicle, where the oocyte increases in size and begins to accumulate polysaccharides between the oocyte and granulosa cells. Secondary follicles are characterized by an increase of granulosa cells, which will determine the production of the main estrogen in females, the 17β-estradiol (E_2), and by the recruitment of interstitial cells that will originate the theca interna. Tertiary or antral follicles are characterized by an increase of granulosa cell layers and the recruitment of more additional interstitial cells that form a layer surrounding the theca interna, leading to the theca externa and the formation of follicular antrum. The preovulatory follicle or de Graaf follicle, after LH-mediated follicular rupture, allows the exit of secondary oocyte from the ovary at ovulation (**Figure 6**). In women, ovulation occurs approximately every 28 days.

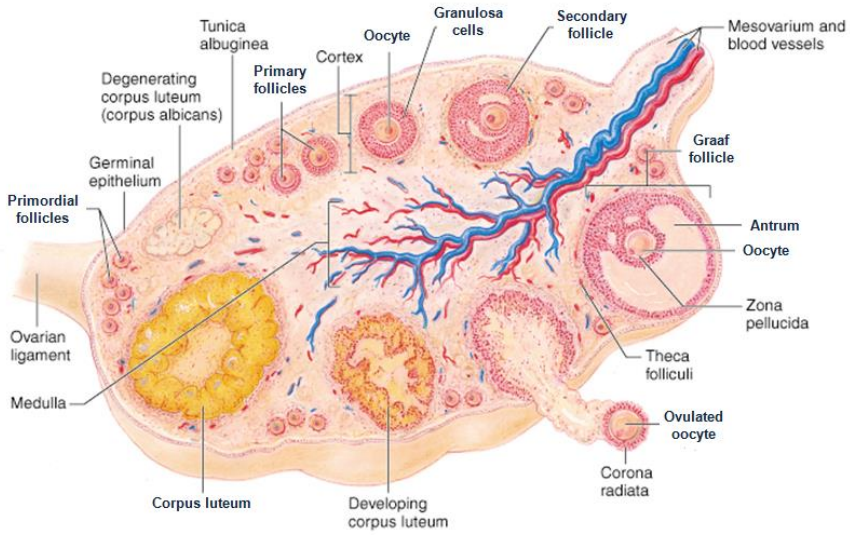

Figure 6. Diagram showing anatomical structure of the ovary. The ovarian cycle consists of the follicular phase, ovulation, and luteal phase. Modified from [43].

Although LH and FSH are crucial in the follicular growth, others paracrine factors are involved in the modulation of follicular growth: E_2, growth factors such as insulin-like growth factor I and II (IGF-I and II), epidermal growth factor (EGF), tumor necrosis factor α and β (TGF-α and β) and fibroblast growth factor β (FGF-β); gonadal peptides (inhibins, activins and follistatins); and cytokines such as interleukin 1 (IL-1) and TNF- α [44-46].

Female rats have cyclic variations in their reproductive activity and changes in vaginal epithelium every 4 days. Thus, estrous cycle of the rats is characterized by the following phases in terms of vaginal cellularity: diestrus-1 or metestrus (D-1), diestrus-2 (D-2), proestrous and estrus, which are consequences of the changes in ovarian steroid secretion (**Figure 7**). Ovulation occurs in estrus phase as result of the preovulatory gonadotropin surge in the afternoon of proestrous [47, 48]. In the female mouse, the duration of the estrous cycles varies from 4 to 6 days depending on the laboratory mouse strains.

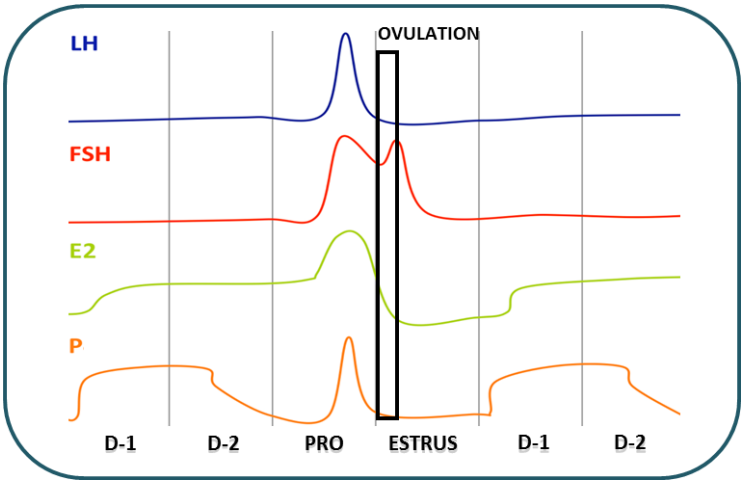

Figure 7. Hormonal profile during the estrous cycle in rat. Phases of the estrous cycle: D-1: diestrus-1 or metestrus; D-2: diestrus-2; PRO: proestrous; y Estrus. E2: estradiol. P: progesterone. Modified from [49].

1.2. REGULATION OF HPG AXIS

GnRH neurons are considered the key hierarchical element in the neuroendocrine control of reproduction, since they act as the final integrators of central and peripheral signals and major output pathway for the brain control of

reproductive function. For this reason, the proper functioning of HPG axis depends on the correct regulation of GnRH neuronal activity.

1.2.1. CENTRAL REGULATORS

The control of GnRH secretion to the portal system depends on the integration of stimulatory and inhibitory central signals. Among them, we highlight the following:

1.2.1.1. STIMULATORY SIGNALS

- **Kisspeptins**

Kisspeptins are considered, especially in mammalian species, as essential primary regulators of GnRH neurons at the hypothalamus and one of the most potent stimulators of the HPG axis. Given the special focus of this Thesis, kisspeptins will be described in detail in section 1.4.

- **Excitatory amino acids (EAA)**

The major excitatory neurotransmitter of the CNS is glutamate (Glu) [50] and its actions are mediated by two receptor types: ionotropic and metabotropic receptors. Ionotropic Glu receptors (iGluRs) are ion channels and are classified according to their ligand binding affinities in: NMDA (N-Methyl-D-Aspartate), AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and KA (kainate) receptors [51, 52]. Electrophysiological techniques have revealed that these receptors may coexist in different neuronal populations [53]. Metabotropic Glu receptors (mGluRs) are GPCRs, with eight different subtypes, classified in three groups. The first group (mGluR1 and -R5) activates PLC, while members of the second (mGluR2 and -R3) and the third group (mGluR4, -R6, -R7 and -R8), except mGluR6 (that activates a cGMP phosphodiesterase) are negatively coupled to adenylate cyclase [54, 55].

GnRH neurons express iGluRs in their axon terminals in the ME [56, 57]. Data obtained *in vivo* indicated that intracerebroventricular (icv) administration of iGluRs agonists induce LH release [58] and *in vitro* studies have been demonstrated Glu direct actions on LH release in the pituitary [59]. Also, NMDA and non-NMDA receptors agonists and antagonists are able to increase or reduce, respectively, the pulsatile

GnRH/LH secretion, in both male and female rats [60-62]. In this context, dual-labeling studies have shown that GnRH neurons express AMPA, NMDA and kainate receptors in a heterogeneous manner, however, the importance of different iGluR signaling on GnRH neurons remains unclear. Thus, whereas *in vivo* studies have indicated the importance of NMDA signaling within the whole of the GnRH neuronal network, electrophysiological studies have showed that AMPA/kainate receptors predominate over NMDA receptors in adult GnRH neurons [53, 63].

In addition, Glu mediates partially the effects of gonadal steroids on GnRH secretion. Thus, immunohistochemistry (IHC) studies demonstrated the colocalization of steroid hormone receptors (estrogen receptor α and β, ERα and ERβ; and androgen receptor, AR) and NMDA and non-NMDA receptors in several hypothalamic nuclei that are involved in the control of GnRH secretion [64]. The importance of glutamatergic pathway mediated by mGluRs on GnRH neurons remains incompletely defined, although stimulatory effects on GnRH secretion have been reported [65, 66].

- **Neurokinin B**

Neurokinin B (NKB), a member of the tachykinin family, is a neuropeptide of 10 aa encoded by *TAC3* gene in humans and *Tac2* gene in rodents. The tachykinins have a common carboxyl terminal aa sequence Phe-X-Gly-Leu-Met-NH2, where X is a hydrophobic residue decisive for the activation of its receptor. Tachykinins act on three types of GPCRs, called NK1R, NK2R and NK3R [67].

NKB exhibits a high-affinity binding for NK3R [68]. NK3R is encoded by *TACR3* gene in humans and *Tacr3* gene in rodents. Given the wide cerebral distribution of NKB/NK3R system, in recent years, NKB has been proposed to be involvement in the control of multiple function systems, such as motor cortex and reproductive function [69, 70]. On the latter, IHC analysis, initially conducted in rats and mice revealed *NK3R* expression in the axon of GnRH neurons, supporting the hypothesis that NKB have a potential direct effect on these neurons [71]. Recent data, however, have questioned the functional relevance of direct KNB actions on GnRH neurons. In this sense, IHC analyses in sheep have demonstrated the existence of NK3R in POA and several hypothalamic regions as ARC and, although GnRH neurons and fibers were in proximity

to NK3R-containing fibers, the lack of NK3R in GnRH neurons suggested that the actions of NKB on GnRH secretory activity are mediated indirectly, [72].

Despite the variable effects of NKB on LH secretion reported in initial studies [70, 73], recent works carried out in different species (such as sheep, rat and monkey) indicate a stimulatory effect of NKB on GnRH/LH secretion [69, 70]. Furthermore, the NKB/NK3R system has been recently implicated in the regulation of reproductive axis in humans, as inactivating mutations in *TAC3/TAC3R* have been found in cases of hypogonadotropic hypogonadism (HH) [74]. As regards the latter, phenotypic discordance between mice and humans have been proposed due to subfertility has been reported in *Tacr3*$^{-/-}$ mice [75]. Thus, although capable of fertility, these animals have central reproductive defects. In this sense, it has been suggest that NKB may activate other tachykinin receptors to partially stimulate GnRH release, in the absence of *Tacr3*, demonstrating that other tachykinin pathways are important in regulating GnRH function [76].

- **Galanin**

Galanin (GAL) is a neuropeptide of 29 aa (30 aa in humans), highly conserved between species [77]. Its effects are mediated by three types of GPCRs: GAL-R1, -R2 and -R3 [78]. It has been described that GAL plays an important role in the regulation of gonadotropin releasing hormone [1] release. Thus, the central administration of GAL enhances GnRH release and increase LH levels in ovariectomized (OVX) rats [79] and it was also confirmed that GAL stimulates GnRH secretion by *in situ* hybridization (ISH) analyses [80]. Moreover, it has been reported in different species, including the rat and sheep, that GAL may act in a subpopulation of GnRH neurons through the GAL-R1 and that its effects is influence by levels of circulating gonadal steroid [81, 82].

- **Galanin-like peptide (GALP)**

GALP, so named for a 10-amino acid region of sequence identity to GAL, is highly conserved between mice, rats and humans [83]. Despite the common amino acid sequence shared by GAL and GALP, these peptides are derived from separate genes [83]. In the mammalian species, expression of GALP is expressed in the ARC and is implicated in the neuroendocrine regulation of metabolism and reproduction [83,

84]. In this regard, it has been described that chronic central administration of GALP increases LH release in male rats [85, 86]. Notably, this peptide is positively regulated by several factors related to energy metabolism such as leptin [84]. In addition, it has been demonstrated that GALP may play a role in readjusting energy balance under changing nutritional circumstances [87]. Thus, this data suggested that GALP is involved in the integrated control of metabolism and reproduction [88].

- **Catecholamines**

It is well-known that catecholaminergic signaling (adrenaline and noradrenaline; NA), via α1- and β-adrenergic receptors, plays an important role in the direct activation of GnRH neurons [89]. The first evidence that suggested the catecholaminergic control of GnRH secretion was obtained after observation that the elimination of NA neurons in the brainstem, as well as the blockade of α-adrenergic receptor, decreased the pulsatile LH secretion [90]. In addition, it was demonstrated the presence of α-adrenergic receptors in GnRH neurons and in a GnRH neuronal cell line, namely the GT1-7 [91]. Specifically, adrenaline seems to be involved in the stimulation of pulsatile and preovulatory GnRH surge, while NA is more related to the control of LH pulse frequency [92].

- **Nitric oxide**

Nitric oxide (NO) is a gaseous neurotransmitter, synthesized by nitric oxide synthase (NOS), known for its important role in regulating neuronal transmission. Recently, it has been implicated in the neuroendocrine control of reproduction and in the regulation of GnRH neuronal activity [93, 94]. In addition, it has been demonstrated that kisspeptin neurons directly act on NO-synthesizing neurons in the POA to promote preovulatory neuronal NOS activation and that these interactions modulate LH secretion [95]. Support this statement, the infusion of a NOS inhibitor, L-NAME, in POA or ME, causes the disruption of estrous cyclicity in rats [96]. However, L-NAME has been also shown to enhance LH secretion at some conditions suggesting some inhibitory action of NO. Moreover, it has been reported that neurons synthesizing NO in the POA act as mediators of the stimulatory effects of leptin to the

reproductive axis [97]. The role of NO signaling as intermediate pathway in the actions of leptin in GnRH neurons will be further described in section 1.5.1.

1.2.1.2. INHIBITORY SIGNALS

- **GABA**

GABA is the most abundant inhibitory neurotransmitter of CNS and its effects are mediated by two types of GABA receptors: ionotropic ($GABA_A$ and $GABA_c$) and metabotropic ($GABA_B$) [98]. Most of the actions described for GABA and its receptors in GnRH neurons and GT1-7 neuronal line indicate that this neuropeptide regulates GnRH secretion both directly and indirectly [99]. While pharmacological studies attribute an inhibitory effect of GABA on GnRH release, electrophysiological studies in adult female mice suggest that GABA may also have excitatory effects [100]. Subsequent studies in the GT1-7 neurons showed a biphasic action of GABA on GnRH secretion, where initial stimulation is followed by a prolonged inhibition [101]. The administration of selective $GABA_A$ and $GABA_B$ agonists *in vivo* indicate that the actions of GABA are mediated mostly by $GABA_A$ receptor, exerting an inhibitory effect of the preovulatory and pulsatile GnRH/LH secretion [102].

- **Endogenous opioids**

The endogenous opioid peptides (EOPs) are classified into three groups: enkephalins, β-endorphins (β-END) and dynorphins (Dyn). They exert their effects in the CNS interacting with three receptor types: μ, κ and δ, acting β-END and Dyn via μ-receptor and κ-receptor, respectively [103].

EOPs are potent physiological inhibitors of GnRH/LH secretion. Thus the β-END/μ-receptor system appears to play a role in the control of the GnRH/LH surge [104], while the Dyn/κ-receptor system has demonstrated an inhibitory effect on the GnRH secretion acting as mediator of the negative feedback exerted by P on pulse LH frequency [105]. Since the expression of EOPs receptors has been not detected in GnRH neurons, but they are located in POA and others hypothalamic areas [106], it has been suggested that the inhibitory effects of β-END and Dyn on GnRH neurons could be mediated through interneurons [90]. Moreover, it has been demonstrated that a

population of Kiss1 neurons of the ARC coexpress *Dyn* and *NKB* genes and also NK3R and κ-receptor. NKB and Dyn act autosynaptically in these neurons to regulate the pulsatile secretion of kisspeptin [70]. The role of these neurons will be described in section 1.4.5

- **Neuropeptide Y (NPY)**

NPY, a peptide of 36 aa highly conserved between species, is one of the most abundant neurotransmitters in the mammalian CNS [107], being considered a key modulator in the control of reproductive function. It is expressed in the ARC and in the brainstem, being its expression partially regulated by estrogens. The NPY effects are mediated by five types of GPCRs called Y1, Y2, Y4, Y5 and Y6. Among these, Y1 and Y5 receptors have been implicated in NPY actions on the HPG axis. The Y5 receptor is expressed in GnRH neurons and it is involved in the inhibitory effect of NPY on LH secretion [108, 109]. The Y1-positive fibers are found in close apposition of GnRH neurons in the POA [110]. It has been reported that central administration of a Y1 receptor antagonist in juvenile rats or monkeys stimulates LH release and advances the timing of puberty onset [111, 112]. Moreover, it has been reported that Y1 knock-out (KO) mice showed an advance in the timing of puberty onset in unfavorable metabolic conditions [113]. The inhibitory effect of NPY on GnRH/LH secretion has been demonstrated in both male [114] and female rats [115, 116]. However, contradictory effects of NPY in the control of HPG axis have been reported depending on the endocrine state, receptor subtype activated and the pattern of administration (acute or chronic). In this sense, it has been demonstrated that central administration of NPY in pubertal female rats is able to induce an advance in the normal age of vaginal opening (VO), an external index of puberty onset, and that NPY administration in 3V stimulates LH release at this age [117]. In addition, it was demonstrated that the central infusion of NPY in OVX females supplemented with E_2, stimulates LH response [118]. A crucial role for the NPY in the mechanisms involved in the regulation of GnRH neuronal activity by metabolic factors has been also reported. This role will be further described in section 1.5.2.

- **Melatonin**

Melatonin is a tryptophan-derived hormone produced in the pineal gland and related with the photoperiodic reproduction. It has been considered an inhibitory neurotransmitter for puberty, as a result of the observation of precocious puberty in humans with pineal tumors [9]. Melatonin levels increase in dark conditions, limiting the sexual behavior in many species. Even so, the nocturnal LH increase in prepubertal stage that happen in many animals, including primates, is favored by the expression pattern of melatonin during postnatal development, which is high during neonatal and early childhood period and then decreases until adulthood, allowing this night activation of reproductive axis [119].

- **Gonadotropin-inhibiting hormone**

For many years it was believed that GnRH was the common final via through the hypothalamic signals controlling gonadotropins secretion. In 2000, was discovered a hypothalamic neuropeptide of 12 aa (SIKPSAYLPLRF-amide) that, acting directly into pituitary, inhibits gonadotropins secretion in birds [120] and for this reason was called gonadotropin-inhibiting hormone (GnIH).

In recent years have been identified GnIH homologs of the avian peptide in other vertebrate species including mammals, reptiles, amphibians and teleosts. Since of they share a common C-terminal sequence, Arg-Phe-NH2 (RFamide), they are included in the family of RFamide-related peptides (RFRPs). In mammals, two GnIH peptides, RFRP-1 and RFRP-3 [114, 121], have been identified, the latter is considered the functional mammalian ortholog for avian GnIH.

In rats and hamsters, the cell bodies of RFRPs neurons are mainly located in DMN and their fibers appear to contact with GnRH neurons, suggesting important functional interactions between both systems. In sheep and hamsters, RFRP-IR fibers have been located in ME [122].

RFRP-3 acts via two types of GPCRs: GPR147, also termed neuropeptide FF receptor type 1 or NPFF-R1, that is the receptor for RFRPs [121]; and GPR74 or NPFF-R2, which has the less affinity [25]. Those receptors are widely distributed in the CNS (including GnRH neurons), pituitary and gonads [123].

The icv or intraperitoneal (ip) administration of avian GnIH reduces LH levels in OVX hamsters [124], while icv administration of RFRP-3 has the same effect in rats [125]. Moreover, studies have been demonstrated that RF9, a selective NPFF-R1 antagonist, induces a strong increase of LH secretion [126], thus supporting the role of RFRPs as central regulators of reproductive axis in mammals. However, recent evidence also suggested that not all the gonadotropin-releasing effects of RF-9 derive from their ability to block NPFF receptors [127].

- **Gonadotropins**

Gonadotropins exert an inhibitory control of their own synthesis through short feedback loops at hypothalamic level and paracrine mechanism acting over adjacent gonadotrope cells at the pituitary level [128].

1.2.2. PERIPHERAL REGULATORS

The function of the HPG axis is specifically regulated by several peripheral signals; among them the signals produced by the gonads and metabolic signals play especially relevant roles.

1.2.2.1. GONADAL REGULATORS

Two groups of gonadal regulators may be distinguished according to their steroid or peptidergic nature:

- **Gonadal steroids**

Sexual steroids (estrogens, androgens and P) are mainly synthesized in gonads, and to a lesser extent, in adrenal glands, brain (neurosteroids) and other tissues. Gonadal steroids and neurosteroids exert a wide variety of effects on CNS, being remarkable their role in the control of HPG axis, where they play a dual role. On one hand, they participate in the control of sexual differentiation of the brain and on the other, they modulate the GnRH/gonadotropin secretion and the activation of neural circuits involved in mating and breeding behavior [129, 130].

Gonadal steroid actions are mediated by their interaction with intranuclear receptors called androgen receptor (AR), progesterone receptors A and B (PR-A and

PR-B) and estrogen receptor α and β (ERα and ERβ). The interaction of estrogens with ERs activates intracellular signaling cascades [131]. It has been reported also that some estrogen effects are mediated by their interaction with ionotropic and metabotropic receptors localized in the cell membrane [132].

ERs are widely distributed in the CNS (including the hypothalamus) and pituitary. In the hypothalamus, ERs are expressed in relevant nuclei involved in the control of energy homeostasis and also in nuclei implicated in the control of reproductive function, including the ARC and the anterior ventral periventricular area (AVPV) [131, 133].

Nowadays, it has been proposed that androgens and estrogens, which interact with AR and ERs in the ARC, exert their negative feedback effects on LH and FSH secretion, while estrogens, which interact with ERs in the AVPV, participate in the positive feedback effects on LH release in female rodents [134]. The direct actions of estrogens on GnRH neurons are mediated by ERβ, the only ER subtype expressed in these neurons, while estrogen actions in GnRH neurons mediated through ERα must be indirect [135, 136]. In this sense, it has been reported that estrogen positive feedback to generate the preovulatory gonadotropin surge was normal in ERβ KO mice, but was absence in ERα mutant mice, demonstrating that ovulation is driven by estrogen actions upon ERα -expressing neuronal afferents to GnRH neurons [137].

- **Gonadal peptides**

Gonadal peptides (inhibins, activins and follistatins) are hormones that regulate gonadotropins release at the pituitary level and exert a paracrine action within the gonads.

Inhibins are proteins produced by Sertoli cells in the testes and by granulosa cells in the ovary. Two types of inhibin have been identified: inhibin A and inhibin B. Both are heterodimeric proteins consisting of two subunits joined by disulfide bonds: a common α subunit and an unique β subunit, being β_A to inhibin A (α, β_A) and β_B to inhibin B (α, β_B) [138]. Inhibins can selectively inhibit FSH secretion in pituitary gonadotropes [139] and also exert a paracrine action on the gonads.

Activins are heterodimeric glycoproteins composed of two inhibin β subunits. It Three types of activins have been identified: activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$) and activin AB ($\beta_A\beta_B$). They stimulate FSH secretion at pituitary level and exert a paracrine action on the gonads, stimulating folliculogenesis in the ovaries [140].

Follistatins are single-chain glycoproteins with a molecular weight between 31 and 49 kDa [141]. The follistatin gene encode two isoforms, follistatin 315 (315 aa) and follistatin 288 (288 aa), resulting from alternative splicing of the precursor messenger mRNA; the 315 aa variant can be subsequently degraded to follistatin 303. Follistatin 315 and 288 inhibit specifically the secretion of pituitary FSH both *in vitro* and *in vivo*, via the blockade of activins [142]. Similarly to activins, follistatins are synthesized in various tissues, including the ovaries and pituitary, where they exert a paracrine action [143].

1.2.2.2. METABOLIC REGULATORS

Reproductive function is a costly process in terms of energy consumption and it is not essential for survival at the individual. Thus, sophisticated mechanisms have been selected during evolution to allow specific inhibition of the HPG axis in unfavorable energetic conditions. For this reason, reproductive axis is highly sensitive to metabolic and energy status of the organism. The different metabolic factors responsible for conveying the metabolic information to the CNS and modulating the reproductive capacity will be described in detail in the section 1.5.

1.2.2.3. OTHERS PERIPHERAL REGULATORS

Other peripheral signals, such as adrenal and thyroid hormones, can influence gonadotropin secretion. Thus, it has been described that glucocorticoids inhibit directly the synthesis and release of GnRH and, in addition, an increase in glucocorticoids levels was followed by a decrease in LH secretion in OVX sheep [143]. On the other hand, it has been reported that thyroid dysfunction affects GnRH secretion, as evidenced by the fact that thyroidectomy and hypothyroidism in rats exert an inhibitory effect on GnRH secretion [144].

1.3. PUBERTY

Puberty is defined as the transition period from juvenile stage to adulthood during which the reproductive capacity is reached. Puberty must be understood not as a one-time event but as a sequence of events leading to full activation of the reproductive axis and the acquisition of reproductive competence. A biological hallmark of puberty is the elevated secretion of gonadal steroid hormones, which result in the development of secondary sex characteristics, such as the growth of genitalia, the appearance of pubic hair in both sexes and facial hair in boys, and breast development in girls, among others. Frequently, puberty are used synonymously with adolescence, however they are not the same. Adolescence is essentially referred to humans and is considered the period between childhood and adulthood, encompassing not only reproductive maturation, but also cognitive, emotional, and social maturation, which leads to the acquisition of adult cognition and social behaviors [145]

During the infantile, juvenile and prepubertal ages, the HPG axis remains completely silent in humans and partially silent in rodents. This is because the secretory activity of GnRH neurons is low and insufficient to support gonadal growth. The onset of puberty and so, the maturation of the HPG axis, are characterized by a gradual increase in the frequency and amplitude of pulses of GnRH secretion [12]. GnRH directs the synthesis and secretion of pituitary gonadotropins, which stimulate the gonadal hormones production and gametogenesis.

1.3.1. NEURONAL AND NON-NEURONAL NETWORKS CONTROLLING PUBERTY ONSET

As mentioned above, the key event required for puberty to occur is an increase in pulsatile GnRH release from GnRH neurons. In primates, these neurons are mostly located in the MBH, while in rodents most of them reside in the POA [146]. Numerous studies have been described that, together with the decrease of sensibility to negative feedback of gonadal steroids, the pubertal increase in GnRH secretion depends on coordinated changes in neuronal and non-neuronal communication [147, 148], consisting of a coordinated increase in excitatory inputs from central and peripheral

origin, combined with a decrease in inhibitory inputs [149]. GnRH secretion is stimulated by excitatory aa, such as Glu, and by the Kiss1/Gpr54 system. The inhibitory input to GnRH neurons is provided by GABAergic, opiatergic neurons or the product of RFRP gene, which may act directly on GnRH neurons or indirectly via inhibition of neurons involved in the stimulatory control of GnRH secretion, such as kisspeptin neurons [150-152]. However, very recent data from our group indicate that NPFFR1 KO mice, do not show detectable alterations in the onset of puberty, suggesting that, a decreased in GnIH signaling is not mandatory for the normal timing of puberty onset [153].

It has been proposed that, in addition to neuronal inputs, the pubertal activation of GnRH secretion also requires information from non-neuronal cells, such as glia [154] and endothelial cells [155, 156]. GnRH nerve terminals are separated from the vascular wall of the portal system in the ME by glial structures belonging to either astrocytes or tanycytes, specialized ependymoglial cells of the ME which line the ventral portion of the 3V. Tanycytes may convey information not only between themselves and GnRH nerve terminals, but also between GnRH terminals and endothelial cells of ME [157]. It has been demonstrated that GnRH secretory activity is modulated, at least in part, by specific cell-cell signaling molecules. Thus, glia-neuronal interactions in the ME regulate GnRH release by the production of different signals, including growth factors, such as epidermal growth factor (EGF)-related peptide, transforming growth factor α (TGF-α), and neuroregulins (NGRs), that act directly and indirectly on GnRH neurons [147, 154, 157], whereas endothelial cells of ME employ NO [95, 157] **(Figure 8)**.

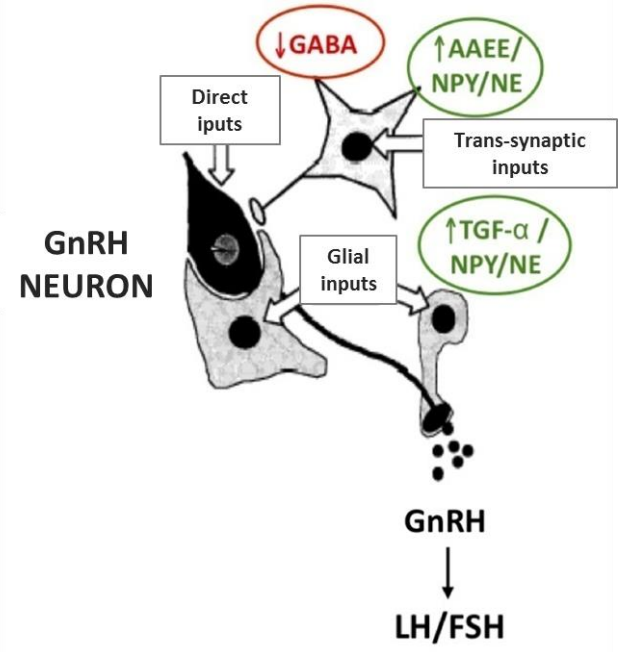

Figure 8. Schematic representation of trans-synaptic and glial inputs to the GnRH network. Modified from [129].

Several studies have suggested that no single pathway or cellular subset is only responsible for the neuroendocrine control of puberty, but there are many genes involved in this control [147, 158]. In support this idea, the Ojeda's laboratory has proposed that puberty is controlled by a set of genes hierarchically arranged within functionally connected networks where GnRH is the final effector [159]. According to this hypothesis, the highest level of network control is provided by transcriptional regulators directing controlling key subordinate genes of the network (such as those operating within kisspeptin, GABAergic, glutamatergic, opioid, and RFRP neurons) in addition to genes expressed in glial cells [147]. Recently it has proposed the concept that a more dynamic and encompassing level of integrative control of puberty is provide by mechanism of transcriptional repression of genes that are stimulatory to the puberty [160, 161]. In addition, microRNAs (miRNAs) have the ability to modulate gene expression at multiple hierarchical levels [162, 163], thus they may act as regulatory system able to integrate the different networks involved in the control of puberty. Recent genome-wide association studies (GWAS) have demonstrated an association of a sequence variation in *Lin28b* (a gene encoding an RNA-binding protein) with early menarche [164-167]. In this line, *Lin28*-related genes (including *Lin28b*) have

been shown to bind to the terminal loops of precursors of the *let-7* family of miRNAs, blocking their processing into mature miRNAs [168]. Given the importance of Lin28/let-7 system in this thesis, their potential role in the timing of puberty will be described in more detail in section 1.7.

1.3.2. PERIPHERAL AND ENVIRONMENTAL SIGNALS FOR THE CONTROL OF PUBERTY

It is well established that activation of the HPG axis during puberty and its maintenance in adulthood, are very sensitive to the action of peripheral endogenous and environmental factors, which interact with central regulators to control the onset of puberty [146]. These are all permissive signals and vary with species and sex, and provide information about the availability of resources and conditions necessary for successful reproduction. Among these factors, internal metabolic cues such as leptin, ghrelin and insulin, relay information about the status of energy reserves and the metabolic state of the organism to CNS [169]. Of note, before the discovery of leptin role in pubertal HPG axis activation, many observations in humans and rodents had pointed out the need for a minimum of energy reserves to achieve reproductive competence. Soon after the discovery of leptin in 1994, it has shown that low leptin levels (hypoleptinemia) induce a delay in the acquisition of reproduction, while high leptin levels (hyperleptinemia) produce an advance in the age of puberty onset [170].

In addition to metabolic cues, environmental factors such as weather or stress conditions and photoperiod (circadian rhythms) [171]also contribute to determine the timing of sexual maturation [172]. In this sense, several studies in humans demonstrated a relationship between the hours of light/season and the timing of menarche. For example, girls living in countries with a large number of daylight hours and warmer temperatures acquired puberty an earlier ages than the girls who live in Nordic countries, with fewer daylight hours and lower temperatures [173]. However, in humans and other mammals, which can breed through the year, these factors are much less important than in animals with fertile periods linked to the season (seasonally breeders).

Finally, epidemiological studies in humans and experimental data in rodents suggest that exposure during the initial stages of development to some natural or synthetic compounds with estrogen mimetic activity (termed as endocrine disruptors) may also influence the age of pubertal onset [174].

As a summary, a schematic representation of the main elements, mentioned above, involved in the control of puberty onset is provided below (**Figure 9)**.

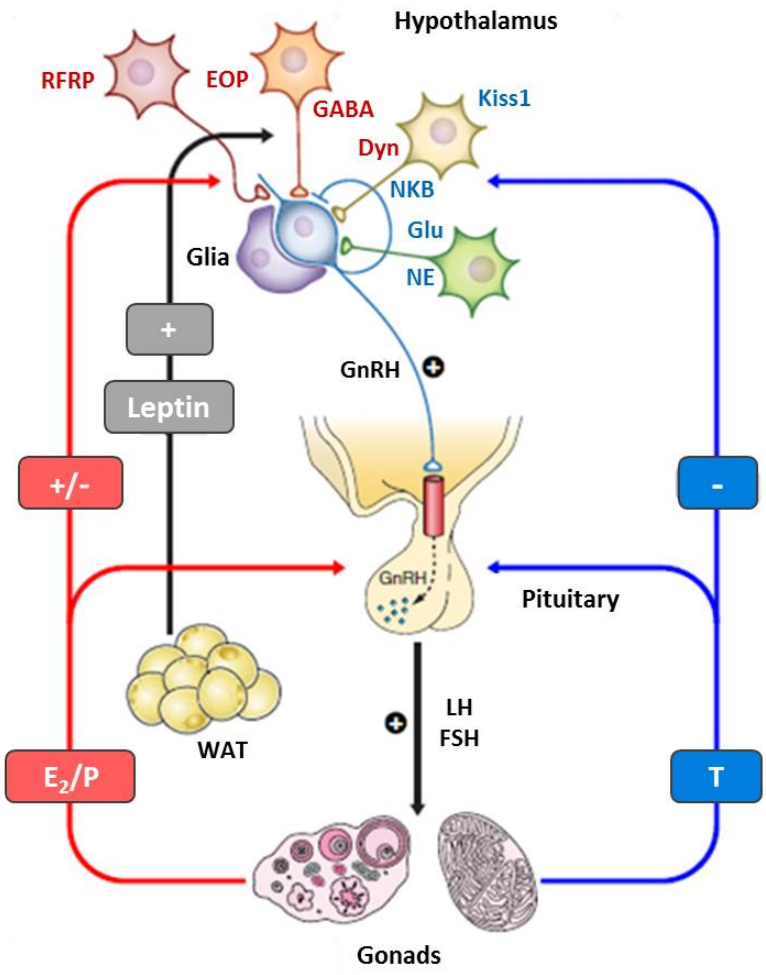

Figure 9. Schematic representation of the major elements involved in the control of puberty onset. GnRH neurons, which receive trans-synaptic and glial inputs, release GnRH to the hypophyseal portal blood system. In turn, GnRH leads the pulsatile secretion of gonadotropins, LH and FSH that stimulate the maturation and regulate the function of the gonads. These major hormonal elements are connected via negative and positive feedback regulatory loops. Moreover, the function of the HPG axis is under the regulation of several peripheral signals, such as gonadal steroids or metabolic hormones. Some of the central inhibitory (red) and excitatory (blue) regulators of the HPG axis are also shown. Modified from [175].

1.4. Kiss1/Gpr54 SYSTEM

As a means of introduction, in this section we will describe the major elements of the Kiss1/Gpr54 system and how the identification of the physiological role of kisspeptins and its receptor, Gpr54, in the control of GnRH secretion have led to significantly advance our knowledge of central mechanisms involved in the control of puberty and reproduction.

In order to unify criteria regarding the nomenclature used in this Thesis, we note here that we use *KiSS1* and *Kiss1* to denote the gene and its transcripts (mRNA) in primates and rodents, respectively. The protein products of *KiSS1*/*Kiss1* genes will be designated as kisspeptins. On the other hand, the receptor of kisspeptins will be referred as GPR54 and Gpr54 in primates and rodents, respectively, while *GPR54* and *Gpr54* will be used to name the corresponding genes/mRNA. To refer to the ligand/receptor system without references to any given species, the term Kiss1/Gpr54 will be used.

1.4.1. HISTORICAL BACKGROUND

The elements (genes and peptides) of this ligand/receptor Kiss1/Gpr54 system were separately identified between 1996 and 2001, but their close association with reproductive physiology came later [176, 177]. In 1996, *KiSS1* gene was originally identified as a metastasis suppressor gene in a melanoma cell line and the mRNA *KiSS1* as a selectively overexpressed transcript in tumor cells with low invasiveness [178]. For this reason, the KiSS1-derived peptide was initially named as metastin. Independently, in 1999 *Gpr54* was identified in the rat brain as an orphan receptor with a significant sequence similarity (>40%) with the transmembrane regions of GAL receptors [179]. Subsequently, the human ortholog of *Gpr54* was cloned, and named *AXOR12* or *hOT7T175* [180]. Finally, in 2001 three independent groups functionally linked the Gpr54 receptor to *Kiss1* gene and metastin, defining the so-called Kiss1/Gpr54 system [171, 181]. One of this report identified several isoforms derived from *Kiss1* with capacity to bind Gpr54 and termed then kisspeptins [171].

It was not until late 2003 when the first evidence of the role of this system in the control of reproductive function was published. In this year, two independent

reports documented the presence of deletions and inactivating mutations of the *GPR54* gene in patients suffering idiopathic hypogonadotropic hypogonadism (iHH). Such findings in humans were reinforced by the report that mice engineered with lack functional *Gpr54* were a complete phenocopy of affected patients [182, 183]. All together, these observations suggested a crucial role for this system in the regulation of HPG axis.

At present, several transgenic (Tg) mouse lines have been generated with mutations in the *Gpr54* gene and in the *Kiss1* gene [184] to reproduce the HH in humans. The nomenclature for these Tg lines is based on the guidelines established by the International Committee on Standardized Genetic Nomenclature for mice (http://www.informatics.jax.org/mgihome/nomen/index.shtml).

The *Gpr54* and *Kiss1* mutant mouse lines show similar phenotypes indicating the absence of compensation by other ligands. All of them are characterized by a lack of normal pubertal maturation and infertility, showing poor development of the gonads with low gonadotropin circulating levels (HH) compared with their respective wild type controls [185]. Males showed disrupted spermatogenesis [183] and females displayed a delay in VO and a disruption of the normal cyclic changes associated with the estrous cycle [183, 186, 187]. In addition, both *Gpr54* and *Kiss1* KO had lower BW than WT, selectively after puberty [183, 186-189].

1.4.2. MAJOR ELEMENTS: *Kiss1*, KISSPEPTINS AND Gpr54

The human *KISS1* gene contains three exons and two introns [190]. An alternative genomic organization, with four exons (the first two being noncoding exons), has been also proposed [191]. The first exon consists of 111 pair of bases and it is not translated, the second exon contains 141 nucleotide (nt), with the star codon at position 39, and the third exon consists of 322 translated nt, followed by the translational stop codon and the polyadenylation sequence [190]. The mouse *Kiss1* gene contain only two translated exons, which correspond to the last two exons of the human gene. The first exon encoded the signal peptide and part of prepro-kisspeptin protein and the second encoded the remaining of the precursor protein including the sequence to kisspeptin-10 (Kp-10) [187] (**Figure 10**).

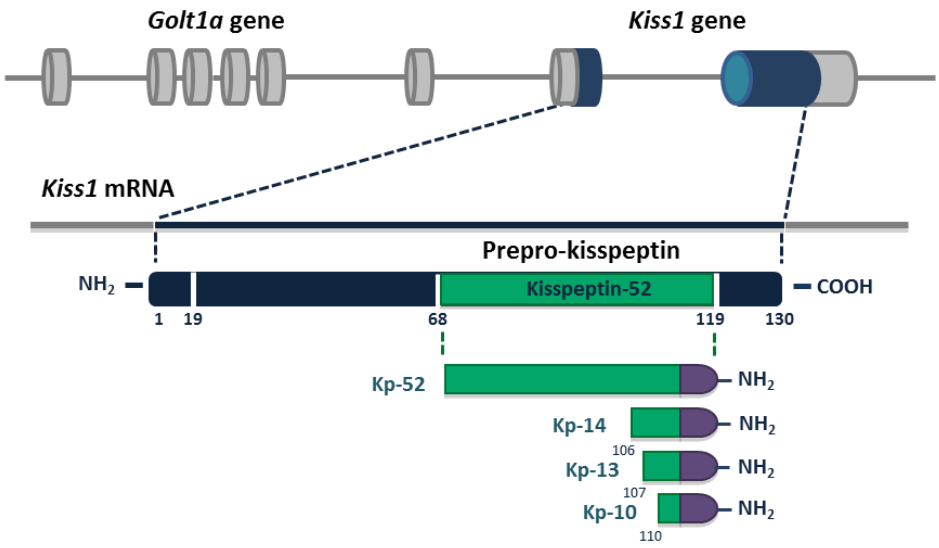

Figure 10. Squematic representation of kisspeptins generation from the *Kiss1* gene in rodents. Different kisspeptins are generated by proteolytic cleavage a common precursor, prepro-kisspeptin, encoded by the *Kiss1* gene. The coding region is composed by two exons, which is close to the *Golt1a* gene. The prepro-kisspeptin contains a central 52 aa region, kisspeptin-52. Lower molecular weight forms of kisspeptins include Kp-14, Kp-13, and Kp-10. Adapted from [192].

Kisspeptins are derived from the differential proteolytic processing of a single precursor. In the human, the kisspeptin precursor (prepro-kisspeptin) comprises 145 aa, with a 19 aa signal sequence, two potential dibasic cleavage sites (at aa 57 and 67), and one site for terminal cleavage and amidation (at aa 121–124) [171, 181], which generate the biologically active kisspeptins (**Figure 10**). Indeed, proteolysis of prepro-kisspeptin gives rise to a 54 aa peptide (kisspeptin-54, Kp-54), which has been considered the major product of the *KiSS1*/*Kiss1* genes. In addition, other peptide fragments of the kisspeptin precursor, such as Kp-14, Kp-13, and Kp-10, have been also identified [171, 181]. All they share the C-terminal region of the Kp-54 molecule, where they contain an Arg-Phe-NH2 motif characteristic of the RF-amide peptide family.

In rats and mice, the largest proteolytic product of the kisspeptin precursor is composed of 52 aa (kisspeptin-52, Kp-52) [193]. Although the degree of sequence homology of the precursor protein between rodents and humans is 54%, the Kp-10 sequence is highly conserved, varying only one aa, Phe, which is substituted by Tyr residue, at position 10, in rodent Kp-10 sequence [194].

The *Gpr54* gene exhibits a high degree of sequence homology between different species. This gene contains 4 introns and 5 exons and codes for a protein of 396 aa, which includes an extracellular amino-terminal domain, responsible for ligand binding, followed by seven transmembrane domains and an intracellular carboxyl-terminal domain, which associate to G-protein [171]. The high degree of conservation of mRNA and protein sequence among species is indicative of the evolutionary importance of this receptor system.

1.4.3. EXPRESSION OF Kiss1/Gpr54 SYSTEM

The Kiss1/Gpr54 system distribution has been studied in a wide variety of species and, although its localization and patterns of expression may vary among them, in general it is closely related to the neuroendocrine regulatory centers of reproductive function.

The first human studies demonstrated *KiSS1* mRNA expression in placenta, pancreas, small intestine, liver, testis and brain, with a scattered distribution throughout the CNS, including the basal ganglia and the hypothalamus [178, 180, 181]. Hypothalamic *Kiss1* expression was also demonstrated by initial studies in other mammalian species, including non-human primates [135, 195, 196]. Although kisspeptin-IR fibers has been located in POA, SON, PVN and DMN [177], studies in rodents have shown that the two main populations of Kiss1 neurons are located in ARC [70, 197, 198] and in the rostral periventricular area of the 3V (RP3V), which includes the AVPV and the periventricular nucleus (PeVN) [195, 196]. In the ARC, a majority of Kiss1 neurons co-express NKB and Dyn and consequently they have been called KNDy neurons [69, 199]. With regard to the population of Kiss1 neurons in the RP3V, it shows a clear sexual dimorphism in AVPV, with females having many more Kiss1 neurons than males [200].

Although hypothalamic Kiss1 neurons localization is coherent with its role as neuroendocrine regulator of reproductive function, the ARC and AVPV populations of Kiss1 neurons display important anatomical differences in terms of projections to GnRH neurons. Thus, direct appositions between Kiss1 and GnRH neurons were initially reported only for Kiss1 neurons from the AVPV [201]. In this regard, studies in

the sheep suggested that ARC Kiss1 neurons do not significantly project to the ventromedial POA, whereas the kisspeptidergic afferents to GnRH neurons are originated from the population located in the POA [202]. However, recent studies in mice have shown that ARC Kiss1 neurons project to the POA, where most cell bodies of the GnRH neurons are located, thus making plausible their direct interaction with GnRH neurons [203]. It should be noted that most of the studies indicate that ARC Kiss1 neurons, and specifically KNDy neurons, project to the ME, where the axon terminals of GnRH neurons are placed, suggesting the existence of interneuronal or even non-synaptic communications between these two neuronal populations [197, 204]. These anatomical differences, in terms of fiber projections, strongly suggest important functional divergences between these two Kiss1 neuronal populations.

The Gpr54 distribution is similar to that described for Kiss1. Thus, *Gpr54* expression is mainly found in placenta, pancreas, liver, skeletal muscle, gonads, pituitary and in different areas of the CNS such as the cerebral cortex, cerebellum, putamen, thalamus, hypothalamus and spinal cord [135, 177, 195, 196, 205]. Also, expression of *Gpr54* mRNA has been detected both in mouse GnRH neurons [206] and in rat pituitary [205].

1.4.4. MECHANISMS OF ACTION OF KISSPEPTINS

Kisspeptins exert their effects by the binding to Gpr54, a $G\alpha_{q/11}$-coupled receptor, which leads to the activation of PLC, with the subsequent stimulation of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) which, in turn, evokes the mobilization of intracellular Ca^{2+} and influx of extracellular Ca^{2+} [171, 180, 207, 208]. In addition, the activation of Gpr54 induces diacylglycerol (DAG) formation following by protein kinase C (PKC) activation which causes activation of protein kinases, such as ERK1/2 and p38 [171, 207].

1.4.5. ROLE OF Kiss1 NEURONS IN THE REGULATION OF GONADOTROPIN SECRETION

By the end of 2004, shortly after the first reports about the reproductive consequences of Gpr54 inactivation in humans and mice, different groups

independently reported the potent stimulatory effect of Kp-10 and Kp-54 on LH secretion in female and male rodents [209-212]. Moreover, subsequent studies demonstrated that kisspeptin administration, both central and systemically, is able to elicit LH and FSH secretion in different stages of development and in different physiological states such as pregnancy and lactation in different species, including rat, monkey and human [207, 209, 213-217].

It is noted that the effects of kisspeptins on gonadotropin secretion differ in terms of duration and sensitivity. In this sense, studies carried out in male rats have demonstrated a rapid (within 5 to 15 min) and robust (up to 10-fold increase over basal levels) responses in terms of LH release after icv injection of Kp-10, while FSH responses were a bit delayed (from 30 min onwards) and of lower relative magnitude (approx. 2-fold increase) after similar dose. Moreover, LH and FSH responsiveness to Kp-10 is different, with EC_{50} of 2 and 400 pmol doses for LH and FSH, respectively [214, 216, 218, 219]. These differences can be explained because the pattern of secretion is more constitutive for FSH than for LH [220] and because the FSH secretion is selectively regulated by gonadal peptides such as inhibins [221].

Another aspect of the pharmacology of kisspeptins is the appearance of desensitization of gonadotropin responses following repeated or continuous exposure. Thus, it has been observed that the stimulatory effects of continuous infusion of Kp-10 in rats, after an early phase of LH hypersecretion, disappeared after 48 h of the beginning of treatment [222, 223].

1.4.5.1. REGULATION OF PULSATILE GnRH SECRETION

Substantial experimental data, obtained mainly in rodents, sheep, and primates, have supported that the actions of kisspeptins are conducted via activation of GnRH neurons at the hypothalamus. Thus, it has been demonstrated that the effects of kisspeptins are completely eliminated by pretreatment with GnRH antagonists in rats, mice, and monkeys [211, 214, 219]. In this line, evidence for a direct effect of kisspeptins on GnRH neurons came from the observation that kisspeptins induced the expression of c-fos, as a marker of early cellular activation, in GnRH neurons in rodents [211] and evoked a potent depolarization responses in GnRH neurons [213, 224]. In

addition, studies using GT1-7 cells, where expression of *Gpr54* gene has been documented, have shown an increase on GnRH secretion after kisspeptin administration [224].

Recently, it has been proposed that signals from ARC Kiss1 neurons participate in the regulation of pulsatile GnRH secretion [192]. This idea was reinforced by studies in female rats, showing that continuous infusion of kisspeptins antagonist in the ARC significantly suppresses the GnRH pulses [225].

As mentioned above (see section 1.4.3.), a population of Kiss1 neurons of the ARC coexpress NKB and Dyn (KNDy neurons), and for this reason, the possibility that these three peptides may jointly participate in the control of the pulsatile GnRH secretion has drawn quite some attention. The most widely accepted hypothesis proposes that NKB and Dyn may operate as positive and negative modifiers, respectively, of the pulsatile release of kisspeptins by KNDy neurons in the ARC [70, 198] (**Figure 11**). According to this model, which is supported by the anatomical demonstration of interconnections among KNDy neurons, these neuropeptides would act autosynaptically on ARC KNDy neurons to modulate kisspeptin release on GnRH nerve terminals at the ME. Note that while expression and functional data support this hypothetical model, some anatomical features of this network are not yet totally clarified, such as, whether the projections of ARC KNDy neurons to GnRH are direct, and if so, whether they target the soma or/and nerve terminals [70, 226].

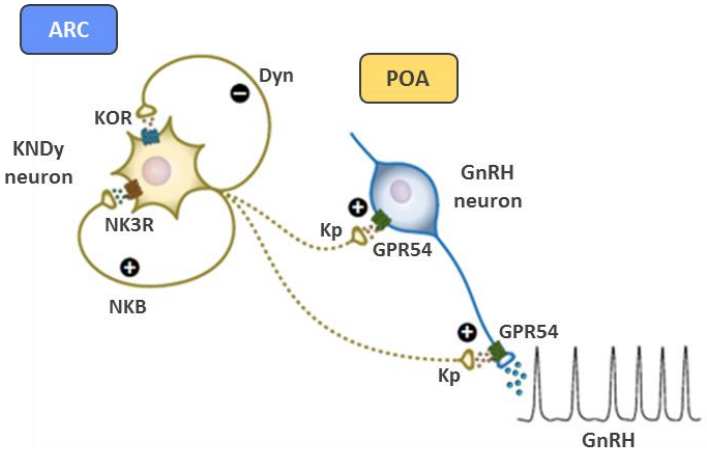

Figure 11. Schematic model to explain the role of ARC KNDy neurons in the control of pulsatile GnRH secretion. KOR (κ-opioid receptor), Dyn receptor; NK3R, NKB receptor; GPR54, kisspeptin receptor. Taken from [175].

The interest to characterize the role that the Kiss1/Gpr54 system play in the regulation of reproduction led to the A.E. Herbison laboratory in association with the G. Schütz laboratory to generated two Tg mice models: the GnRH-Cre;Gpr54$^{f/f}$ model, which present a specific ablation of *Gpr54* on GnRH neurons; and the Gpr54$^{-/-}$;Tg model, generated via a selective reintroduction of *Gpr54* expression on GnRH neurons of *Gpr54* KO mice. The phenotype of the latter has been characterized in our laboratory [153]. These Tg mice models provide a useful tool to further understand the function of Gpr54 and kisspeptins in mammalian reproduction *in vivo.*

1.4.5.2. THE NEGATIVE FEEDBACK CONTROL OF GONADOTROPIN SECRETION

The first evidence for the involvement of hypothalamic Kiss1 neurons in the negative feedback of gonadotropins by gonadal steroids was obtained from rodent studies in models of gonadectomy (GNX), with or without sex steroid replacement. The elimination of gonadal factors in adult male and female rats evoked a robust increase in *Kiss1* mRNA levels, which was also accompanied by an increase in circulating gonadotropin levels. By contrast, the replacement with sex steroids, T in orchidectomized (ORX) male rats and E_2 in OVX female rats, blockade the increase of both *Kiss1* expression levels and gonadotropin levels following the GNX [135, 196, 212, 217]. In addition, ISH studies in rats and mice have been demonstrated that hypothalamic *Kiss1* mRNA expression changes indicated above take place specifically in the ARC. These observations have been confirmed subsequently in other mammalian species, including humans [227-229].

In relation to the receptors involved, almost all Kiss1 neurons express ERα, as well as PR y AR [199, 230], while only a part express ERβ [135]. Functional genomics and pharmacological studies have demonstrated that ERα is the main mediator of the negative feedback that estrogens exert in ARC *Kiss1* expression [135, 136]. In this way, it has been recently documented that the selective lack of ERα in ARC kisspeptidergic neurons was followed by an increase of *Kiss1* mRNA expression in prepubertal mice [231]. In addition, studies about the effect of T and DHT, a non-aromatizable androgen, on GnRH release, suggest that the control of ARC *Kiss1* expression is mediated via AR and ERα (after the aromatization of T) [135].

1.4.5.3. ROLE OF Kiss1/Gpr54 SYSTEM IN THE CONTROL OF PREOVULATORY GnRH/LH SURGE

As described above, besides the ARC population of Kiss1 neurons, a second population of Kiss1 neurons in the AVPV, which also express ERα, has been reported in rodents [196]. It has been demonstrated that, contrary to the ARC, neurons at the AVPV displayed decreased *Kiss1* mRNA levels after GNX and increased expression following sex steroid replacement. Moreover, expression analyses in cyclic and E_2-primed OVX female rats demonstrated that there is an increase in *Kiss1* mRNA expression at the AVPV during the preovulatory LH surge [134, 232]. Additionally, pharmacological studies have documented that selective blockade of ERα blunted the endogenous preovulatory LH surge and induced a suppression of LH responses to exogenous Kp-10, when administered during the proestrous [233]. All these observations have consolidated the notion that Kiss1 neurons in the AVPV mediate the stimulatory effects of estrogen (positive feedback) on gonadotropin secretion in rodents (**Figure 12**).

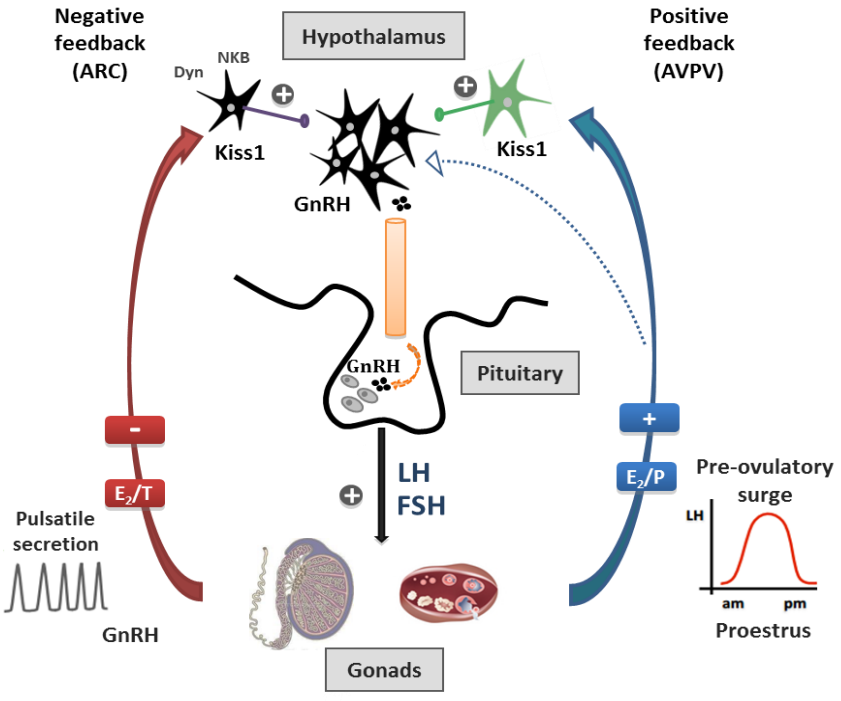

Figure 12. Schematic representation of the regulatory effects of gonadal steroids (E_2, P and T) on Kiss1 neurons in rodents. Estrogen (E_2) input exerts a predominant inhibitory action on KNDy neurons in the ARC, which contributes to negative feedback control of GnRH (left). In contrast, the stimulatory effects of E_2 to induce the preovulatory surge of gonadotropins (positive feedback) are mediated by Kiss1 neurons in the AVPV. Adapted from [175].

1.4.6. Kiss1 NEURONS IN THE CONTROL OF PUBERTY ONSET

As highlighted previously, puberty is the crucial developmental period when reproductive capacity is achieved. A key event in the onset of puberty is the progressive increase of the neurosecretory activity of GnRH neurons [147, 172]. It is thought that pubertal changes in the pattern of GnRH secretion are the result of the combined increase in excitatory inputs and decrease in inhibitory inputs projecting to GnRH neurons [147, 234]. In this context, recent data have documented an important role of kisspeptins in the control of puberty in mammals.

The first evidence for the essential role of kisspeptin signaling in the control of puberty onset came from the studies describing the impaired pubertal progression and sexual immaturity of humans and mice with genetic inactivation of *GPR54*/*Gpr54* [183, 188]. Later, in order to characterize the physiological significance of the Kiss1 system in puberty onset, hypothalamic expression analyses of the mRNA of both *Kiss1* and *Gpr54* were implemented at different stages of postnatal maturation. Studies in male and female rats showed a clear increase in *Kiss1* and *Gpr54* mRNA levels in the hypothalamus during pubertal progression [212]. This phenomenon was later confirmed in non-human primates, where it was shown that *Kiss1* and *Gpr54* mRNA expression in the hypothalamus of female monkeys increases approximately threefold during the transition from the juvenile to the pubertal stage [235]. More specifically, IHC studies in rats and mice have documented an increase in the number of Kp-IR neurons in the AVPV during pubertal maturation, although this increase was higher in females than in males [204, 236]. The functional relevance of such rise of kisspeptin tone in the AVPV for the timing of puberty is supported by the simultaneous increase of Kp-IR projections originating from AVPV to GnRH neurons [236]. Nonetheless, studies conducted in the ARC have demonstrated a rise in *Kiss1* mRNA levels during the pubertal transition [204, 226], although with a magnitude more modest compared with the reported in the AVPV, especially in female [204].

In addition to changes in the ligand, studies to evaluate potential modifications in Gpr54 signaling showed an increase in *Gpr54* mRNA expression during puberty at the hypothalamus of rats and monkeys [212, 235]. Moreover, ISH analyses and electrophysiological recordings suggested that this phenomenon was not primarily

caused by an increase in the expression levels of *Gpr54* mRNA per cell, but a gradual increase of GnRH neurons expressing *Gpr54*, as well as, an enhancement in the efficiency of this receptor to couple with its effector systems during the pubertal transition [213].

The above data suggest a rise of the kisspeptinergic tone during puberty, supported by the observation in monkeys of an increase in the pulsatile release of Kp-54 in the ME during this stage, which is coincident with the elevation of GnRH pulse frequency seen at puberty [237]. In support of the essential role of kisspeptins in the acquisition of reproductive function, pharmacological studies have demonstrated that central repeated administration of Kp-10 in prepubertal female rats induced precocious VO and earlier activation of the HPG axis, with the consequent gonadotropins response [151]. In fact, it has been demonstrated that Kp-10 administration in immature rats was followed by an increase on LH circulating levels [207].

The functional relevance of kisspeptin signaling in the pubertal maturation has been recently confirmed by the observation of the delayed puberty in pubertal female rats after the central infusion of an effective kisspeptins antagonist, peptide 234 [238]. However, recent data suggested that pubertal maturation in female mice can be attained even after congenital ablation of *Kiss1*- or *Gpr54*-expressing neurons. These observations could be indicative of the activation of compensatory mechanisms and/or the involvement of other neuronal populations with an important role in the activation of HPG axis during puberty [239].

1.5. METABOLIC CONTROL OF PUBERTY

The notion that reproduction requires of the amount of energy reserves dates back thousands of years. However, despite this intuitive knowledge, it was not until the 1960s and 70s when the works of Kennedy and Frisch formulated, on a scientific basis, the interactions between nutrition and reproduction. They established “the critical (fat) mass hypothesis” that proposes the need to have a critical body weight (BW) as a physiological requirement for menarche and maintaining of reproductive function in women [240-243]. Moreover, not only situations of energy insufficiency

induce serious alterations of reproductive function, conditions of severe energy excess, such as morbid obesity, are also coupled to reproductive disorders [244, 245].

Many signals, of both central and peripheral origin, are responsible to relay information about energy and metabolic state to the neural centers controlling reproductive function. In general, it is considered that signals of energy sufficiency inhibit food intake (anorexigenic signals) and stimulate reproductive function, while signals of insufficiency energy stimulate food intake (orexigenic signals) and inhibit the function of the HPG axis.

1.5.1. PERIPHERAL SIGNALS INVOLVED IN THE INTEGRAL CONTROL OF ENERGY BALANCE AND REPRODUCTION

Several peripheral signals transmit the metabolic information to the neural centers involved in the control of reproduction. These signals are mainly originated from the adipose tissue, pancreas and gastrointestinal tract and are described in detail below.

- **Leptin**

Leptin is considered an essential signal in the integrative control of BW, energy homeostasis and reproductive function. Leptin, also known as OB protein, is a 16 kDa peptide hormone encoded by the *obese* (*ob*) gene that was identified and cloned in 1994 [246]. It is mainly produced by adipocytes and is present in serum, in both rodents and humans, at levels directly related with the amount of adipose tissue [247]. Leptin effects are mediated by its interaction with leptin receptors (Lep-R or Ob-R) which are expressed in several hypothalamic nuclei involved in the control of BW, energy homeostasis and reproduction.

Leptin has a key role in the control of energy balance as anorexigenic signal, suppressing appetite and increasing energy expenditure. Thus, the leptin-deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) mice exhibit obesity due to hyperphagia, insulin resistance and infertility [248]. In addition, it has been demonstrated the peripheral and central administration of exogenous leptin to *ob/ob* mice reduces obesity and restores reproductive function [245]. Moreover, leptin exert a permissive

effect on puberty onset, as its lack or deficiency produces pubertal failure and HH [249-251].

Although leptin acts centrally to promote puberty onset and to maintain fertility, the apparent lack of functional Ob-R in GnRH neurons suggests the existence of afferent pathways that would convey its action onto GnRH neurons [252]. In this sense, leptin could act via different neuronal populations involved in the metabolic control of reproduction, such as NPY neurons [253-256], POMC neurons [257], GALP neurons [258] and NO neurons [93, 97], the latter also intermediate of kisspeptin neurons [95]. These intermediate pathways whereby leptin indirectly modulates GnRH function will be detailed in the next sections.

- **Insulin**

Insulin is a peptidergic hormone produced by β-cells of the pancreas. Insulin circulating levels increase after food intake, following the rise of the immediate principles in blood, mainly glucose. Insulin exerts an anorexigenic effect and is involved in the control of GnRH/LH secretion, thus participating in the metabolic control of the HPG axis. This argument is supported by the identification of insulin receptors (IR) in the hypothalamus of several species, specifically in neurons close to GnRH neurons in ME and ARC [259]. In addition, it has been demonstrated the presence of IR in cultured GnRH neurons [260]. Insulin administration to diabetic sheep model restored the pulsatile LH secretion, suggesting a central effect on GnRH secretion [261]. In line with above data, the neuronal-specific inactivation of IR in mice resulted in metabolic alterations and infertility associated with a decreased on GnRH levels [262], suggesting the critical role of insulin signaling in the brain for the metabolic control of the HPG axis. Besides the direct effects of insulin on GnRH secretion, it has been proposed also an indirect effect of insulin on GnRH/LH secretion mediated by NPY inhibition [256].

- **Ghrelin**

Ghrelin is a 28 aa peptide hormone secreted, mainly, by specific endocrine cells of the gastric mucosa, which was identified in 1999 as the endogenous ligand of the GH secretagogue receptor (GHS-R) [263]. Besides its stimulatory role on GH release, one of the most remarkable characteristics of ghrelin is its capacity to increase food

intake and to adjust reproductive function to the availability of energy reserves [264, 265]. Due to its orexigenic effects, this hormone has been considered as a functional antagonist of leptin in the control of food intake [266]. Circulating ghrelin levels are inversely correlated with the body mass index; thus, it has been considered as a signal of energy insufficiency. In good agreement, it has been described that ghrelin operates as a negative modifier of gonadotropin secretion and, in rats, chronic central administration that this hormone produces a delay of puberty onset and a decrease on LH circulating levels [267].

- **Peptide YY**

Peptide YY (PYY) is a hormone consisting of 36 aa with structural homology to NPY. The two major forms, PYY_{1-36} and PYY_{3-36}, are synthesized by L cells in the mucosa of the gastrointestinal tract. PYY_{3-36} binds to Y_2 and Y_5 receptors in the hypothalamus, mainly in the ARC, pituitary and intestine to control food intake, as signal of energy sufficiency. Additionally, PYY_{3-36} participates in the control of the HPG axis, stimulating LH and FSH releases by increase GnRH secretion and by acting at pituitary level [268].

1.5.2. CENTRAL SIGNALS FOR THE INTEGRATIVE CONTROL OF ENERGY BALANCE AND REPRODUCTION

The central signals that participate in the control of energy balance modulate the activity of GnRH neurons directly or via hypothalamic interneurons which project to GnRH neurons. The main neurons involved in the metabolic control of reproduction are located in the ARC and express kisspeptins, NPY, orexins, NO, GALP and POMC and its peptides products (α-MSH and β-END).

- **Kiss1/Gpr54 system**

The Kiss1/Gpr54 system has recently been proposed as a key integrative element that links energy homeostasis and reproductive function [269]. Several studies documented that pubertal rats and adult mice of both sexes subjected to fasting showed a decrease in the *Kiss1* hypothalamic expression levels, which was associated with a decrease in circulating LH levels [270, 271]. Similarly, it has been demonstrated that central administration of Kp-10 to rats subjected to fasting revert

the reduction of gonadotropins induced by the state of negative energy balance. In addition, in female rats submitted to chronic subnutrition during the peripubertal period, repeated administration of Kp-10 rescued the negative effects on the puberty onset and LH secretion caused by chronic subnutrition [271].

Few years ago, it was demonstrated Kiss1 neurons in the ARC (and in the POA in ewes) express Ob-R. Since Ob-R are not present in GnRH neurons, it was proposed that leptin could act directly on Kiss1 neurons to mediate its effects on the puberty onset and maintaining of reproductive function [272, 273]. In good agreement, lower *Kiss1* levels have been found in leptin-deficient mice that were recovered after administration of the hormone [272]. Moreover, rats subjected to food restriction during lactation displayed reduced hypothalamic expression of *Kiss1* mRNA and Kp-IR in the ARC associated to hypoleptinemia [274, 275]. In addition, female rats subjected to postnatal overnutrition showed an advance in puberty onset associated with an increases in circulating leptin levels, *Kiss1* mRNA levels and Kp-IR fibers in the AVPV [274]. However, against the putative role of Kiss1 neurons as direct mediators of leptin effects on HPG axis, it has been recently documented that the specific ablation of Ob-R on Kiss1 neurons does not prevent fertility, suggesting that leptin may act indirectly (and/or independly) on Kiss1 neurons to control the onset of puberty and reproductive function [276].

- **NPY/AgRP neurons**

In rats, NPY neurons are located in different brain areas, including a large population of neurons in the ARC that coexpress AgRP (Agouti-Related Protein) and that has been postulated as key element in the metabolic control of the HPG axis.

NPY acts as an orexigenic neuropeptide and it has been demonstrated that the activation of ARC NPY/AgRP neurons stimulates feeding in mice [277]. With regard to the potential role of NPY in reproduction, since ARC NPY neurons project to GnRH perikarya and nerve terminals, it has been suggested that they participate in conveying information about energy insufficiency to the reproductive axis. In fact, it has been shown that food restriction increases NPY expression and reduces LH release [278]. Additionally, the infertile phenotype displayed by ob/ob mice has been associated with

high levels of *NPY* mRNA and indeed, deficiency of either NPY or its Y1 or Y4 receptors rescues fertility in these animals [279]. Moreover, given that GnRH neurons do not express Ob-R and IR, several studies suggested that NPY neurons can act as mediator for leptin and insulin actions on GnRH secretion [256].

- **POMC neurons**

The neurons that coexpress POMC and CART (Cocaine and Amphetamine-regulated transcript) in the ARC are also involved in the integrative control of metabolism and reproduction. Peptide products resulting from processing of the POMC precursor, such as α-MSH and β-END, exert different effects in food intake and gonadotropin release. Thus, while α-MSH, a signal of energy sufficiency, reduces food intake and stimulate LH release in adulthood [280], β-END, a signal of energy insufficiency, stimulates food intake and inhibits LH secretion [281]. The role of POMC and melanocortins as metabolic regulators of reproduction will be explained in more detail in the section 1.6.

- **RFRP/NPFF1R system**

Recent data propose that the RFRP/NPFF1R system participates in the integrative control of energy homeostasis and reproduction. In addition to its role as inhibitor of the reproductive axis [125, 126] (see section 1.2.1.1.), pharmacological studies in birds, mice, ewes and non-human primates demonstrated that this system likely participates in the control of food intake, exerting an orexigenic effect [125, 282-285]. Moreover, it has been documented that RFRP neurons project into NPY/AgRP and POMC/CART neurons in the ARC [286], suggesting that RFRP/NPFF1R system could be involved in the metabolic control of reproduction via these neurons. In this sense, recent data showed an inhibitory effect of GnIH on POMC neurons [153, 287].

- **Orexins**

Orexins (A and B) belong to a family of neuropeptides mainly expressed in the LHA. Several studies have demonstrated that central administration of orexins increases food intake, in particular, orexin A showed the most potent orexigenic effects [288]. Surprisingly, although orexins are signals for energy insufficiency, several

lines of evidence suggest a putative role of orexins as stimulators of the HPG axis. Accordingly, it has been shown that hypothalamic levels of orexins change during the estrous cycle with the maximal levels at proestrous phase [289]. In addition, data obtained in different laboratories indicated that orexins stimulate LH secretion in OVX rats supplemented with E_2 and P [290-292] and also, that central administration of orexins recovered the LH pre-ovulatory surge in rats subjected to fasting [293].

- **NO neurons**

As was mentioned above, in addition to its role in the neuroendocrine control of reproduction, neuronal NO signaling acts as intermediate pathway of leptin actions to GnRH neurons. Thus, it was demonstrated that the deletion of the gene encoding NOS or its pharmacological inhibition in the POA, prior to peripheral leptin administration, suppress the leptin-induced release of LH in mice and prevent the restoration of fertility in leptin-deficient female mice by leptin treatment. In addition, it was shown that the inhibition of nNOS activity abolished the ability of exogenous leptin to rescue fertility in fasting conditions [97]. These data show that part of the actions of leptin in the regulation of HPG axis are mediated by the activation of nNOS in the POA.

Despite progress made in the last few years in the identification of new central and peripheral signals involved in the integrative control of energy balance and reproduction, our knowledge in the area remains incomplete.

1.6. THE MELANOCORTIN SYSTEM: ROLE IN THE HPG AXIS REGULATION

As discussed above, many factors are involved in the metabolic control of the reproductive axis. In this sense, a bidirectional interaction between GnRH neurons and key metabolic neuropeptides, such as α-MSH, has been recently proposed. In this section we will be described in detail the role of the central melanocortin system in the regulation of HPG axis and its interaction with leptin and GnRH neurons.

1.6.1. COMPONENTS OF THE MELANOCORTIN SYSTEM

The melanocortin system is a ligand/receptor system composed by four POMC-derived peptides, five melanocortin receptors (MC1-R to MC5-R) and two endogenous antagonist, agouti and AgRP.

POMC-derived peptides include ACTH and α-, β- and γ-melanocyte stimulating hormone (α-, β- and γ-MSH), collectively known as melanocortins. All melanocortins are unified by the fact that they contain the aa sequence His-Phe-Arg-Trp, which is key for their biological activity [294]. Particularly α-MSH, of particular interest for this Thesis, is phylogenetically a remarkably well-conserved molecule and one of the first peptide hormones to be isolated [295, 296]. Besides the above mentioned peptides, cleavage of the POMC precursor also originates the corticotrophin-like intermediate peptide (CLIP), β- and γ-lipotropin, β-END and Met-enkephalin peptides (**Figure 13**).

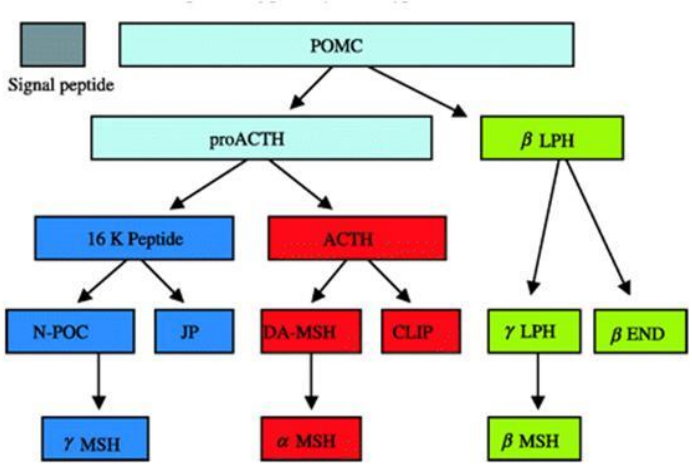

Figure 13. Processing pathway of pro-opiomelanocortin (POMC) in the hypothalamus and pituitary.

POMC-derived peptides are synthesized from a single gene, *POMC*, mainly expressed in the pituitary, CNS and peripheral tissues. In the pituitary, the *POMC* gene is expressed by two cell types: corticotropic and melanotropic cells, while in the CNS, the *POMC* gene is mainly expressed in the hypothalamus, and in peripheral tissues, it is abundantly expressed in the skin.

The melanocortins were primarily recognized for their role in the regulation of adrenal steroid production and skin pigmentation. However, it is now well-known that

melanocortins are also involved in other functions as the regulation of food intake, reproduction or inflammation.

The action of the melanocortin peptides is mediated by a family of five subtypes of the melanocortin receptors (MC-R) namely MC1-R to MC5-R. These are G protein-coupled receptors that, following ligand binding, induce an increase in cAMP in target cells. Additionally, MC-R signaling has also been associated with increases in intracellular Ca^{2+} concentration to activate PKC pathways. The MC1-R is mainly expressed by melanocytes, where it has a key role in determining skin and hair pigmentation. In addition, MC1-R is expressed in other cell types as keratinocytes or endothelial cells in the skin, and leukocytes, where it mediates the antiinflammatory and immunomodulatory actions of melanocortins. The MC2-R is expressed in the adrenal cortex where it mediates the effects of ACTH on steroid synthesis. This receptor is pharmacologically distinct from the other MC-R subtypes as it does not bind the MSH peptides but has high affinity for ACTH [297]. The MC5-R is primarily expressed in peripheral tissues, including the adrenal gland, adipocytes and leukocytes and it participates in exocrine function, particularly, in sebaceous gland secretion [298, 299] (**Table 1**).

The MC3-R and MC4-R are involved in the central regulation of energy balance and reproduction and they are the most abundant MC-R in the brain. In detail, MC3-R is mostly confined to the hypothalamus, particularly high expression of *MC3-R* mRNA has been found in the ARC, where the main population of POMC neurons is also located. In addition, these receptors are also expressed in other areas of the CNS, including the cortex, hippocampus and thalamus, and in several peripheral human tissues, such as gastrointestinal tract and placenta [300]. Regarding MC4-R, it is only expressed in the CNS, where it is widely distributed, including the cortex, thalamus, brain stem and spinal cord and in the VMN and PVN in the medial hypothalamus [301]. Given the special interest in this Thesis to identify and analyze signals involved in the metabolic control of puberty, the role of MC3 and MC4 receptors in this function is described in detail in the section 1.6.2.

Table 1. The main sites of expression, preferred endogenous agonist and antagonist, and the physiological roles for the five MC-R subtypes.

Subtype	Expression sites	Endogenous agonists	Endogenous antagonists	Physiological role
MC1-R	Skin	α-MSH (ACTH)	Agouti	Pigmentation Inflammation
MC2-R	Adrenal gland	ACTH		Steroid production and secretion
MC3-R	Brain	α-MSH (γ-MSH)	AgRP	Energy balance
MC4-R	Brain	α-MSH (β-MSH)	AgRP (Agouti)	Food intake and energy balance
MC5-R	Brain and a number of peripheral sites	α-MSH		Exocrine gland secretion

One of the most interesting aspects of the melanocortin system is that it comprises also agouti and AgRP, two endogenous antagonists of the MC-Rs [302]. Both peptides show structural homology and they have selectivity MC-R subtype antagonism (see **table 1**). Pharmacologically, agouti shows a high-affinity to antagonize the effect of melanocortin peptides at MC1-R and MC4-R while AgRP is equipotent antagonist of MC3-R and MC4-R. Mice with mutations in the *agouti* gene show yellow fur, obesity, insulin resistance and increased somatic growth. AgRP is expressed primarily in the ARC of the hypothalamus and in the adrenal cortex. In the hypothalamus, AgRP acts as a potent orexigenic factor due to its ability to antagonize melanocortins at MC3-R and MC4-R.

1.6.2. PHYSIOLOGICAL ROLES OF THE CENTRAL MELANOCORTIN SYSTEM

Given its importance in this Thesis, the physiological role of the central melanocortin system in energy homeostasis and regulation of the HPG axis is detailed below.

1.6.2.1. POMC NEURONS IN THE CONTROL OF METABOLIC HOMEOSTASIS

POMC neurons act as key elements in the control of food intake and energy expenditure to keep BW.

In the brain, two different populations of POMC neurons have been identified. The main POMC neuronal population is located in the ARC. These neurons coexpress

neurotransmitters, including GABA and Glu (274, 275). In addition, these neurons express a wide variety of receptors such as Ob-R, IR and NPY Y1-R, that confer them the ability to sense different peripheral and central signals involved in the control of energy homeostasis. Additionally, a second small neuronal population has been located in the nucleus of solitary tract [274], whose which function is not yet well-known.

A recent study has demonstrated that the selective ablation of ARC POMC neurons mimics the obese phenotype of the POMC-null mice, as consequence of an increase in food intake and a reduction on energy expenditure. In contrast, the selective ablation of NTS POMC neurons did not reproduce the obese phenotype, suggesting that POMC neurons in NTS may have a minor contribution in the control of energy homeostasis exerted by the POMC system [303].

1.6.2.2. ROLE OF POMC NEURONS IN THE CONTROL OF REPRODUCTIVE AXIS

In the last few years, several research groups tried to document whether GnRH neurons are direct targets for melanocortin actions. Recent data have demonstrated that GnRH neurons express *MC3/MC4-R*, supporting the direct effect of melanocortins in the control of GnRH secretion. In good agreement, IHC analyses showed that POMC neurons establish synaptic contacts with GnRH perikarya and nerve terminals, suggesting a direct effect of POMC-derived peptides on GnRH excitability [304] and, accordingly, electrophysiological recordings of GnRH neurons demonstrated that α-MSH increases the cell firing in a majority of GnRH neurons through postsynaptic activation of both MC3-R and MC4-R [305].

Pharmacological studies showed that the administration of α-MSH induces an increase of LH secretion in different mammalian species [306] and also stimulates sexual behavior in female rats. Moreover, it was demonstrated that melanotan II (MT II), a MC3-R and MC4-R agonist, increased GnRH release in goats [307]. Furthermore, subsequent data showed that, in addition to increased fat mass and reduced lean body mass, *MC3-R* KO female mice show a certain degree of subfertility [308]. In good agreement, it was demonstrated that mice with functional blockade of both MC3-R and MC4-R, by overexpression of *AgRP*, are infertile [309]. Finally, although data

demonstrated that MC4-R deficient male mice are fertile, females are poor breeders and exhibited reduced ovulation rates [310].

1.6.2.3. ROLE OF POMC SYSTEM IN THE INTEGRATIVE CONTROL OF ENERGY BALANCE AND REPRODUCTION

Since, as mentioned above, GnRH neurons do not express either Ob-R or IR but they express MC3-R and MC4-R, it has been suggested that POMC neurons could transmit leptin and insulin metabolic information to GnRH neurons to modulate their secretory activity.

Although, initial studies documented that independent ablation of Ob-R or IR on POMC neurons did not disrupt fertility [304], more recently, analyses carried out in mice lacking both Ob-R and IR in POMC neurons, demonstrated that these animals display reproductive deficiencies that resulted in reduced fertility [311]. Moreover, ob/ob mice showed reduced *POMC* mRNA expression in the ARC and this expression was rescued after leptin administration [312]. Additionally, it was shown that Ob-R deletion from POMC neurons induces hyperleptinemia and disrupts BW homeostasis [313]. In this regard, it has been shown that leptin infusion in the POA and ME produces an increase in α-MSH secretion followed by GnRH/LH release [314]. Accordingly, the restoration of melanocortin indirect signaling in db/db mice recovers the normal timing of puberty onset and fertility, suggesting that in such extreme conditions of leptin signaling deficiency melanocortin actions can overcome the functional deficit of GnRH neurons [315].

Despite all these evidence, the role of POMC neurons, and particularly α-MSH, in the regulation of puberty, as well as the existence of indirect intermediaries between POMC and GnRH neurons, remain unclear. For this reason, one of the main objectives of this Thesis was further to explore the role of α-MSH signaling on puberty onset as well as its role as mediator of the leptin effects in this maturational event.

1.7. NEW SIGNALS INVOLVED IN THE CONTROL OF PUBERTY: MICRORNAs

As mentioned previously, puberty onset depends on complex neural networks whose concerted action determines the full activation of GnRH secretion from

hypothalamus. However, at the moment, the molecular events that precisely control GnRH neuronal activation at puberty remains poorly understood. Nonetheless, accumulating evidence suggests that the spatial and temporal control of gene expression can play a crucial role on the onset of puberty [316]. In this sense, miRNAs, which can modulate gene expression at multiple hierarchical levels, have been suggested as a potential regulatory system able to integrate the different networks involved in the control of puberty [162, 163].

1.7.1. miRNAs

miRNAs are small non-coding RNA molecules of 18-24 nt in length first discovered in 1993 by Lee et al. in *C. elegans* [317]. In the larval stage of this nematode, these researchers found a 18-24 molecule partially complementary to a sequence in the 3´ untranslated region (3´UTR) of the *lin-14* mRNA. The binding of this sequence at the 3´UTR region of *lin-14* mRNA inhibited the production of LIN-14 protein, as consequence, the correct transition from larvae into adult worm in the nematode was prevented [318, 319].

After the identification in *C. elegans* of a second miRNA, namely lethal-7 (let-7) [318, 320], many groups of researchers began to seek, and actually found, other small non-coding RNAs. Presently, the primary miRNA sequence repository (http://www.mirbase.org/) contains more than 24000 miRNA loci present in unicellular eukaryotic organisms, some viruses, plants and numerous animal species [321]. Specifically in the human genome, currently more than 1000 miRNAs have been identified [321] and it has been recognized that, at least, one-third of all human genes is regulated by miRNAs.

From initial earliest work, miRNAs were grouped into 48 families. This classification is based on the similarities in 2-8 nt in the 5' end region. This region, known as the "seed sequence", is used to seek complementarity inside mRNA and predict possible miRNAs targets [322, 323].

At first, it was thought that miRNAs only acting as negative regulators of gene expression and exerting their effects by binding to the 3´UTR region of their target mRNAs. However, recent data showed that the post-transcriptional control exerted by

miRNAs is much more complex and there is now consensus that miRNAs can not only decrease, but also increase gene expression binding to their target mRNAs both to the promoter and coding regions, as well as to 3´UTR region [324-326].

It should be noted that miRNAs themselves can be regulated. This regulation can take place at transcriptional and processing level by autoregulatory feedback loops and, at least in mammals, by epigenetic mechanisms, including DNA methylation and histone modifications [324, 325]. As further some of complexity, computational and experimental approaches have revealed that a single miRNA can regulate entire networks of genes and also, each mRNA may be regulated by more than one miRNA [327].

1.7.1.1. SYNTHESIS OF miRNAs

The synthesis of miRNAs is a complex process that involves two distinct steps, the first one takes place in the nucleus and the second one in the cytoplasm, where a mature single-stranded miRNA of about 18-24 nt is finally generated [328].

The miRNA synthesis begins with the transcription of genes encoding miRNAs and requires the participation of RNA polymerase II (Pol II) and transcriptional factors such as c-Myc or p53. It is interesting to note that mammalian miRNA genes can be independent genes or portions of exons or introns of other genes. The initial transcriptional process generates molecules of about 100-1000 nt length called primary miRNAs (pri-miRNA).

Once the pri-miRNA is generated, a microprocessor complex, which consists of the RNase III endonuclease Drosha and its partner DGCR8 (DiGeorge syndrome chromosomal region 8), cleaves the pri-miRNA and reduces it into a molecule of about 70 nt, namely the precursor miRNA (pre-miRNA). Then, this pre-miRNA is transported from the nucleus to the cytoplasm by the Exportin-5/Ran-GTP complex (**Figure 14**).

In the cytoplasm, the pre-miRNA is further processed by the RNase III Dicer, its cofactor TRBP (Tar RNA binding protein) and the argonaute protein (Ago), resulting in a miRNA/miRNA* duplex of about 22 nt.

Usually, only one strand of this duplex, the mature miRNA, is maintained and is integrated with the miRNA-induced silencing complex (miRISC). The passenger miRNA*

is subsequently degraded [329]. However, exceptionally, the miRNA* strand is also maintained and it can even be more functional relevant than the guiding strand. This fact has allowed suggesting a biological role for miRNA* [330].

The mature miRNA after integration in the miRISC complex in cytoplasm, regulates its mRNA target by one of two mechanisms. When it binds its target with perfect complementarity, it leads to the target mRNA cleavage. Nevertheless, if it binds its target with imperfect complementarity, promotes mRNA degradation and translational suppression of the latter (**Figure 14**).

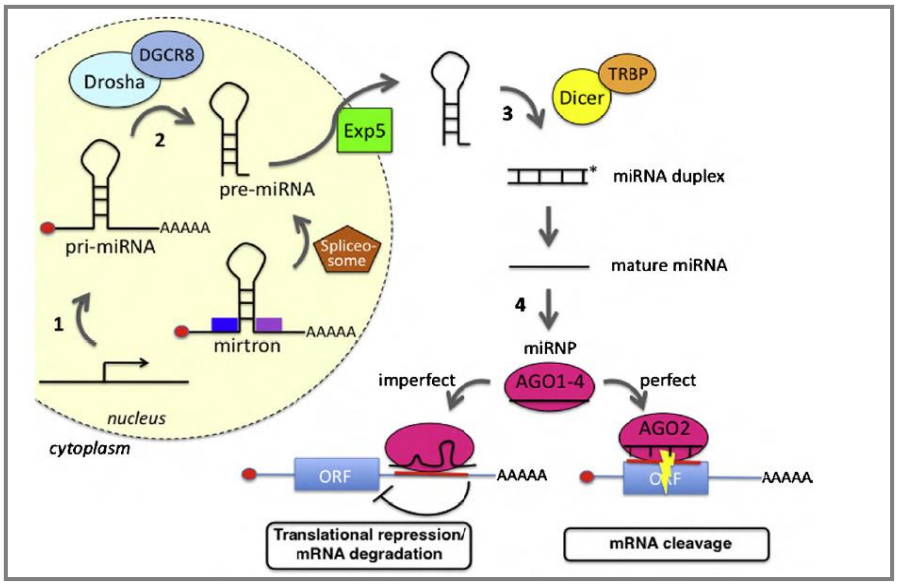

Figure 14. Biogenesis de microARNs. Taken from [324]

1.7.1.2. REGULATION OF miRNAs SYNTHESIS

The miRNAs synthesis is a regulated process at both transcriptional and post-transcriptional level. Given the special interest of this Thesis, some of these regulators will be detailed in the following sections. In addition, to ease understanding of this phenomenon, some of the main regulators of this process are summarized in **table 2**.

Although at transcriptional level, Pol II provides the main point of control of biosynthesis of miRNAs, transcription can be also regulated by multiple other mechanisms.

Among the transcriptional factors involved in the regulation of miRNAs synthesis, it has been demonstrated that c-Myc, interacting miRNA promoters,

modifies the synthesis of several miRNAs including mir-15a, -29, -34 and members of let-7 family [331].

Regarding epigenetic control of miRNA biosynthesis, different studies have demonstrated that about 5-10% of miRNAs in mammals are regulated by epigenetic mechanisms such as DNA methylation and histone modifications. Accordingly, recent data showed that the major enzymes involved in the DNA methylation, DNMT1, DNMT3a and DNMT3b, can modify the expression of some miRNAs such as mir-148a, mir-34b, mir-9 and let-7a [332, 333]. In addition, expression of mir-127, among many other miRNAs, was altered after treatment with 5-Aza-Cdr, a potent DNA methylation inhibitor, and PBA, a histone deacetylase inhibitor [334]. As index of this complex regulation, it has been also published that, in mammals, the mir-34 family is regulated by both transcription factors, as c-Myc and p53 and epigenetic mechanisms.

Besides transcriptional regulation of miRNAs, their post-transcriptional regulation is also important. In this regard, the miRNA processing in the cytoplasm is regulated, either positively or negatively, by a number of factors interacting with Drosha [335], with miRNA precursors [336] and/or with Dicer [337] (**Figure 15**).

Among positive regulators that increase the mature miRNA production, several studies have been identified the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), the KH-type splicing regulatory protein (KSRP) and the p68/p72 helicases (see **table 2**).

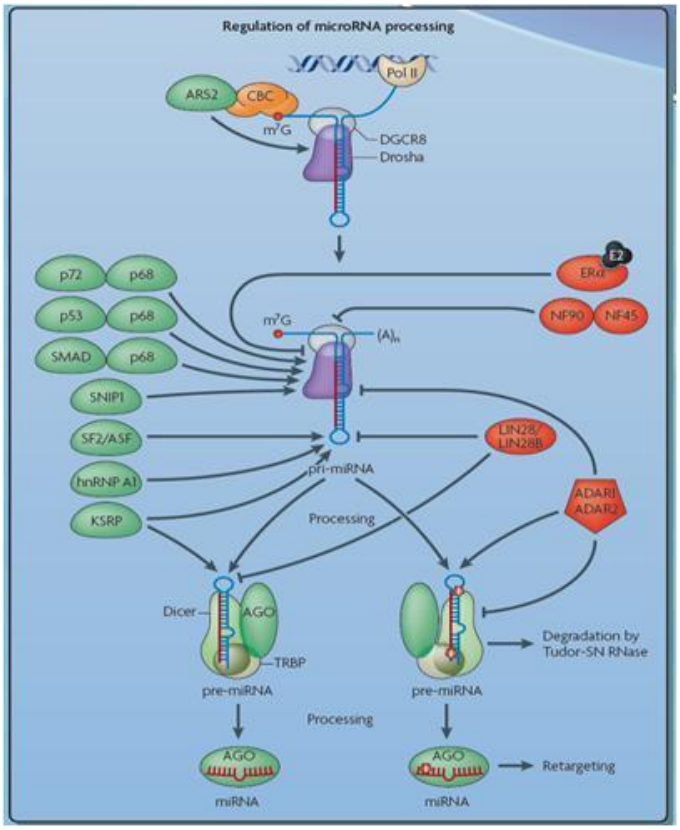

Figure 15. Activators and repressors of microRNA processing. Taken from [338].

With regard to negative regulators that decrease the production of mature miRNAs adenosine deaminase acting on RNA enzymes 1 and 2 (ADAR1 and 2) have been identified, since they can affect the accumulation of mature miRNAs. ADARs can change the adenosine by inosine residues in the double-stranded RNA-binding domain (dsRNA), resulting, for example, in the inhibition of the correct processing of pri-mir-142 by Drosha [339]. Also, ADARs can affect the correct processing of some miRNAs by Dicer, inhibiting for example the complete maturation of mir-151 [340, 341].

One of the best-studied negative regulator of the miRNAs is Lin28, which has important roles in regulating the levels of mature miRNAs of the let-7 family acting, at both at Drosha and Dicer levels. In view of the importance of Lin28 in this Thesis, its role in the regulation of miRNAs biogenesis will be described in detail in the section 1.7.3.

Table 2. Main regulators of miRNAs biogenesis in animals.

Regulator	Regulation site	Known Activity	Mechanism	Target miRNA
c-Myc	DNA binding (promoter site)	Protooncogene	Inhibiting tumor suppressor miRNAs	mir-15a,-29,-34 let-7
p53	DNA binding (promoter site)	Tumor suppressor	Promoting Drosha cleavage Enhancing tumor suppressor miRNAs	mir-16,-34-143
ERα	DNA binding	Nuclear ER	Inhibiting Drosha cleavage	A subset
p68/p72	Microprocessor complex	Components of microprocessor	Promoting Drosha cleavage	A subset
hnRNP A1	RRM, M9	Pre-mRNA splicing	Chaperone for Drosha/DGCR8 binding; Enhancing mature miRNA production	mir-18a
KSPR	KH	Pre-mRNA splicing	Promoting Drosha and Dicer processing	pre-mir-16,-20,-21, pre-mir-26b, -106a, pre-let-7
Lin28	CCHC-type zinc finger	Promoting pluripotency Inhibition	Inhibition of Drosha and Dicer processing	let-7 family
ADARs	dsRBD	Adenosine-Inosine RNA editing	Inhibition of Drosha and Dicer processing	Pri-mir-142, pri and pre-mir-151
DGCR8	dsRBD	Binding to Drosha	Stabilizing Drosha	Global
Exportin-5	RanBP	Binding to tRNAs and pre-miRNAs	Nuclear transport of pre-miRNAs	Global
HuR	RNA-binding protein	Binding to 3' UTR region of mRNA target	Inhibiting miRNA inhibition	mir-122
Dnd1	RNA-binding protein	Selective RISC complex-competitive	Inhibiting miRNA inhibition	mir-430, -221, -1, mir-372, -206
Importin 8	RNA-binding (P bodies)	Components of RISC	Enhancing RISC complex and argonaute activity	Global
Argonaute	RNA-binding (P bodies)	Components of RISC	Stabilizing associated miRNAs	Global

ADARs, adenosine deaminase acting on RNA enzymes; dsRBD, double-stranded RNA-binding domain; DGCR8, DiGeorge syndrome critical region gene 8; ERa, estrogen receptor a; hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; RRM, RNA recognition motifs; M9, nucleocytoplasmic shuttling signal sequence; KSRP, KH-type splicing regulatory protein; RanBP, ran-binding protein; Dnd1 (RNA-binding protein dead end homolog 1).

Finally, there are several proteins that interfere with miRNA actions. For instance, it has been demonstrated that RNA-binding proteins, such as HuR [342] and Dnd1[343], block the capacity of some miRNAs to inhibit the expression of their mRNA targets and, in contrast, importin 8, an essential component of miRISC complex, facilitate the recruitment of Ago proteins and for this reason, the binding between miRNA and the 3'UTR region of mRNA targets [344] (see **table 2**).

1.7.2. THE LET-7 FAMILY OF miRNAs

As mentioned previously, the *let-7* gene was initially discovered as an essential developmental gene in *C. elegans* and, later, as a gene related with the synthesis of a family of miRNAs highly conserved across evolution, called the let-7 family [345-347].

Regulation of let-7 family of miRNAs in mammals is controlled, in part, at a post-transcriptional level. Recent studies have found that Lin28 is directly involved in this regulation. The role of Lin28 in mammals and the involvement of Lin28/let-7 system in the control of puberty will be described in section 1.7.3.2.

Numerous members of the let-7 family have been identified in various species and different members are found in different species [348]. Thus, the multiple copies of let-7 miRNA variants in the genome are represented with a letter located after let-7 to distinguish between multiple isoforms, which slightly differ in sequences, whereas a number at the end denotes that the same sequence is present in multiple genomic locations. At present, 10 mature subtypes of the let-7 family have been identified in humans, which are produced from 13 precursor sequences. Three separate precursors produce the mature let-7a sequence (let-7a-1, let-7a-2 and let-7a-3) and precursors from two different genomic locations produce the let-7f (let-7f-1 and let-7f-2) sequence [348].

The let-7 family is involved in many biological functions including the regulation of stem cell differentiation in C. elegans [345], neuromusculature development and adult behaviors in flies [349], as well as limb development in chickens and mice [350, 351]. In addition, let-7 family is involved in cell proliferation and differentiation in different species [352-354]. Accordingly, recent data demonstrated the increase in pri-let-7a, pri-let-7e, let-7a, let-7c and let-7e during brain development in mice [335]. In

addition, many let-7-family members act as tumor suppressors in a diversity of cancers [348, 355].

1.7.3. LIN28

Lin28 is a RNA-binding protein which was first identified in *C. elegans* as a heterochronic gene which plays an essential role during development [356, 357]. In mammals, two *Lin28*-related genes, named *Lin28* (also termed *Lin28*a) and *Lin28b*, have been described [358, 359]. *Lin28* paralogs are highly conserved across evolution [360], therefore suggesting similar regulatory functions. These genes encode two different proteins, Lin28 and Lin28b, with two types of domains to bind to RNA targets, a cold shock domain in the N-terminal and pair of retroviral-type CCHC zinc finger motifs in the C-terminal.

In mammals, Lin28 is widely distributed during embryonic maturation, but its expression becomes restricted to specific tissues in adulthood [361, 362]. Lin28b is expressed in the testis, placenta, fetal liver and hepatocellular carcinoma [359]. Both Lin28 homologs are predominantly located in the cytoplasm and P bodies [363] and, consistent with this location, act to regulate mRNA translation or stability.

It is known that Lin28 has a putative role as a pluripotency factor and, together with OCT4, SOX2, and NANOG, was used for reprogramming adult human somatic cells into pluripotent stem (iPS) cells [364]. Accordingly, mutations in *Lin28* gene alter the normal timing of maturational events in the nematode, resulting in the appearance of specificities of the larval period in adulthood or vice versa [319, 357]. However, its molecular function remains unclear.

1.7.3.1. LIN28 AND LET-7

Years ago, several researchers found in mice that, at postnatal day 19 (PND-19), both embryonic stem cell (ESCs) and embryonic carcinoma cells, showed abundant expression primary-let-7g. However, in these undifferentiated cell types, mature let-7g expression was not detected whereas when these cells had differentiated, this let-7 family member was abundantly expressed [365, 366]. This observation indicated that the processing of pri-let-7g was under post-transcriptional regulation [335, 367].

Later, four independents groups identified Lin28 and Lin28b as factors that prevent the let-7 processing in undifferentiated cells [365, 368-370]. In this regard, it has been demonstrated that Lin28 proteins bind to the terminal loops of precursors of the let-7 miRNAs, either pri- or pre-let-7 depending on the let-7 isoform, blocking their processing into mature miRNAs [365, 369]. This regulation is possible because Lin28 is known to transport between the nucleus and cytoplasm. Thus, in the nucleus, Lin28 binds to the loop region of pri-let-7, blocking the interaction with Drosha, while into the cytoplasm, these proteins block Dicer processing by binding to the pre-let-7 [370]. Accordingly, Heo et al. suggested that Lin28 promote uridylation of pre-let-7 in *C. elegans* and mammalian cells by recruiting the terminal uridylyl transferase 4 (TUT4) enzyme, which adds multiple U residues to the 3' end of pre-let-7, preventing Dicer processing and inducing precursor degradation [368] (**Figure 16**).

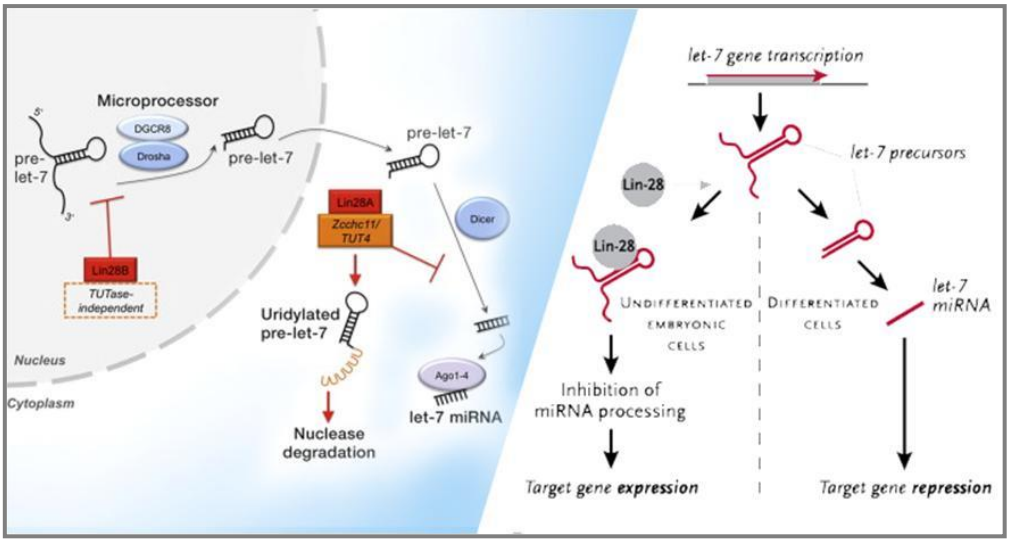

Figure 16. Lin28 selectively blocks let-7 biogenesis. Taken from [168].

Besides the fact that Lin28/Lin28b represses the synthesis of mature let-7 miRNAs, it has been demonstrated that let-7 miRNAs are able to suppress Lin28 levels, thereby creating a double-negative feedback loop (**Figure 17**).

To add further complexity in the relationship between Lin28/let-7, it has been observed that c-Myc transcriptionally activates both Lin28 and Lin28b expression and in turn, let-7a represses c-Myc expression in a Burkitt lymphoma cell line [371]. These data suggest a double-negative feedback loop in which c-Myc inhibits let-7 synthesis

via activation of Lin28 transcription, and let-7 negatively regulates c-Myc [168, 372, 373] (**Figure 17**). In this way, deregulation of c-Myc/Lin28/let-7 network can explain in part the alteration of several miRNAs, included members of let-7 family, observed in many cancers [365, 374].

In addition, numerous upstream regulators of the c-Myc/Lin28/let-7 have been identified recently. For example, it has been shown that mir-145 inhibits c-Myc expression at the post-transcriptional level [375]. Moreover, by the use of bioinformatic algorithms, mir-132 and mir-9 have been predicted as putative miRNA repressors of Lin28 our own data [376] (**Figure 17**).

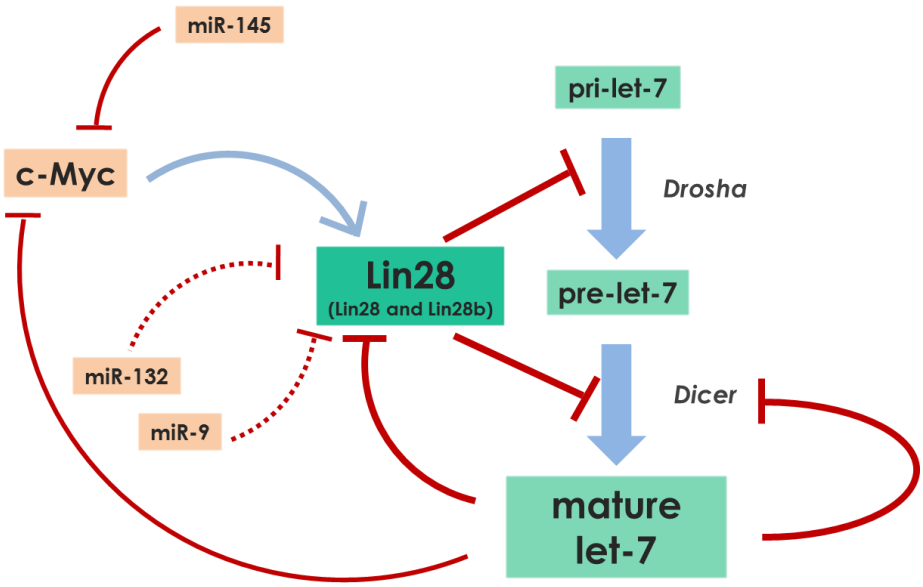

Figure 17. Squematic representation of the regulatory interactions between c-Myc/Lin28/let-7and upstream regulators.

1.7.3.2. ROLE OF LIN28/LET-7 SYSTEM IN THE REGULATION OF POSTNATAL MATURATION AND PUBERTY

In the last few years, different GWAS have shown the putative link between variations in or around the *Lin28b* locus and changes in key developmental events in humans. Specifically, in 2008, Lettre et al. reported 12 loci, including *Lin28b* and several let-7 targets, that were strongly associated with variation around 2% in human height [377]. Independly, in 2009, four GWAS found an association of human growth, the age of menarche and other specific characteristics of the puberty with changes in or around the *Lin28b* locus [164-167]. These data, together with the observations in

nematodes regarding the essential role of Lin28 through development [356, 357], suggested that Lin28/let-7 system could play an important role in the normal development and the somatic cell reprogramming throughout the evolution, and alterations in this system could carried out to oncogenesis.

In 2010, Zhu et al explored the function of the Lin28/let-7 pathway in a model of Tg mice that overexpressed *Lin28* [378]. Although, they observed that the WT and Lin28 Tg pups had the same size, Tg animals showed an increased growth rate and, in adulthood, were heavier and larger than control animals. Of note, the increased body size observed in Lin28 Tg mice was similar to phenotype previously described in humans associated with genetic variation in the *Lin28b* locus. Moreover, Lin28 Tg animals showed an increase in relative organ weight, suggesting a role for Lin28 in the regulation of organ size [377].

In relation with the putative role of Lin28 in the control of puberty and reproductive function, it has been documented that these Lin28 Tg female mice display delay in the age of VO and first estrous combined, with a decrease in ovarian and uterus weights (OW and UW). Additionally, those animals had a delay in the age date of their first litter.

Moreover, Lin28 expression has been found in reproductive tissues as testis. In this regard, Lin28 is differentially expressed in spermatogonia of mouse and primate, suggesting a role in the maintenance of spermatogonial stemness [379]. In fact, despite its wide expression during embryonic development, the testis is one of the few tissues where Lin28 is persistently detected in adulthood of several species, including the mouse, monkey, and human [379]. Recent works has been suggested a role for Lin28/let-7 system in fertility and spermatogenesis [380, 381] [382]. Thus, Chakraborty et al., using a conditional Lin28 KO in adult germ line stem cells, showed that the loss of Lin28 reduced testicular weight (TW), sperm number and impaired spermatogonial cell proliferation without compromising their differentiation capacity [382]. Nowadays relatively little is known about the role of the Lin28/let-7 system in the male reproductive system.

In the work of Zhu et al, mentioned above, the potential involvement of metabolic mechanisms in the overgrowth of Lin28 Tg mice was also analyzed. In detail,

Zhu and coworkers observed that Lin28 Tg mice showed lower glucose levels than WT, both in fasting and normal fed conditions and also increased insulin sensitivity. They suggested that overexpression of Lin28 in muscle alters the metabolic state, increases glucose uptake and promotes glucose metabolism. Additionally, the same group has reported both *Lin28* and *Lin28b* Tg mice were resistant to obesity and showed increased insulin sensitivity and enhanced glucose tolerance. In contrast, muscle-specific *Lin28* KO and inducible *let-7* Tg mice displayed glucose intolerance [383]. Thus, *let-7* upregulation inhibited normal glucose metabolism, supporting the idea that Lin28 and Lin28b exert their metabolic effects, at least in part, via *let-7* suppression. Data showed by Zhu's group reinforced the hypothesis that *Lin28/let-7* system is a key regulator in modulating glucose metabolism in mammals.

Despite all these evidences, the potential involvement of miRNA regulatory pathways, and in particular of the Lin28/let-7 system, in the control of the central and peripheral developmental events that lead to puberty onset and metabolic homeostasis remains ill defined and warrant future analysis.

OBJECTIVES

2. OBJECTIVES

Puberty is defined by a sequence of events leading to full activation of reproductive axis and the acquisition of fertility. The regulatory signals controlling the HPG axis include, among others, peripheral factors that transmit nutritional information to CNS, where they interact with central regulators to control the onset of puberty [146]. As mentioned in the introduction of this Thesis, the key event required for puberty to occur is an increase in pulsatile GnRH release from GnRH neurons, the main hierarchical element in the neuroendocrine control of reproduction, which act as the final integrator of central and peripheral regulators of the HPG axis.

In spite of the key role of GnRH neurons to integrate metabolic information to regulate the reproductive axis, different studies suggest that there is not a direct interaction between GnRH neurons and leptin. In this sense, recent evidence suggests that POMC neurons act as intermediary element to convey metabolic information to GnRH neurons [312, 314, 315]. Furthermore, a bidirectional interplay between α-MSH pathways and kisspeptins, indispensable signals in the control of puberty onset, has been suggested in adulthood [202, 273]. However, the role of α-MSH in the regulation of puberty, and its interaction with kisspeptins during this key maturational event, remain virtually unexplored.

In addition, numerous studies have unveiled that the pubertal increase in GnRH secretion is controlled by a set of genes, hierarchically arranged, within functionally connected networks where GnRH is the final effector [159]. In this sense, recent evidence has indicated a role for epigenetic mechanisms, including miRNAs, in the control of the timing of female puberty [161]. In this context, previous data from GWAS demonstrated an association between early menarche and a sequence variation in or close to the *LIN28B* locus [164-167]. The major known role of Lin28b is the blocking of the processing of mature miRNAs of Let-7 family [365, 369]. However, despite the potential involvement of Lin28/let-7 system in the control of puberty [164-167, 383], relatively little is known about the function of this system in pubertal maturation.

On the above basis, the **GENERAL OBJECTIVE** of this Thesis was to investigate the role of neuropeptidergic systems, particularly α-MSH and Kp, and novel regulatory mechanisms, specifically miRNAs, in the physiological control of puberty. To cover this general objective, the following **SPECIFIC OBJECTIVES** were addressed:

1. To characterize the potential function of α-MSH signaling in the control of puberty onset and its potential interaction with leptin and kisspeptins.

2. To define the profiles of expression of the Lin28/let-7 system, and several associated factors, during pubertal maturation and study the changes in their expression patterns in preclinical models of altered puberty at different sites of the HPG axis, including the hypothalamus and testis.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. ANIMALS

Male and female Wistar rats bred in the vivarium of Faculty of Medicine at the University of Córdoba were used in this Thesis. After birth, the rat pups were cross-fostered and reared in litter sizes of 10-12 pups, unless otherwise stated. Hypophysectomized (HPX) rats were purchased from Charles River (Barcelona, Spain). In addition, CB57B/6 mouse strain and genetically modified, Gpr54 null mice were used. Mice were bred and housed in the Animal Experimentation service Center of the University of Córdoba.

The day the animals were born was considered day 1 of age. Animals were housed under constant conditions of temperature (22°C) and light (14-hours from 07.00 h), unless otherwise indicated. Animal were weaned at postnatal day (PND) 21 and were provided with a free access to tap water and fed a standard soy-free diet. All experiments and animal protocols included in this Thesis were approved by Córdoba University Ethical Committee and were conducted in accordance with European Union normative for the use and care of experimental animals (Directive 2010/63/UE on September 2010).

3.2. DRUGS

MT II (MCR-3/4 agonist), the selective MC4-R agonist (Cat. 043-31), SHU9119 and Kisspeptin $_{110–119}$-NH2 (termed Kp-10) were purchased from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA). The selective MC3-R agonist (Ref. 56-0-13) was obtained from American Peptide Company Inc. (Sunnyvale, CA, USA). Recombinant rat leptin was obtained from ProSpec-Tany TechnoGene Ltd. (Ness Ziona, Israel). GnRH (Ref. L7134), estradiol benzoate (EB), testosterone propionate (TP) and 17β-estradiol (E_2) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). FSH and human chorionic gonadotropin (hCG), a superagonist of LH, were purchased from Merck-Serono (Madrid, Spain). All drugs were dissolved in physiological saline (0,9% NaCl) with the exception of EB, TP and E_2 which were dissolved in olive oil.

3.3. GENERAL EXPERIMENTAL PROCEDURES

3.3.1. CANNULATION AND ICV ADMINISTRATION

In order to allow the administration of different compounds in the lateral cerebral ventricle, rats and mice were cannulated 24 hours before the beginning of the different studies. Cannulae (INTRADEMIC polyethilene Tubing, Becton Dickinson, Sparks, MD, USA) were inserted to a depth of 2 mm beneath the surface of the skull, with an insert point at 1 mm posterior and 1.2 mm lateral to Bregma following standard procedures. Once the animals were cannulated, they were housed in individual cages until the end of the experiments. The doses of the different drugs were administrated in a final volume of 10 µl for rats and 5 µl for mice.

3.3.2. GONADECTOMY AND HORMONAL SUPPLEMENTATION

To avoid the potential confounding factor of changes in circulating estrogen, in some experimental designs, female rats were subjected to bilateral OVX on PND-25 and then, they received a subcutaneous implant of a SILASTIC brand silicone tubing (Dow Corning, Midland, MI) of 10 mm in length, 1.98 mm in inner diameter and 3.18 mm outer diameter containing E_2 (solution of 10 µg/ml in olive oil).

3.4. GENERAL ANALYTICAL PROCEDURES

3.4.1. REVERSE TRANSCRIPTION (RT) AND POLYMERASE CHAIN REACTION (PCR) FOR mRNA AND miRNA.

3.4.1.1. TOTAL RNA EXTRACTION FROM TISSUES

Total RNA was extracted from different tissues using TRIsure™ reagent (Bioline, London, UK), in accordance with the conditions lay down by the manufacturer. RNA concentration of each sample was determined by measuring the absorbance at 260 nm using a spectrophotometer (DU-530, Beckman). The quality samples were analyzed by electrophoresis in a 1% agarose gel to observe 28S, 18S and 5S ribosomal RNA bands. In those samples that did not meet the established quality criteria, the extraction was repeated. Up to this point, the steps to be followed for mRNAs and miRNAs analyses are the same.

3.4.1.2. REVERSE TRANSCRIPTION FOR mRNA QUANTIFICATION

To remove any traces of genomic DNA, RNA samples received a DNase treatment (Promega, WI, USA) prior to RT-PCR. RNA samples were treated with 2 units of RQ1 DNase and were incubated at 37°C for 30 min. Then, 1 µl of RQ1 DNase Stop solution was added and the samples were incubated at 65°C for 10 min to terminate the reaction.

RT reaction for the synthesis of cDNA from RNA molecules was carried out by the mix of following components:

- 2 µg of total RNA
- 6 µl of AMV RT 5X reaction buffer (250nM Tris-HCl, pH 8.3, 250 mM KCl, 50 mM MgCl2, 50 nM DTT, 2.5 mM; Promega)
- 2 µl of dNTP mix 5 mM (Promega)
- 0.5 µl of Random Primers (Promega)
- 0.5 µl (20 units) of RNasin Ribonuclease Inhibitor (Promega)
- 7.5 units of AMV RT (Promega)
- Nuclease-free water to final volume of 30 µl

The reaction mixture was performed in a thermo-cycler (iCycler iQ®5; Bio-Rad, Hercules, CA, USA) which carried out the following cycles: 25°C for 5 min, 42°C for 60 min and 85°C for 5 min. Once RT was over, the reaction volume was diluted with Nuclease-free water to final volume of 100 µl. The cDNA samples were stored at -20°C.

3.4.1.3. QUALITATIVE FINAL TIME RT-PCR FOR mRNA

For the final time PCR were used 10 µl of the diluted cDNA samples following the protocol and conditions lay down by the manufacturer (Promega). They were amplified by the use of specific primers (**Table 3**) designed from the different sequences genes of study published in GenBank. The final volume of PCR reaction was 50 µl. In addition to the cDNA samples, negative controls for genomic DNA, RT and PCR were included. The thermal cycling conditions for DNA polymerase-mediated PCR amplification were as follows: i) initial denaturation at 95°C for 5 min; ii) 25-36 cycles, depending of gene, at 95°C for 30 seconds (denaturation), 58-63°C, depending of the primer Melting Temperature (Tm), for 30 seconds (annealing) and 72°C for 10 seconds

(extension); and iii) final extension at 72°C for 5 min. Expression of ribosomal protein S11 (RP-S11) in rats and L19 (RP-L19) in mice were used as endogenous control genes (**Table 3**). PCR products were visualized by 2% agarose gel with ethidium bromide (1µg/ml) in 1X TBE (10X TBX: 10% Tris-HCl, 5% boric acid and 4% EDTA) electrophoresis buffer and their identity confirmed by direct sequencing in the Nucleic Acids Unit of the Research Support Services Center (SCAI) at the University of Córdoba.

3.4.1.4. SEMIQUANTITATIVE REAL-TIME PCR FOR mRNA

The real time PCR (qPCR) is used to amplify and simultaneously detect and quantify the presence of nucleic acid sequences compared to standard PCR, where the product of the reaction is detected at its end. For the qPCR the detection system used was CFX96^{TM} Real-Time PCR (BIO-RAD Laboratories, Hercules, CA). The PCR reaction included 5 µl of the diluted cDNA samples in duplicate. These samples were amplified by the use of specific primers for different genes (**Table 3**) and GoTaq® qPCR Master Mix (Promega). The final volume of PCR reaction was 15 µl. In addition to the cDNA samples, negative controls for genomic DNA, RT and PCR were included. Moreover, for data analysis, were included relative standard curves constructed from serial dilutions of one reference cDNA sample in the same plate. The thermal cycling conditions were as follows: i) one cycle of 10 min at 95°C; ii) 40 cycles of 15 seconds at 95°C, 35 seconds at 58-63°C (depending of the primer Tm) and 10 seconds at 72°C; and iii) one hold of 72°C for 10 min. The dissociation (Melting) curve was used to assess the quality of the PCR.

The iCycleriQ qPCR system collects, with each amplification cycle, the increase in fluorescence intensity which is proportional to the increase in amplicon concentration. The cycle number at which an amplification plot crosses a threshold fluorescence level established is called the threshold cycle (Ct). The Ct values of the standard curve obtained were analyzed in logarithmic scale and then, the Ct value for each unknown target sample was directly correlated with the calibration curve obtained. To determine the relative concentration, the value of the target gene obtained was standardized to endogenous control genes, HPRT in rats and RP-L19 in mice (**Table 3**), with reference value at 100.

Table 3. Primers list used for PCR analysis of mRNA.

Gene	GenBank	Primers	Amplicon size	Tm (°C)
Lin28 (rat)	NM_001109269.1	FW: 5'-CCCGGTGGACGTCTTTGTG-3' RV: 5'-CACTGCCTCACCCTCCTTGA-3'	76 bp	60
Lin28b (rat)	XM_001069344.4	FW: 5'-GGATCAGATGTGGACTGTGAGAGA-3' RV: 5'-GGAGGTAGACCGCATTCTTTAGC-3'	102 bp	60
Lin28 (mouse)	NM_145833.1	FW: 5'-GTCTTTGTGCACCAGAGCAA-3' RV: 5'-CTTTGGATCTTCGCTTCTGC-3'	194 bp	63
Lin28b (mouse)	NM_001031772.2	FW: 5'-TGGTTCAACGTGCGCATGGGA-3' RV: 5'-CCACTGGCTCTCCTTCTTTCAAGCT-3'	145 bp	63
c-Myc (rat)	NM_012603.2	FW: 5'-AGCTCCTCGCGTTATTTGAAG-3' RV: 5'-GAGTCGTAGTCGAGGTCATAGTTCCT-3'	117 bp	60
RP-S11 (rat)	NM_031110.1	FW: 5'-CATTCAGACGGAGCGTGCTTAC-3' RV: 5'-TGCATCTTCATCTTCGTCAC-3'	240 bp	58
HPRT (rat)	NM_012583.2	FW: 5'-AGCCGACCGGTTCTGTCAT-3' RV: 5'-GGTCATAACCTGGTTCATCATCAC-3'	72 bp	60
RP-L19 (mouse)	NM_009078.2	FW: 5'-GAAATCGCCAATGCCAACTC-3´ RV: 5'-ACCTTCAGGTACAGGCTGTG-3'	290 bp	55

3.4.1.5. REVERSE TRANSCRIPTION FOR miRNA QUANTIFICATION

The most commonly used method to detect specific miRNAs is qPCR analysis. A miRNA real-time reaction starts with reverse transcribing RNA into cDNA. The limited length of the mature miRNA (about 22 nt), the lack of a common sequence feature like a poly(A) tail and the fact that the mature miRNA sequence is also present in the pri- and pre-miRNA transcript raise several problems for appropriate RT.

In our method, miRNAs were reverse transcribed individually by using stem–loop–specific RT primers. They are designed to have a short single-stranded region that is complementary to the known sequence on the 3' end of the miRNA, a double-stranded part (the stem) and the loop that contains the universal primer-binding sequence (TaqMan MicroRNA RT; Applied Biosystems, Foster City, CA).

Each RT reaction combined: 5 µl of total RNA sample (2ng/µl), 3 µl of the TaqMan-specific RT primers (Applied Biosystems) and 7 µl of the RT master mix that contained the following reagents:

- 4.16 µl of Nuclease-free water
- 1.5 µl of 10X RT Buffer (Applied Biosystems)
- 0.15 µl of dNTP mix (100mM total) (Applied Biosystems)

- 0.19 µl of RNase Inhibitor (20 U/µl) (Applied Biosystems)
- 1 µl of MultiScribe™ RT enzyme (50 U/µl) (Applied Biosystems)

The reaction mixture (15 µl) was performed in a thermo-cycler (iCycler iQ®5; Bio-Rad, Hercules, CA, USA) with the following conditions: 16°C for 40 min, 42°C for 40 min and 85°C for 5 min. Once RT was over, the reaction volume was diluted with Nuclease-free water to final volume of 215 µl. The cDNA resulted was then used as a template for qPCR.

3.4.1.6. REAL-TIME PCR FOR miRNA QUANTIFICATION

Each PCR reaction contained 1.33 µl of RT product (dilution 1:15) and the following reagents (Applied Biosystems):

- 1 µl of TaqMan® Small RNA Assay 20X
- 10 µl of TaqMan® 2X Universal PCR Master Mix (No AmpErase®UNG)
- 7.67 µl of Nuclease-free water

The final volume for PCR reaction was 20 µl. The thermal cycling conditions were as follows: i) 2 min at 50°C; ii) 10 min at 95°C and iii) 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Data analyses were carried out in the same way than mRNA but, in this case, RNU6 (Applied Biosystems) was used as internal reference. The predesigned primers used were let-7a and let-7b, as two representative miRNAs of the let-7 family, miR-132, miR-145 and miR-9 (Applied Biosystems).

3.4.2. ISH ASSAYS

3.4.2.1. ISH TO DETECT mRNA

For the analysis of gene expression and neuroanatomical distribution of *Kiss1* in hypothalamic tissues obtained from different experimental groups, ISH technique was performed. Five sets of coronal sections 20-µm-thick were generated and thaw mounted in SuperFrost Plus slides (VWR Scientific). The samples were stored at -80°C until their analyses. Standard procedures of tissue collection were applied, starting in a fixed coordenate in the rostral hypothalamic area up to the ARC, to encompass equivalent areas including anterior and medial portions of this nucleus, where Kiss1 neurons are abundantly located (Paxinos and Franklin, 2001).

For the ISH technique, a specific antisense riboprobe for *Kiss1* rat mRNA (5'-TGG TGA ACC CTG AAC CCAC A-3) located between 328-367 nt of the cDNA sequence (GenBank NM_181692.1) was used. This probe was generated using T7 RNA polymerase (Promega) and *Kiss1* cDNA template containing T7 polymerase region. The radiolabeled RNA probe was synthesized by adding the following ingredients in a volume of 25µl: 250 Ci [33P]-UTP (Perkin Elmer, Massachusetts, USA), 1 µg of template product (0.5 mM rATP, rCTP and rGTP) and 40-U of T7 RNA polymerase. The residual DNA was digested with 4-U DNase (Bio-Rad) and the reaction was terminated by addition of 2 µl 0.5 M EDTA, pH 8.0. The labeled riboprobe was purified using Illustra^{TM}ProbeQuant^{TM} G-50 MicroColums (GE Healthcare, UK).

A single set of sections was used for ISH (adjacent sections 100-µm apart). These tissue sections were: i) fixed in paraformaldehyde at 4% for 15 min; ii) stabilized with 0.1 M phosphate buffer (pH 7.4) at room temperature for 20 min; iii) treated with saline triethanolamine and acetic anhydride to prevent non-specific binding of probes; iv) dehydrated in increasing concentrations of ethanol; v) delipidated with chloroform and vi) air dried at room temperature for 1 h.

After these steps, the *Kiss1* riboprobe hybridization was performed during 16 h at 55°C. The hybridization solution contained: 4X saline-sodium citrate (SCC) buffer, 50% deionized formamide, 1X Denhardt's solution, 50% dextran sulfate and 0.03 pmol/ml of radiolabeled riboprobe along with yeast tRNA (10 mg/ml).

After hybridization, slides were: i) washed with 4X SSC for 30 min; ii) incubated in RNase-A buffer (Roche Biochemical) at 37°C (10mg/ml) for 1 h; iii) washed in 0.1X SSC for 1 h at 65°C; iv) dehydrated in increasing ethanol series and v) air dried at room temperature for 1 h.

Finally, slides were dipped in Kodak Autoradiography Emulsion type NTB (Eastman Kodak) and exposed for 1 week at 4°C in a dark room. After this period, the sections were developed and fixed following the manufacturer instructions (Kodak; Rochester, NY): 3 min in Kodak Dektol Developer; 10 second in distilled water; 5 min in Kodak Fixer and 5 min in distilled water. For the mounting, the sections were previously dehydrated and rinsed with citrasol (Sigma) and then, was used Permaslip (Sigma) as mounting medium.

For the analysis, 50-60 sections from each animal (9-10 slides; 6 sections/ slide) were evaluated, with 5 animals per group. Slides from all of the animals were read under dark-field illumination with custom-designed software enabled to count the total number of cells (grain clusters). Cells were counted as *Kiss1* mRNA positive when the number of silver grains in a cluster exceeded that of background.

3.4.2.2. ISH TO DETECT miRNA

For detection and distribution of *let-7b* and *miR-145* miRNAs in testicular tissues, ISH analysis was carried out. Testes from adult rats were obtained from different experimental groups and were formalin-fixed and paraffin-embedded in our laboratory. The ISH was performed in collaboration with Dr. Susana Sangiao-Alvarellos (University of Santiago de Compostela). The samples were sectioned at 10 µm thickness and placed on glass slides. For this analysis, double digoxigenin (DIG)-labeled miRCURY LNA™ *let-7b, mir-145* and *RNU6* detection probes (Exiqon, Denmark) were employed as was previously described [384].

3.4.3. IHC ASSAYS

IHC analyses carried out in this Thesis were conducted through two different IHC staining methods: immunoenzymological staining and immunofluorescence.

3.4.3.1. IMMUNOENZYMOLOGICAL STAINING

Mice testes were removed immediately following the decapitation of animals and fixed in Bouin's solution (formalin/picric acid) for 24 h. Then, tissues were embedded in paraffin and were cut to a thickness of 5 µm. The paraffin sections were processing to study by IHC assays the Lin28 and Lin28b protein distribution, using specific antibodies against each target.

Sections were deparaffinized, incubated in 2% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase, and after rehydration in graded ethanol, rinsed in PBS. In the case of Lin28 immunostaining, was necessary antigen retrieval by autoclaving at 1.5 atmospheres for 5 min in 0.1M citrate buffer (pH 7.6).

Thereafter, sections were incubated overnight at 4°C with primary antibodies: anti-Lin28, dilution 1:200 (Abcam, UK; ref. ab115698), and Lin28b-specific polyclonal

anti- body, dilution 1:25 (ProteinTech Group, Inc; Chicago, Illinois; ref. 16178-1-AP). Then, bound antibodies were revealed by the avidin-biotin-peroxidase complex method, as specified by the manufacturer. In both cases, sections were incubated for 30 min using a VECTASTAIN ABC Kit, dilution 1:1000 (Rabbit IgG; Vector Laboratories, Burlingame, CA, USA). Finally, sections were stained with hematoxylin and eosin and examined under the microscope for their morphometric analysis. Tissue samples from different ages were run in the same assays to assess age-related changes in the intensity of immunostaining, using a semiquantitative scale. In addition, negative control sections were run in parallel by replacing primary antibodies by nonimmune serum or PBS.

3.4.3.2. IMMUNOFLUORESCENCE

Perfused brains from pubertal female rats were removed and post-fixed in the 4% PFA solution at 4°C for 24 h and then, were directly transferred into a PBS solution. Fixed brains were then impregnated with 30% sucrose in PBS at 4°C**.** When brains sunk, were cut coronally at 30 µm thicknesses and stored in cryoprotectant.

The immunofluorescence analyses were carried out in collaboration with Dr. Rafael Pineda (University of Edinburgh). The samples were mounted on SuperFrost Plus slides (VWR International, Cat. No.631-0108). After drying for 1 h at 37°C, sections were washed in PBS-T (0.1% Tween-20 - Sigma-P9416-) for 10 min at room temperature and then, heated in 10 mM sodium citrate (pH 6) (Sigma-S4641) for 10 min at 90°C to induce the epitope retrieval. Afterwards, sections were cooled to room temperature and then washed 5 min in PBS. Sections were blocked in blocking buffer (3% Donkey Serum, Sigma-D9663; 0.4% Triton X-100, Sigma-T8787) in PBS for 45 min at room temperature. Next, sections were incubated with primary antibody against sheep Kp (approved by Professor Caraty), 1:10000 dilution, and with primary antibody against rabbit α-MSH (Peninsula Labs-T4434), in 1:1000 dilution, overnight at 4ºC. Then, sections were washed in PBS. Finally, sections were incubated with secondary antibodies Alexa Fluor® 488 Donkey Anti-Sheep IgG (H+L) (Invitrogen-A11015), 1:500 dilution, and with Alexa Fluor® 555 Donkey Anti-Rabbit IgG (H+L) (Invitrogen-A31572), 1:500 dilution, respectively, for 1 h at 37°C and later, washed in PBS. Slides were immersed for 30 seconds in double distilled water to remove salts and the coverslips

were mounted using PermaFluor Aqueous Mounting Medium (Thermo Scientific, TA-030-FM). No signal was detected after applying secondary antibodies in absence of primary antibody. Immunoreactivity analyses were imaged on the Nikon A1R FLIM confocal system. Images were viewed and Z stacks condensed to 2D projections using NIS-Elements Viewer software.

3.4.4. RADIOIMMUNOASSAY (RIA)

Serum LH levels in rat and mice were determined in a volume of 25-50 µl using a double antibody method and RIA kits supplied by the National Institute of Health, NIH (Dr. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Bethesda, MD, USA). Hormonal determinations were performed in duplicate in the case of rats. Hormone provided by NIH (LH-I-10) was labeled with ^{125}I using Iodo-gen® method (Pierce, Rockford, IL). The hormone concentrations were expressed using reference preparations of LH-RP-3. The sensitivity of the assay was 75 pg/ml. Intra- and inter-assay coefficients of variation (CV) were lower than 8%. The reliability of the hormone determinations was confirmed by measurement of rat serum samples with known concentrations of hormone of interest.

3.5. EXPERIMENTAL DESIGNS

3.5.1. CHARACTERIZATION OF THE ROLE OF MC3/4-R SIGNALING IN THE CONTROL OF THE GONADOTROPIC AXIS AT PUBERTY AND ITS POTENTIAL INTERACTION WITH LEPTIN AND KISSPEPTIN

As was mentioned in the introduction of this Thesis, recent evidence suggest that POMC neurons, known particularly for their key role in the control of energy homeostasis, could act as intermediary element to convey metabolic information to GnRH neurons. In addition, bidirectional interplay between MC3/4-R and kisspeptin neurons has been suggested. Nevertheless, most of data in the literature about the effect of α-MSH in the reproductive axis have been carried out in adults animals, thus the role of α-MSH in the regulation of puberty remains virtually unexplored.

In order to analyse the putative function of central melanocortin signaling in the control of puberty onset and to explore its potential interaction with leptin and kisspeptins, three sets of experimental designs were carried out.

3.5.1.1. ANALYSIS OF EFFECTS OF ACUTE CENTRAL ADMINISTRATION OF MT II ON LH SECRETION

In the first set of experiments, the acute effects of central administration of MT II (a MC3/4-R agonist) on LH secretion were monitored.

In **experiment 1**, peripubertal male (PND-43) and female (PND-29) rats (n=10-12 animals/group) fed *ad libitum* or fasted for 48h, were icv injected with vehicle (0.9% NaCl) or MT II (1 nmol/rat). Animals were underwent with light ether anesthesia and blood samples were obtained by jugular puncture at before (0), 15- , 30- and 60-min (only male rats) after injection (**Figure 19**).

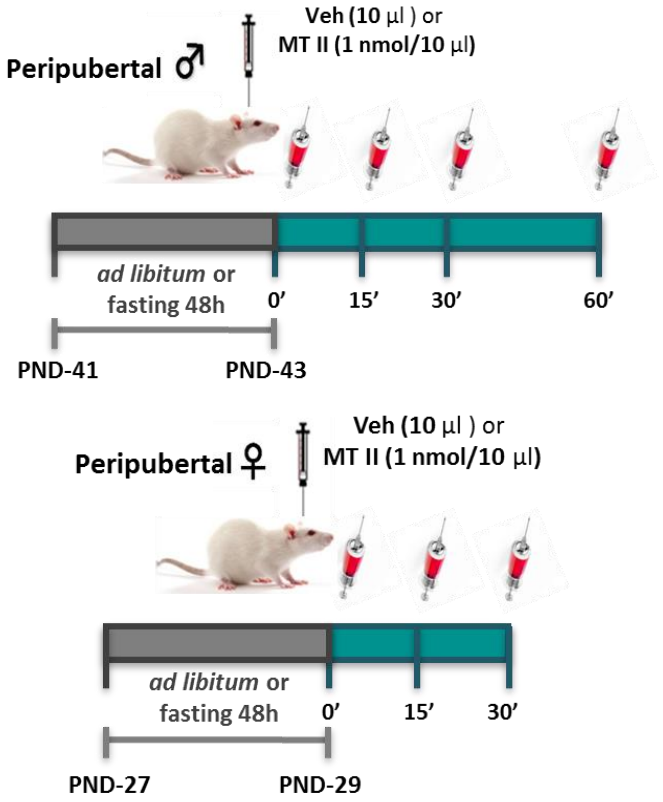

Figure 19. Schematic representation of the experimental protocol used in experiment 1.

In addition, in **experiment 2**, infantile male and female rats (PND-15; group size: n=10-12) were icv injected with vehicle (0.9% NaCl) or MT II (1 nmol/rat). Animals were killed by decapitation 15 min after the injection and blood samples were recorder (**Figure 20**).

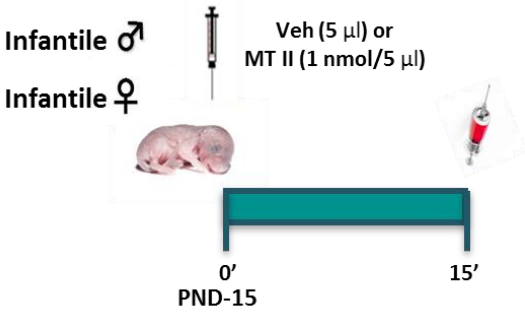

Figure 20. Schematic representation of the experimental protocol used in experiment 2.

Since MT II bind both MC3-R and MC4-R, in order to know which receptor mediated the effect of MT II on LH secretion, in **experiment 3**, peripubertal male (PND-43) rats (n=9-11 animals/group) were icv injected with vehicle (0.9% NaCl) or a selective MC3-R or MC4-R agonist (0.2-, 1- or 5-nmol/rat). Blood samples were obtained by jugular venipuncture after light ether anesthesia, before (0) and 15-, 30- and 60- min after injections (**Figure 21**).

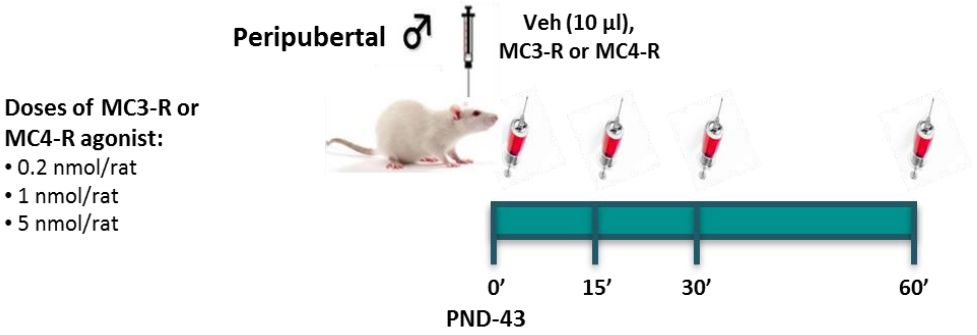

Figure 21. Schematic representation of the experimental protocol used in experiment 3.

3.5.1.2. ANALYSIS OF THE EFFECTS OF CHRONIC BLOCKADE OF MC3/4-R DURING PUBERTAL TRANSITION

In a second set of studies, the effects of chronic blockade of MC3/4-R during the pubertal transition were explored. To this end, in **experiment 4**, female rats (n=10-11) received two daily icv injections of vehicle (0.9% NaCl) or SHU9119 (1nmol/rat), a MC3/4-R antagonist, between PND-28 to -35. Since treatment with SHU9119 increases daily food intake, in order to avoid the possible influence of BW on puberty onset, SHU9119-treated animals were fed with the same amount of food eaten the vehicle-treated animals (*pair-feeding*). BW and VO were daily monitored during treatment.

Animals were killed by decapitation 60 min after the last injection and blood samples, ovaries and uterus were collected (**Figure 22**).

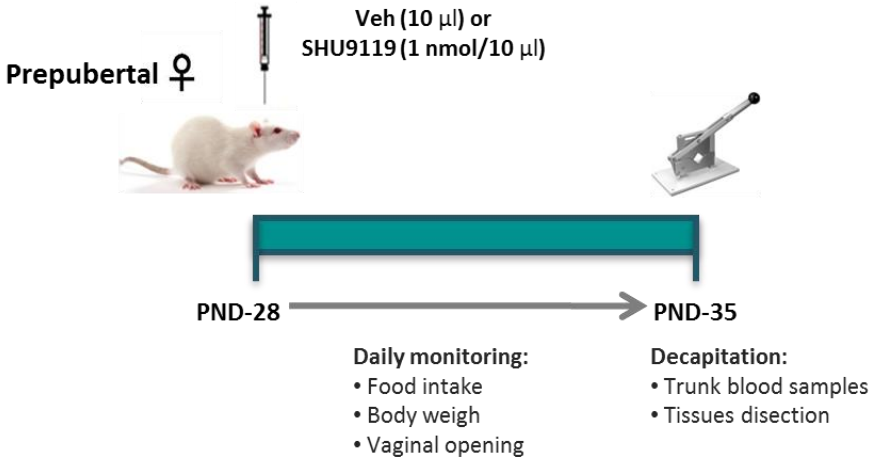

Figure 22. Schematic representation of the experimental protocol used in experiment 4.

In order to explore the putative role of MC3/4-R signaling on the permissive effect of leptin on puberty onset, in **experiment 5**, peripubertal female rats were submitted to a 20% restriction in daily food intake from PND-23 (UN) received two daily icv injections of vehicle (0.9% NaCl), leptin (3.1 µg/12 h/rat) alone or in combination with SHU9119 (1 nmol/12 h/rat) between PND-29 to -35 (n=9-12). A groups of animals fed *ad libitum* (NN) that received two daily icv injections of vehicle served at controls. BW and VO were daily monitored. On PND-35, animals were killed by decapitation 60 min after the last injection and trunk blood, ovaries and uterus were collected (**Figure 23**).

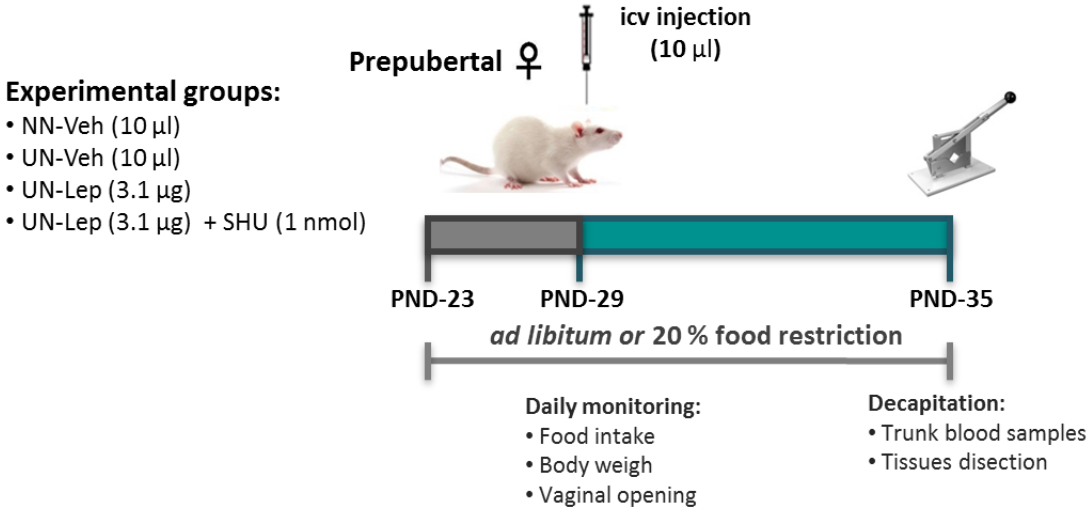

Figure 23. Schematic representation of experimental protocol used in experiment 5.

3.5.1.3. CHARACTERIZATION OF THE POTENTIAL MC3/4-R AND KISSPEPTIN INTERACTIONS IN THE CONTROL OF PUBERTY ONSET

To study the potential MC3/4-R and kisspeptin interactions in the metabolic control of puberty, the third set of studies was carried out in two ways. Firstly, in experiment 6, it was studied the possible role of MC3/4-R as mediators of kisspeptins effects on LH secretion. Then, in experiment 7 and 8 was explored the possible kisspeptidergic mediation of the effects of MC3/4-R on LH secretion.

In **experiment 6**, the effect of Kp-10 on LH secretion after the blockage of MC3/4-R signaling was analyzed. To this end, two groups of peripubertal male rats (groups size: n=30) were icv injected with vehicle (0.9% NaCl) or SHU9119 (1 nmol/rat) at -120 min and -60 min before administration of a third bolus with vehicle or SHU9119 alone or combined with Kp-10 (100 pmol/rat) or MT II (1nmol/rat). Blood samples were obtained before (0) and 15- and 60-min after last injection (**Figure 24**).

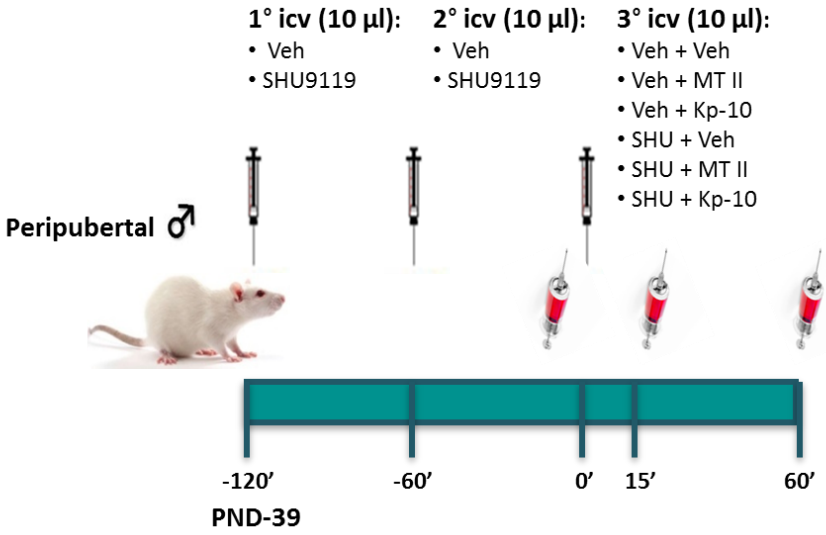

Figure 24. Schematic representation of the experimental protocol used in experiment 6.

In **experiment 7**, the effect of MT II on LH secretion in the absence of kisspeptidergic signaling was studied using a Gpr54 null mouse model. The Gpr54 KO mouse is a model of congenital hypogonadism of central origin as a result of the lack of kisspeptin signaling. Detailed description of the generation and phenotypic characteristics of the Gpr54 KO mouse line has been recently described by our group [65]. Adult male mice (>2-month-old) of both genotypes ($Gpr54^{-/-}$, $Gpr54^{+/+}$) were used. To exclude the possibility that defective gonadotropin responses to the stimuli can be derived from insufficient pituitary responsiveness to GnRH due to their low endogenous tone, Gpr54 KO mice were subjected to a protocol of GnRH priming during two days before the test. The priming protocol consisted of five successive ip boluses of a low dose of GnRH (0.15 µg/mouse), with the following schedule: at 10.00 h, 17.00 h, and 00.00 h on day 1; and at 08.00 h and 16.00 h on day 2. $Gpr54^{+/+}$ mice were submitted to a similar protocol but with vehicle injections served as controls. On day 3, $Gpr54^{+/+}$ (n=12) and $Gpr54^{-/-}$ (n=7) male mice received a single icv injection of vehicle (0.9% NaCl) or MT II (1nmol) and blood samples were collected 15 min after injection (**Figure 25**).

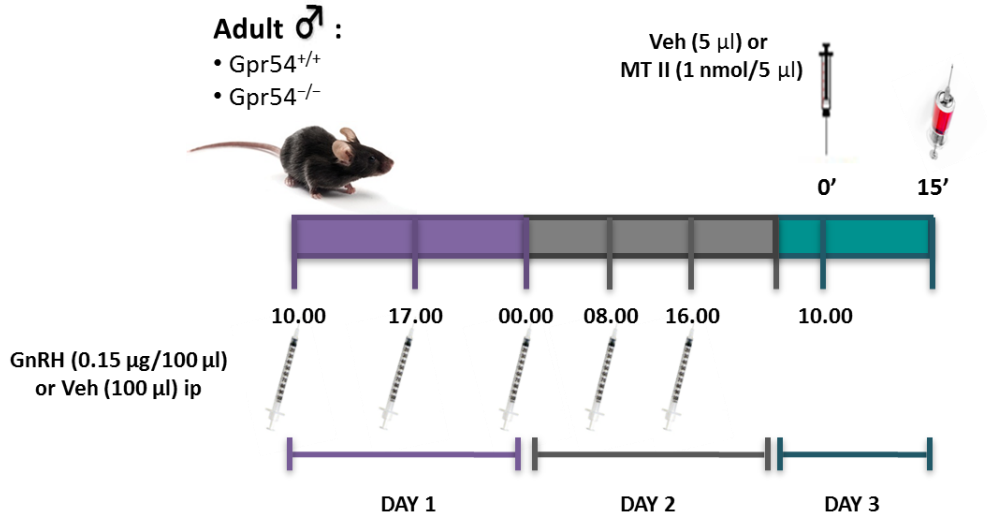

Figure 25. Schematic representation of the experimental protocol used in experiment 7.

In **experiment 8**, hypothalamic *Kiss1* mRNA levels were assayed after blockade of MC3/4-R. To avoid the potential confounding factor of changes in circulating estrogens, 25 day-old female rats were subjected to bilateral OVX and supplemented with E_2, following standard protocol (see section 3.3.2.). This dose of replacement was selected to achieve moderate levels of circulating E_2. Animals received two daily icv injections of vehicle (0.9% NaCl) or SHU9119 (1nmol/rat) between PND-28 to -32. SHU9119-treated animals were pair fed to vehicle-treated rats, as was described in experiment 4. BW was daily monitored and, at the end of the experiment, animals were euthanized by decapitation and brains removed, frozen on dry ice and stored at -80°C for ISH analyses (**Figure 26**).

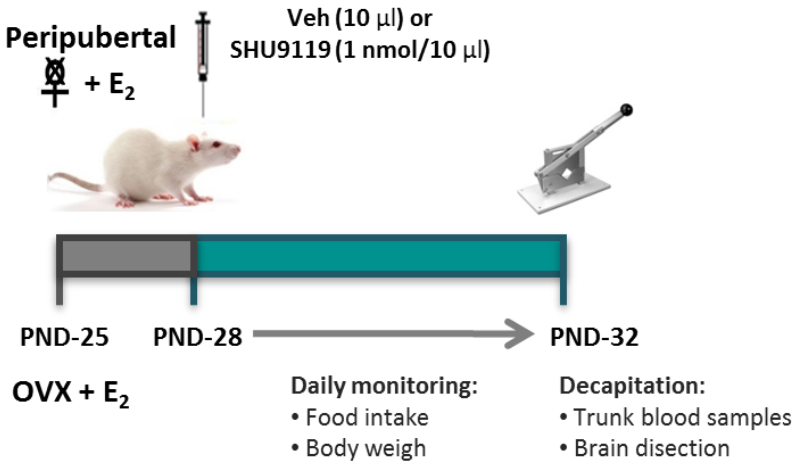

Figure 26. Schematic representation of the experimental protocol used in experiment 8.

In order to analyze the localization of POMC fibers and Kiss neurons, in **experiment 9**, pubertal female rats were injected with an effective dose of anaesthetic (pentobarbital) and intracardially perfused with saline solution (0.9% NaCl) followed by 4% PFA in PBS (pH 7.4). Brains were removed and post-fixed for immunofluorescence analyses (see section 3.4.3.2.).

3.5.2. CHARACTERIZATION OF THE PUTATIVE ROLES OF miRNAs IN THE CONTROL OF PUBERTY ONSET

Previous data from GWAS and functional genomic studies suggest the potential involvement of miRNA regulatory pathways in general and of Lin28/let-7 system in particular, in the central control of developmental events that lead to pubertal activation. However, in spite of these recent studies, relatively little is known about the function of Lin28/let-7 system in the pubertal maturation. In order to extend our knowledge about the participation of Lin28/let-7 system in the control of puberty, a series of experimental designs were carried out. The let-7a and let-7b were analysed as two representative miRNAs of the let-7 family belonging to different clusters [348]. In addition, c-Myc, miR-145 [375, 385, 386], as well as miR-9 and miR-132, miRNAs predicted as potential regulators of the Lin28/let-7 system by the use of bioinformatic algorithms in this Thesis, were also studied.

3.5.2.1. CHARACTERIZATION OF THE TISSUE DISTRIBUTION AND DIFFERENTIAL EXPRESSION OF *LIN28* AND *LIN28B* GENES

In **experiment 10**, the expression profiles of *Lin28* and *Lin28b* mRNAs was explored in a broad panel of tissues from adult rats (>75-days-old, n=3) and mice (>2-month-old, n=3). Different tissues were collected immediately after decapitation of animals. On the basis of the expression results of this experiment, the following studies were conducted in the hypothalamus and testes of rats and testes of mice.

3.5.2.2. CHARACTERIZATION OF THE PROFILE OF EXPRESSION OF *LIN28/LET-7* SYSTEM AND RELATED miRNAs IN HYPOTHALAMUS AND TESTES IN RATS

3.5.2.2.1. ANALYSIS OF THE PROFILES OF HYPOTHALAMIC AND TESTICULAR EXPRESSION OF *LIN28/LET-7* AND RELATED miRNAs DURING POSTNATAL MATURATION

In **experiment 11**, the expression profiles of *Lin28*, *Lin28b* and *c-Myc* mRNAs, as well as *let-7a*, *let-7b*, *mir-145*, *mir-132*, and *mir-9* miRNAs were determined in testes and hypothalamus, both males and females, in rats at different age during postnatal maturation (n=7-9/group of age). The ages of studies were selected according to the classification proposed by Ojeda et al. [387]: neonatal (PND-1), late neonatal (PND-7), infantile (PND-15), juvenile (PND-24 in females; PND-30 in males), early pubertal (PND-30 in females; PND-38 in males), pubertal (PND-35 in females; PND-45 in males), and adult (>PND-75) ages. Pubertal ages were determined by the observation of external index of puberty onset, VO in females and balano-preputial separation (BPS) in males.

In **experiment 12**, neonatal (PND-1) and pubertal male (PND-45) and female (PND-35) rats were killed by decapitation and cortex and hypothalamus were collected. In these samples, Lin28b expression was analyzed.

In addition, in **experiment 13** neonatal, infantile, juvenile and pubertal male and female rats (n=7-9) were euthanized by decapitation and hypothalami were obtained. *Lin28*, *Lin28b*, *let-7a*, and *let-7b* expression were analyzed in two main areas related with reproductive function, in the POA region and in the MBH.

3.5.2.2.2. ANALYSIS OF CHANGES IN THE PROFILES OF HYPOTHALAMIC AND TESTICULAR EXPRESSION OF LIN28/LET-7 AND RELATED miRNAs IN MODELS OF PERTURBED PUBERTY

In order to provide further evidence for the putative role of Lin28/let-7 system in the maturational program leading to puberty, the next set of experiments were carried out in various preclinical models of disturbed puberty.

It is known that alterations of the sex steroid milieu during the critical neonatal period of sexual differentiation disrupt pubertal maturation and gonadotropic function later in the life [388]. In this line, in **experiment 14**, male and female rats (n=10 per group) received at PND-1 subcutaneous (sc) injection of EB (100 µg/rat in females; 500

µg/rat in males) or vehicle (olive oil). An additional group of females (n=10) was sc injected on PND-1 with TP (1.25 mg/rat) (**Figure 27**). In order to verify the perturbed pubertal maturation in these animals, hormonal and phenotypic indices of puberty were studied. Trunk blood samples, testes and hypothalamus, both male and female, were obtained from peripubertal (PND-45 in males; PND-35 in females) animals. Tissue samples were stored at -80°C until further processing.

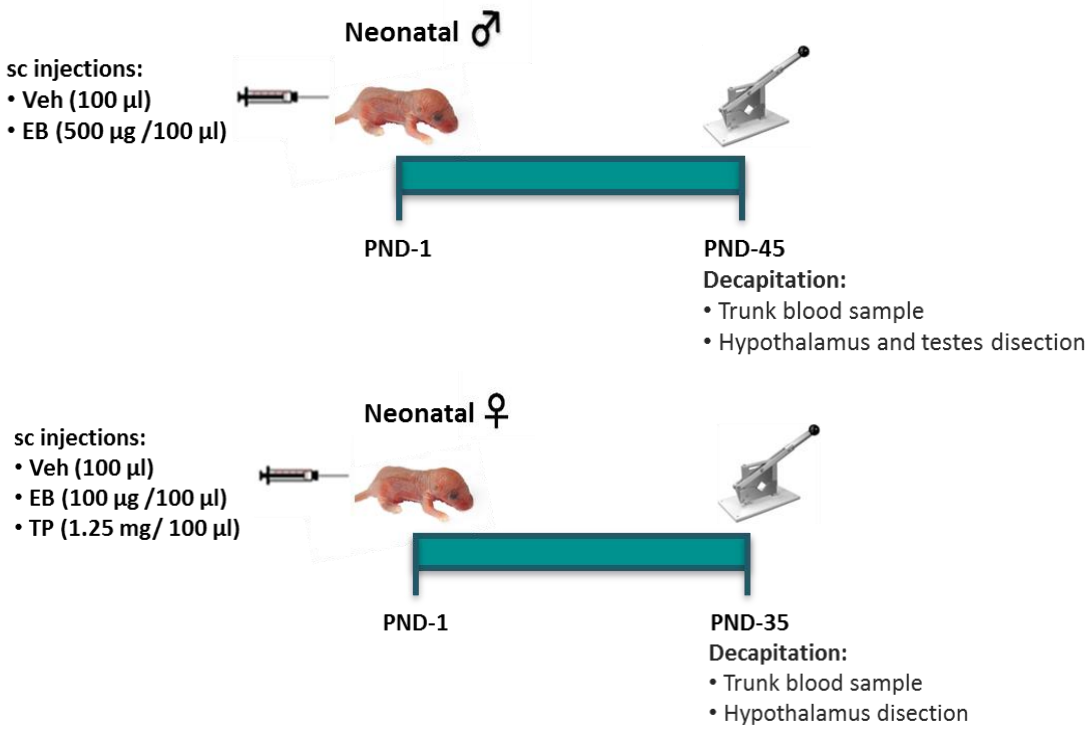

Figure 27. Schematic representation of the experimental protocol used in experiment 14.

On the basis of previous evidence showing that either changes in melatonin levels or photoperiod/day length modify the normal timing of puberty [389, 390], in **experiment 15**, photoperiodic manipulation was used as another model of perturbed puberty. In this respect, male and female rats were submitted to constant darkness (CD), between PND-10 to -15. , Male and female rats (n=8–12) were euthanized and testes and hypothalamus, were collected at PND-15 (immediately after completion of CD) and at puberty (PND-35 in females; PND-45 in males) (**Figure 28**). Pubertal maturation was monitored by detecting VO in females and BPS in males. Groups of rats reared in standard photoperiod conditions (14-h light/10-h dark) served as controls.

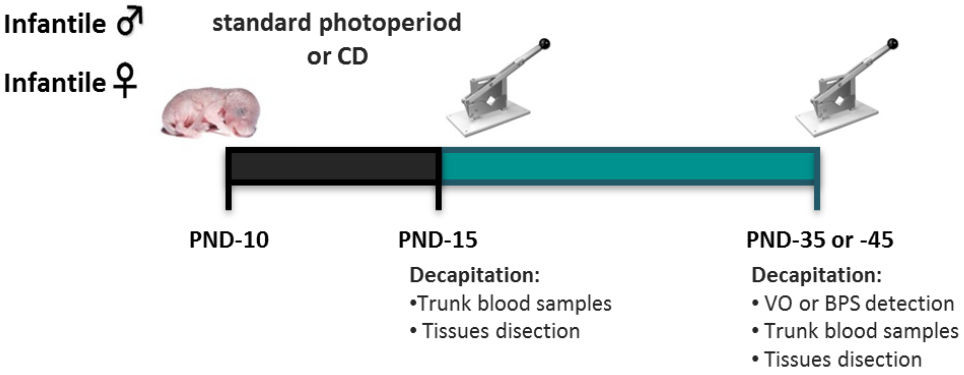

Figure 28. Schematic representation of the experimental protocol used in experiment 15.

In **experiment 16**, the impact of undernourishing during lactation on hypothalamic and testicular expression of the *Lin28/let-7* system was explored in male and female rats bred in large litters (20 pups/litter; UN) [274]. This model has been proposed to mimic subnutrition conditions during the last trimester of human gestation [391]. After weaning, rats were fed *ad libitum*. Subsets of rats were killed at representative ages of postnatal maturation, PND-5, -15, and -35 (in females) or -45 (in males) and hypothalamic and testicular samples were collected (**Figure 29**). Animals bred in normal litters (12 pups/litter) were used as model of normonutrition (NN). Phenotypic parameters such as BW, testicular weight (TW), BPS and VO were studied.

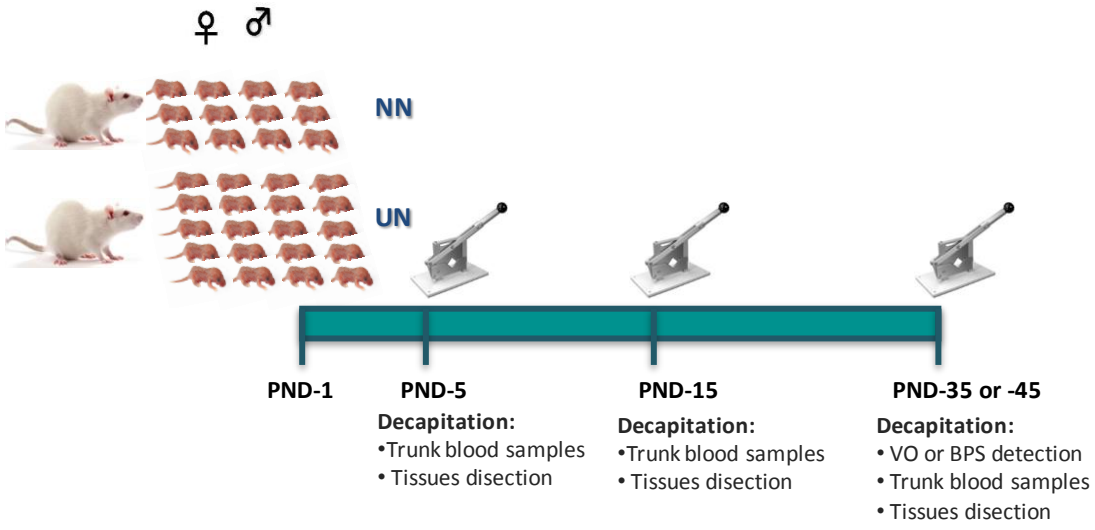

Figure 29. Schematic representation of experimental protocol used in experiment 16.

In **experiment 17**, another form of metabolic distress, involving persistent caloric restriction after weaning was used in order to discriminate between early (large

litter model) and late metabolic insults. Male and female rats were submitted to a 30% reduction in their daily food intake from PND-23 to puberty. Females and males were euthanized at about the time of puberty (PND-35 and PND- 45, respectively) and the hypothalami was removed (**Figure 30**). Rats fed *ad libitum* were used as control group.

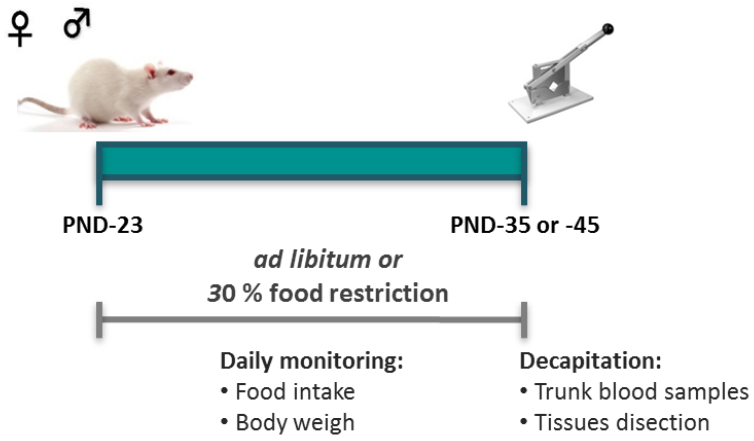

Figure 30. Schematic representation of experimental protocol used in experiment 17.

3.5.2.2.3. ANALYSIS OF CHANGES IN TESTICULAR EXPRESSION OF LIN28/LET-7 SYSTEM AND RELATED miRNAs IN MODELS OF DISTURBED NEUROENDOCRINE AXIS

In **experiment 18**, regulation by gonadotropins of testicular *Lin28* and *let-7* expression were explored using HPX male rats as experimental model. Groups of long-term HPX adult rats (4 weeks after HPX) received for 7 days a daily ip injection of vehicle (0,9% NaCl), hCG (50 IU/24 h, n=8), FSH (12.5 IU/24 h, n=7) or a combination of both (n=8) (**Figure 31**). At the end of the treatments, animals were killed by decapitation and testes were removed. Groups of intact animals injected with vehicle (100 µl) served as controls. After the surgery, rats HPX were provided with a bottle of isotonic saline (0.9%).

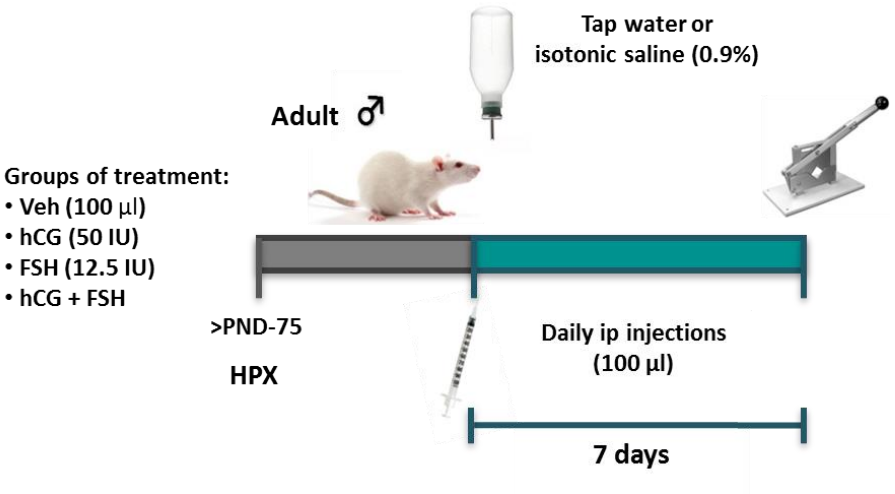

Figure 31. Schematic representation of the experimental protocol used in experiment 18.

3.5.2.3. CHARACTERIZATION OF THE TESTICULAR EXPRESSION OF LIN28/LET-7 SYSTEM IN MICE

Based on the abundant mRNA expression of both Lin28 and Lin28b in the testis (see experiment 10), the following mice studies were conducted in this tissue. Firstly, in addition to RNA data, in **experiment 19,** the expression of Lin28 and Lin28b peptides in testes from adult mice was studied. Testes form adult (2-month-old) mice from the C57B/6 strain (n=4) were dissected and fixed overnight in Bouin solution for IHC analyses. The results obtained lead to analyze the expression pattern of Lin28/let-7 system in testes during postnatal maturation and in a mouse model of hypogonadism.

3.5.2.3.1. ANALYSIS OF THE EXPRESSION OF LIN28/LET-7 SYSTEM DURING POSTNATAL TESTICULAR DEVELOPMENT

In **experiment 20** the expression profiles of Lin28, Lin28b as well as let-7a and let-7b miRNAs were determined in testicular samples from mice at three representative ages of postnatal testicular maturation: neonatal (PND-1), infantile (PND-15), and pubertal/early adult (PND-45) stages (n=6–7 animals per age-point). Testes were obtained following decapitation of animals and stored at -80°C until use for RNA/miRNA analyses.

3.5.2.3.2. ANALYSIS OF TESTICULAR EXPRESSION OF LIN28 AND LIN28B IN A MOUSE MODEL OF HYPOGONADOTROPIC HYPOGONADISM

In **experiment 21**, the testicular expression of Lin28/let-7 system was analyzed in Gpr54 KO mice, a model of congenital hypogonadism (see experiment 7). This mouse line displays markedly decreased circulating LH and FSH levels, as well as severely reduced testis weights in adulthood [65]. WT animals, harboring two alleles for Gpr54, served as controls. Adult (>2-month-old) WT and Gpr54KO mice were sacrificed and testicular samples were removed for IHC and mRNA studies of Lin28 and Lin28b. In addition, the relative levels of *let-7a* and *let-7b* miRNAs were also analyzed.

In order to provide an insight into the hormonal regulation of the testicular expression patterns of Lin28 and Lin28b in Gpr54 null mice, in **experiment 22**, adult Gpr54 KO mice were subjected or not to standard protocols of gonadotropin priming. Groups of Gpr54 KO mice (n=4) received a daily ip injection of recombinant hCG (5 IU/100 µl) or FSH (3.75 IU/100 µl) for 7 days (**Figure 32**). Animals injected with vehicle served as controls. Testicular samples were removed and fixed overnight in Bouin solution for IHC analyses.

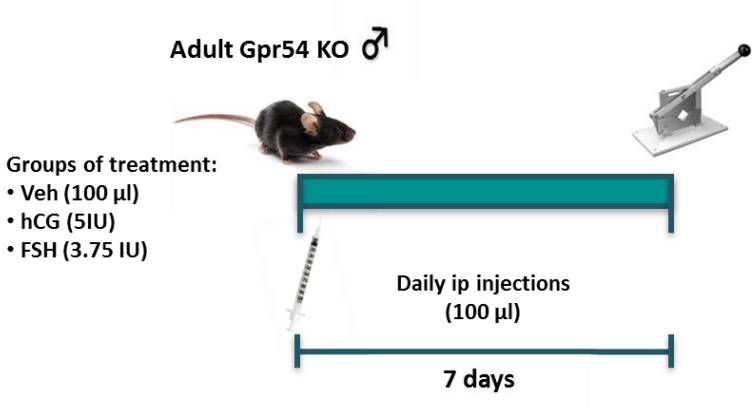

Figure 32. Schematic representation of the experimental protocol used in experiment 22.

3.6. BIOINFORMATIC ANALYSIS

Bioinformatic analyses used to search the potential regulatory miRNAs of Lin28 and Lin28b were carried out in collaboration with Dr. Susana Sangiao-Alvarellos (University of Santiago de Compostela) and were used four different algorithms: TargetScan [392]: http://www.targetscan.org/; miRanda [393]: http://www.

microrna.org/microrna/home.do; PicTar [394]: http://pictar.mdc-berlin.de/; and Diana Lab [395]: http://diana.cslab.ece.ntua.gr/microT/.

3.7. STATISTICAL ANALYSIS

Data of BW, absolute and relative organ weight, ISH and hormonal levels are expressed as the mean ± SEM (standard error of the mean). Both time-course data and integral LH levels of different experimental groups relative to the control groups were estimated as area under the curve (AUC) by the trapezoidal rule. Expression data were expressed as percentage of the control group in each experiment and presented as mean ± SEM. Hormonal determinations were conducted with a minimal total number of 7-12 determinations per group. RNA analyses were performed in duplicate from at least 6 independent samples per group. ISH assays were conducted with 5 animals per group. Results were analyzed using Student t test and ANOVA followed by Student-Newman-Keuls multiple range tests or post hoc Tukey test (Prism GraphPad 5.0 software; GraphPad Software Inc., La Jolla, CA). Significance level was set at $P \leq 0.05$ and different letters or asterisks indicate statistical significance.

RESULTS

4. RESULTS

4.1. CHARACTERIZATION OF THE ROLE OF CENTRAL MELANOCORTIN SIGNALING IN THE CONTROL OF THE GONADOTROPIC AXIS AT PUBERTY AND ITS POTENTIAL INTERACTION WITH LEPTIN AND KISSPEPTIN

With the aim to analyze the putative function of MC3/4-R signaling in the control of puberty onset and to explore their potential interaction with leptin and kisspeptins, three sets of experimental studies were performed.

4.1.1. EFFECTS OF CENTRAL ACTIVATION OF MC3/4-R SIGNALING ON LH SECRETION

In the first set of experiments, we studied the effect of acute activation of MC3-R and MC4-R on LH secretion, as surrogate marker of GnRH function.

Firstly, we examined the effect of central administration of an effective dose of MT II (1 nmol/rat), a MC3/4-R agonist, on peripubertal male (PND-43) and female (PND-29) rats fed *ad libitum* or fasted for 48h. We observed that in males fed *ad libitum*, MT II evoked a significant increase in LH secretion at 15 min with persistently elevated LH levels at least up to 30 min after injection. In animals subjected to fasting for 48h basal LH levels were significantly decreased. In fasted males, MT II induced a significant increase in serum LH levels only at 30 min (**Figure 33,** upper panels). In the case of females, we found that a single bolus of MT II elicited a rapid and robust response in terms of LH secretion that extended for at least 30 min, despite the unfavorable metabolic conditions (**Figure 33**, lower panels).

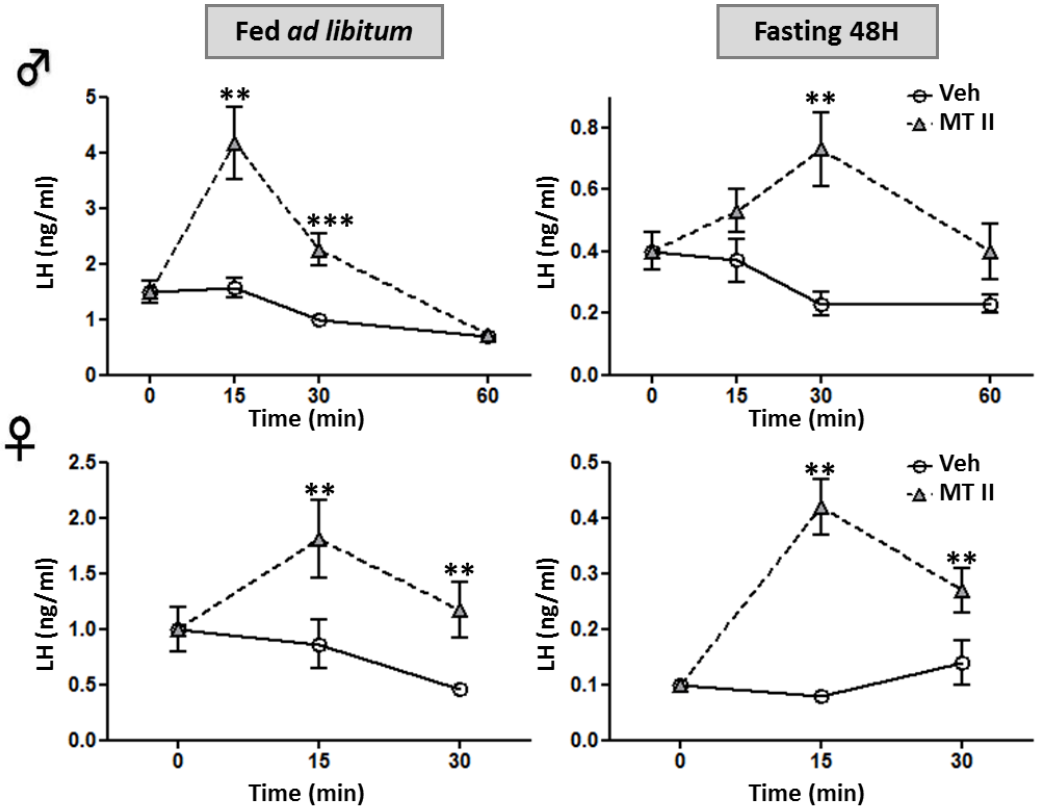

Figure 33. Serum LH levels (ng/ml) after central MC3/4-R activation. Time course for the effects of vehicle (0,9% NaCl solution, Veh) or MT II (1nmol/rat) icv administration to peripubertal male (*upper panels*) and female (*lower panels*) rats fed *ad libitum* or fasted for 48 h are shown. LH levels were assayed before (0 min) and at 15-, 30- and 60-min (only in males) after the icv injection. Group sizes: n=8-10. Data are presented as mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 vs. corresponding vehicle-injected group, followed by Student t test.

We also examined the LH response after same doses of MT II in infantile (PND-15) male and female rats. Our data indicated that, MC3/4-R activation failed to induce a significant increase in LH secretion 15 min after injection of MT II, either in males and females (**Table 4**).

	Males	Females
Vehicle	0,39 ± 0,07	0,69 ± 0,08
MT II	0,50 ± 0,10	0,85 ± 0,09

Table 4. Effects of acute administration of vehicle (0,9% NaCl solution) or MT II (1nmol/rat) on LH secretion (ng/ml) in 15 day-old male and female rats at 15 min after icv injection are shown. Group sizes: n=10. Data are presented as mean ± SEM, followed by Student t test.

Finally, we further examined whether the effects of MT II on LH secretion are mediated through MC3-R and/or MC4-R. To this end, peripubertal male rats received a single bolus of different doses (0.2-, 1- or 5-nmol) of a selective MC3-R or MC4-R agonists. We found that activation of both MC3-R and MC4-R produces significant changes in LH release in a dose-dependent manner. Thus, we detected that 0.2- and 1-nmol doses of the MC3-R agonist evoked a significant increase LH levels, at 15 min after the icv injection, while a high doses caused a significant decrease LH secretion at 60 min (**Figure 34A**). In contrast, while doses of 0.2- and 1-mnol of the MC4-R agonist did not cause changes on LH secretion, animals injected with 5 nmol displayed a significant increase in LH levels at 15 min after the icv injection, which remained elevated for at least 60 min (**Figure 34B**). Interestingly, the profile of LH increase was similar to that found after MT II administration (see **Figure 33A**).

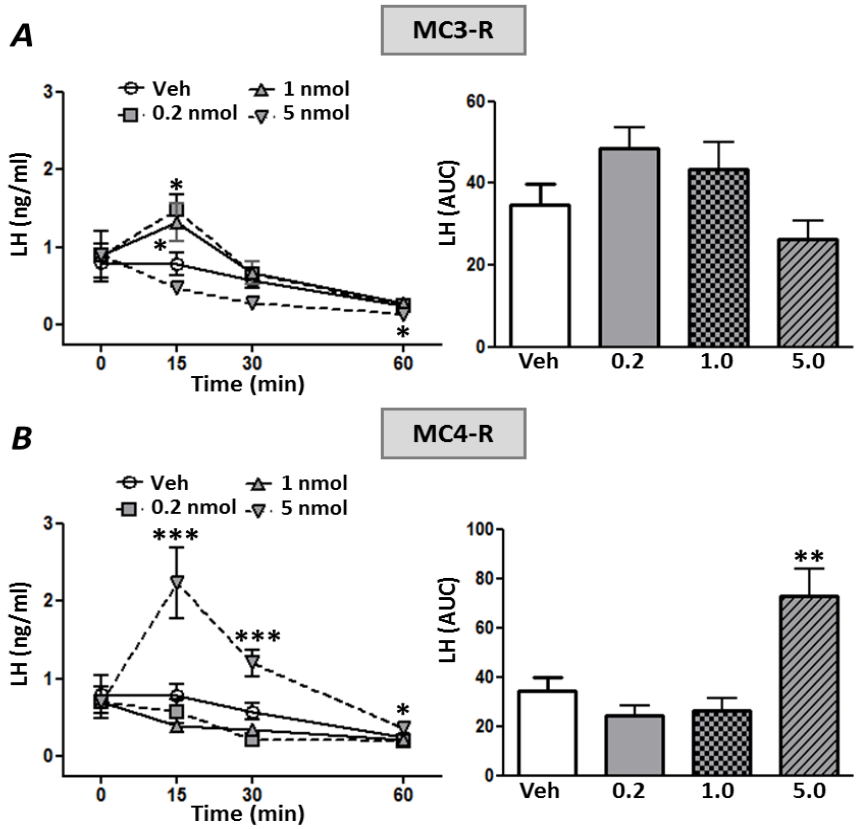

Figure 34. Secretory profiles of LH responses (ng/ml) to different doses of selective MC3-R or MC4-R agonists in peripubertal male rats. Animals were icv injected with vehicle (0,9% NaCl solution) or a single dose of 0,2-, 1- or 5-nmol of MC3-R (*A*) or MC4-R (*B*) agonists. LH levels were assayed before (0 min) and at 15-, 30- and 60-min after the icv injection. Both time-course data and integral LH levels, estimated as area under the curve (AUC) over the 60 min after injection by the trapezoidal rule, are shown. Group sizes: n=8-10. Data are presented as mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 vs. corresponding vehicle-injected group; ANOVA followed by Student-Newman-Keuls multiple range test.

4.1.2. EFFECTS OF CENTRAL INACTIVATION OF MC3/4-R SIGNALING DURING THE PUBERTAL TRANSITION

In the second set of experiments, we evaluated in female rats the effects of central chronic blockade of MC3/4-R during pubertal transition through administration of SHU9119 (1 nmol/rat). Initial experiments evidenced that repeated icv administration of SHU9119 caused an increase in daily food intake. Thus, in order to avoid the possible influence of changes in BW on puberty onset, SHU9119-treated animals were pair fed to control animals. As we expected, no differences in BW between both groups were observed at PND-35 (**Figure 35**, left upper panel). However, repeated injections of SHU9119 between PND-28 to PND-35 caused a marked delay on puberty onset. In fact, at PND-35 less than 13% of SHU9119-treated group showed complete VO versus 75% of vehicle-treated group (**Figure 35,** right upper panel). This effect was associated to a reduction in relative UW and OW at the end of experiment (**Figure 35**, left and middle lower panels). At this time, in the SHU9119 group, LH levels tended to be lower than in the vehicle-treated group. However, these differences were not statistically significant (**Figure 35**, right lower panel).

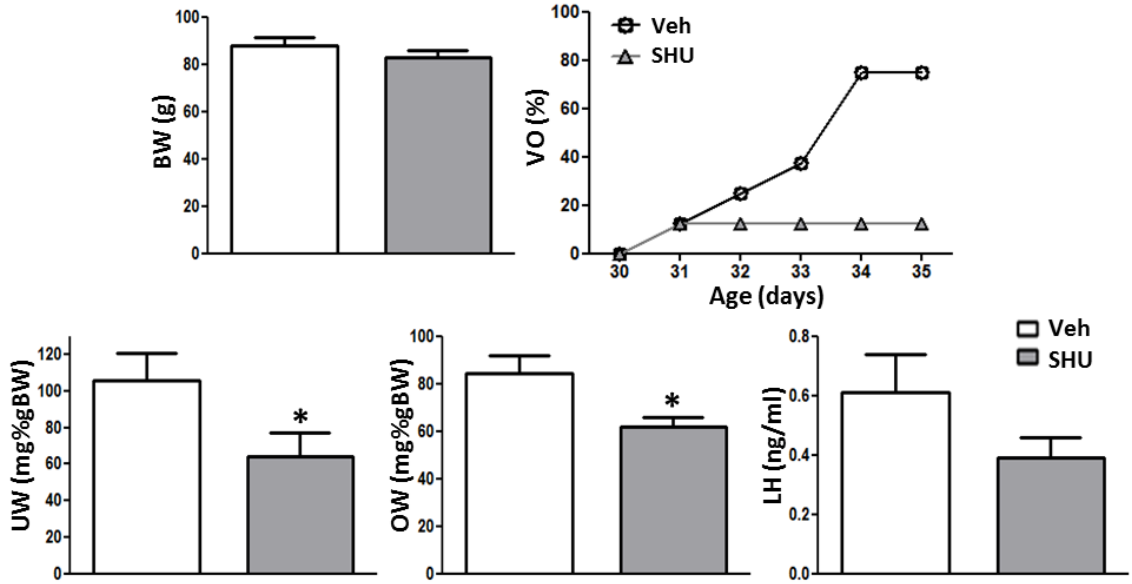

Figure 35. Effects of central inactivation of MC3/4-R signaling between PND-28 to -35 in female rats. BW (g), VO (%), relative UW and OW (mg%gBW) and serum LH levels (ng/ml) are presented from animals subjected to repeated icv administration of vehicle (0,9% NaCl solution) or SHU9119 (1 nmol/rat) for 7 days. Group sizes: n=9-11. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. vehicle-injected group, followed by Student t test.

Based on the above data, we further evaluated whether the permissive effect of leptin on puberty onset depends on proper MC3/4-R signaling, using female rats subjected to moderate food restriction (20%) from PND-23 (UN) as model. To this end, food restricted animals were subjected to repeated central injection of vehicle or, leptin alone or in combination with SHU9119 from PND-29 to -35. Females fed *ad libitum* (NN) and chronically treated with vehicle served as controls. As we expected, chronic subnutrition produced a significant reduction on BW, relative UW, delayed puberty onset and a decrease in relative OW and LH circulating levels (**Figure 36**). Repeated administration of leptin to UN animals was able to partially rescue the negative effect of the subnutrition on the age of puberty onset and completely reversed the decrease in relative UW (**Figure 36**). Interestingly, concomitant blockade of central MC3/4-R signaling prevented the rescue effect of leptin on the timing of VO without affect its on UW. Moreover, no significant differences in terminal serum LH levels were detected among any of the groups subjected to food restriction (**Figure 36**).

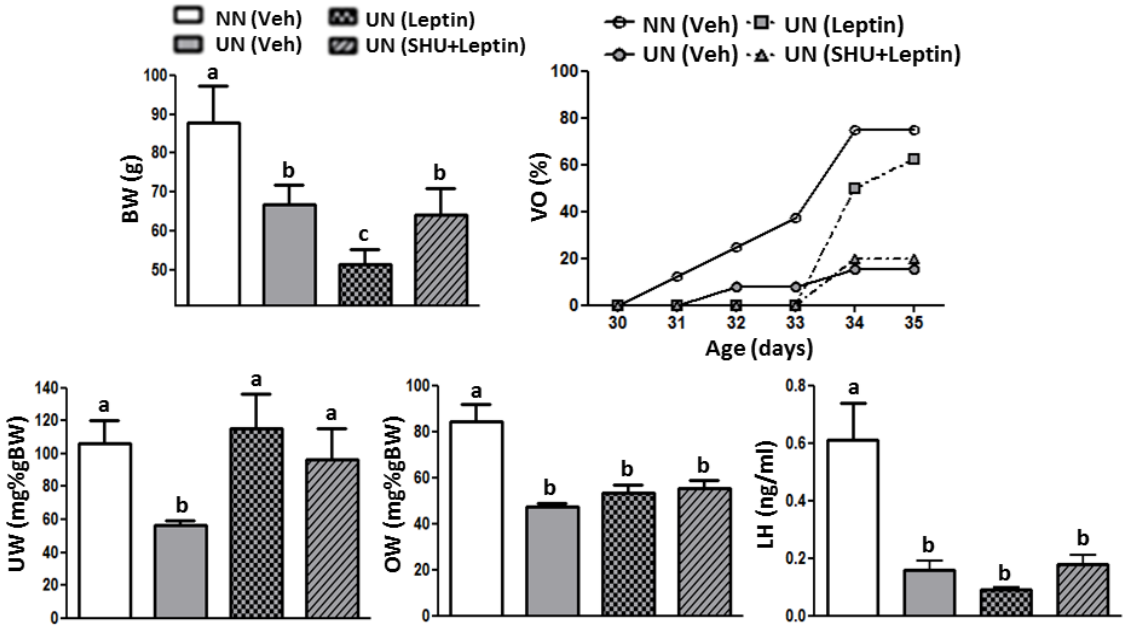

Figure 36. Central inactivation of MC3/4-R signaling prevents the permissive effect of leptin on puberty onset in female rats. BW, VO, relative UW, relative OW and terminal LH levels are presented from animals under moderate restriction (20%) in daily food intake (UN) and submitted to repeated icv administration of vehicle (0,9% NaCl solution) or leptin (3,1 µg/rat) alone or in combination with SHU9119 (1 nmol/rat). UN females group, as well as NN female rats, chronically treated with vehicle served as controls. Group sizes: n=9-11. Data are presented as mean ± SEM. Groups with different superscript letters are statistically different, $P \leq 0.05$; ANOVA followed by Student-Newman-Keuls multiple range tests.

4.1.3. CHARACTERIZATION OF THE POTENTIAL MC3/4-R AND KISSPEPTIN INTERACTIONS IN THE CONTROL OF PUBERTY ONSET

In the third set of studies, we evaluated whether MC3/4-R signaling is required for kisspeptin effects in the control of puberty onset or vice versa.

Firstly, we examined the effect of Kp-10 on LH release after blockade of central MC3/4-R signaling by SHU9119. Interestingly, we observed that central administration of two boluses of SHU9119 (1 nmol/rat), at 120- and 60-min before the analysis, caused a significant decrease of LH levels (**Figure 37A**). As we expected, SHU9119 administration completely blocked the effect of MT II on LH secretion at 15 min (**Figure 37B**). In contrast, melanocortin blockade by SHU9119 did not affect LH response to Kp-10 at 15- and 60-min post-injection (**Figure 37C**).

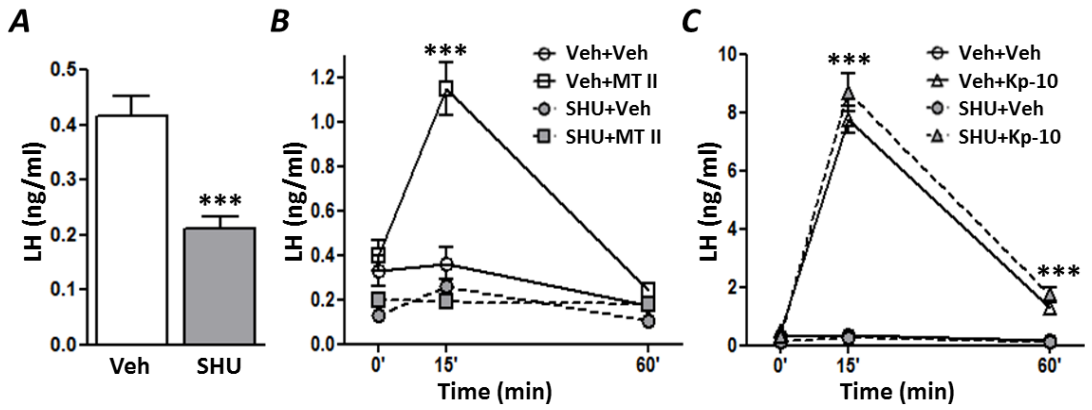

Figure 37. Secretory profiles of LH in response to MT II and Kp-10 after blockade of MC3/4-R in peripubertal male rats. (*A*) LH levels after the pretreatment, at 120- and 60-min before, with vehicle (0,9% NaCl solution) or SHU9119 (1 nmol/rat) are presented. Group sizes: n=26-30. Data are presented as mean ± SEM. ***, P ≤ 0.001 vs. corresponding veh-treated group, followed by Student t test. LH levels immediately before (0 min) and at 15- and 60-min after the last icv injection with Veh- or SHU-alone or co-administered with MT II (*B*) or Kp-10 (*C*) are shown. Group sizes: n=8-10. Data are presented as mean ± SEM. ***, P ≤ 0.001 vs. corresponding veh-injected group with the same pre-treatment; ANOVA followed by Student-Newman-Keuls multiple range test.

Secondly, we also evaluated the effect of MT II on LH secretion in the absence of kisspeptin signaling using a Gpr54 KO mice model, which had been subjected to a protocol to GnRH priming previously. As we expected, WT animals showed a significant increase on LH secretion 15 min after icv administration of a single bolus of MT II (1 nmol/mouse). However, LH response to MT II was markedly attenuated, although not totally eliminated, in Gpr54-deficient mice (**Figure 38**).

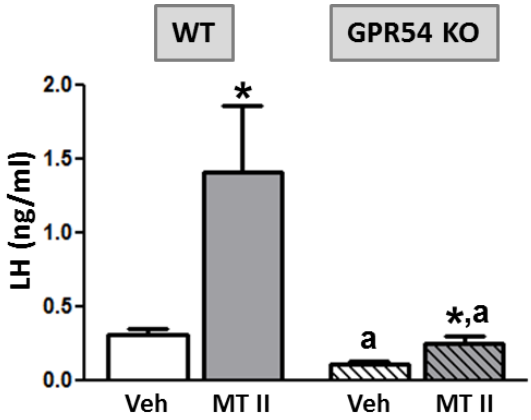

Figure 38. Effects on LH secretion of central activation of MC3/4-R signaling in the absence of kisspeptin signaling. Adult WT and Gpr54 KO male mice were treated with vehicle (0,9% NaCl solution) or MT II (1nmol/mouse) and LH levels (ng/ml) were assayed at 15 min after the icv injection. Group sizes: n=6-9. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. corresponding vehicle-injected group; a, P ≤ 0.01 vs. corresponding WT values, followed by Student t test.

In the next experiment, we studied in female rat the effect of the chronic blockade of endogenous MC3/4-R signaling between PND-28 to -32 on hypothalamic *Kiss 1* mRNA expression. To avoid the potential confounding factor of changes in circulating estrogen on *Kiss1* expression, female rats were subjected to bilateral OVX and supplemented with moderate doses of E_2 to mimic physiological levels. In addition, in order to avoid the possible influence of changes of BW on puberty onset, SHU9119-treated animals were pair fed to control animals. In keeping with previous studies, two main hypothalamic populations of *Kiss1*-expressing neurons were located in the ARC and AVPV. Our ISH analyses detected that chronic administration of SHU9119 induced a significant suppression (45%) of *Kiss1* mRNA levels in the ARC of peripubertal females, whereas *Kiss1* expression in AVPV was not affected by SHU9119 treatment (**Figure 39**).

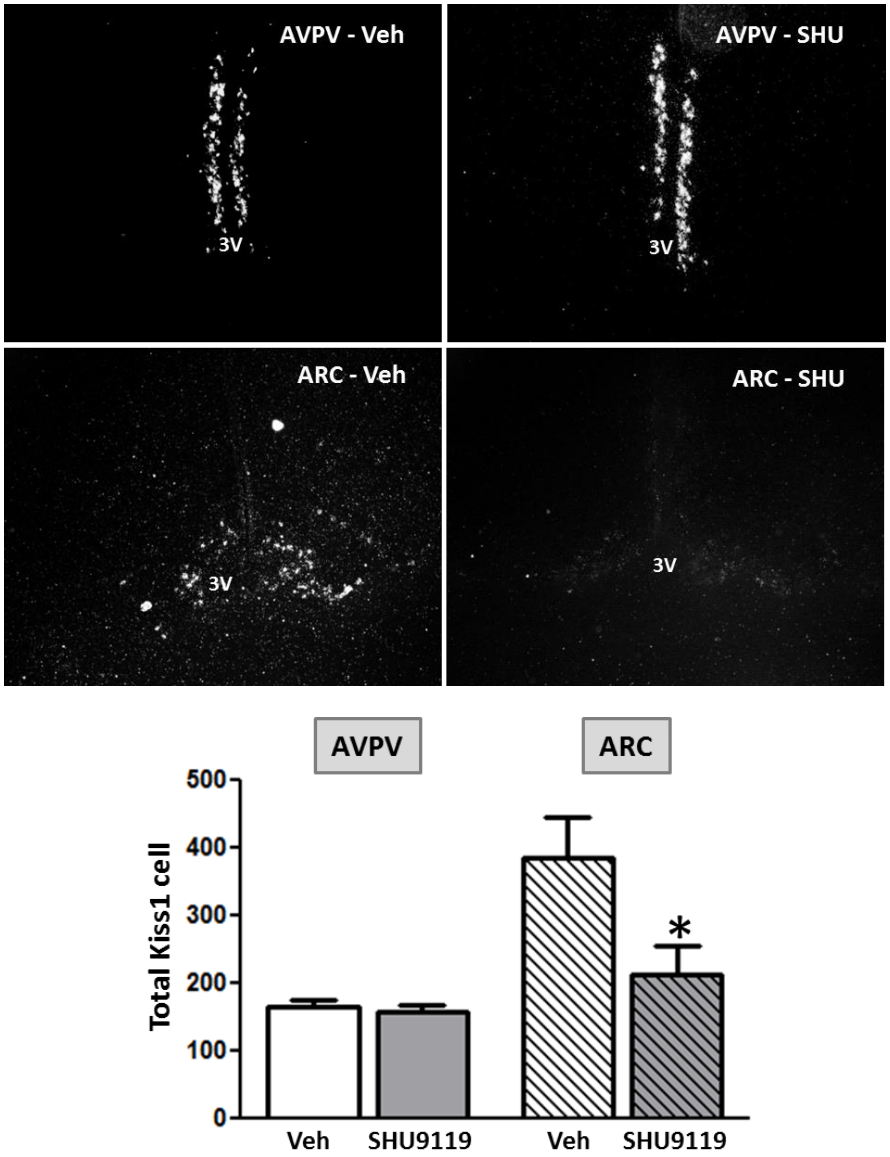

Figure 39. Hypothalamic expression of *Kiss1* after central MC3/4-R blockade. In the *upper panels*, representative *in situ* hybridization photomicrographs showing *Kiss1* mRNA expressing-neurons in the AVPV and ARC of peripubertal OVX+E2 female rats after repeated icv administration of vehicle (0,9% NaCl solution) or SHU9119 (1nmol/rat) between PND-28 to -32. In the *lower panel*, the total number of *Kiss1*-expressing cells (defined as grain in clusters) in the AVPV and ARC subjected to analysis are presented. Group sizes: n=5. *, P ≤ 0.05 vs. corresponding vehicle-injected group followed by Student t test.

Finally, we further evaluated the possible physical contacts between POMC and kisspeptin neurons in the ARC of pubertal female rats by neuroanatomical studies. We found that α-MSH-IR fibers were located in close apposition to kisspeptin cell bodies in the ARC.

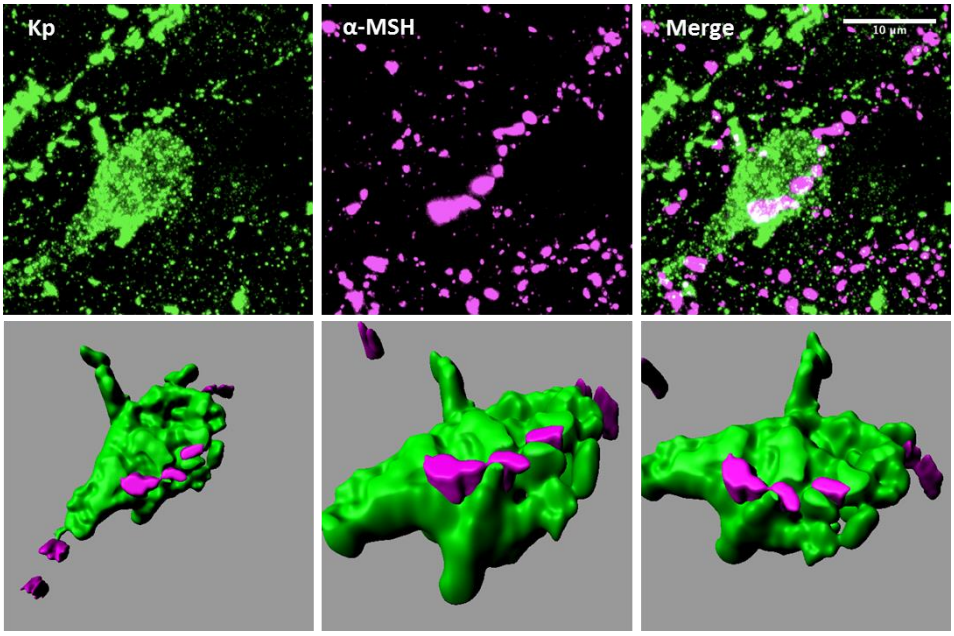

Figure 40. Illustration of localization of α-MSH-IR fibers and kisspeptin cell bodies in the ARC of pubertal female rats. Immunofluorescence using antibodies against Kp (*green*) and α-MSH (*magenta*) is presented.

4.2. CHARACTERIZATION OF THE PATTERNS OF EXPRESSION OF LIN28/LET-7 SYSTEM AND RELATED FACTORS DURING PUBERTAL MATURATION

In order to analyse the putative role of Lin28/let-7 system in the control of puberty, a series of expression analyses were carried out. The *let-7a* and *let-7b*, as two representative members of the let-7 family, *c-Myc*, *mir-145*, as well as *mir-9* and *mir-132*, miRNAs predicted as potential regulators of the Lin28/let-7 system by the use of bioinformatic algorithms, were studied (see **Figure 17**).

4.2.1. TISSUE DISTRIBUTION AND DIFFERENTIAL EXPRESSION OF *LIN28* AND *LIN28b* GENES

In a first approach, we explored the expression profiles of *Lin28* and *Lin28b* mRNAs in different tissues from adult rats and mice (**Figure 41**).

In the case of adult male and female rats, our analyses showed that *Lin28* mRNA is prominently expressed in placenta, testes, ovary and pituitary, with modest expression in the hypothalamus and weak expression in adipose tissue, heart, lung, liver and stomach. No expression was detected in kidney, spleen and skeletal muscle

(**Figure 41A**). Likewise, we detected that *Lin28b* mRNA is abundantly expressed in testes and placenta. In addition, remarkable expression was observed in the hypothalamus and modest but detectable levels of *Lin28b* mRNA were found in skeletal and cardiac muscle. The pituitary, pancreas and stomach showed very low or undetectable *Lin28b* mRNA levels (**Figure 41A**).

Screening of adult male and female mouse tissues for expression of *Lin28* and *Lin28b* genes revealed that *Lin28* is only prominently expressed in the testis, whereas weak to null expression was detected in a panel of tissues including the pituitary, ovary (both with low but detectable mRNA levels), skeletal muscle, liver, brown adipose tissue, lung, spleen, heart and hypothalamus (data not shown). Similarly, we observed that *Lin28b* transcript was abundantly expressed in adult mouse testes and hypothalamus, whereas modest expression was observed at the pituitary and skeletal muscle and weak expression in the ovary. No expression of *Lin28b* was detected in liver, brown adipose tissue, lung, spleen and heart (data not shown). **Figure 41B** shows the expression patterns of *Lin28* and *Lin28b* transcripts in selected tissues, including testis, hypothalamus, liver and muscle.

Based on these expression analyses, showing the lack of expression of *Lin28b* in rat ovary and the weak expression of *Lin28* and *Lin28b* in mouse ovary, we carried out our studies in the hypothalamus and the testis of rats and testis of mice.

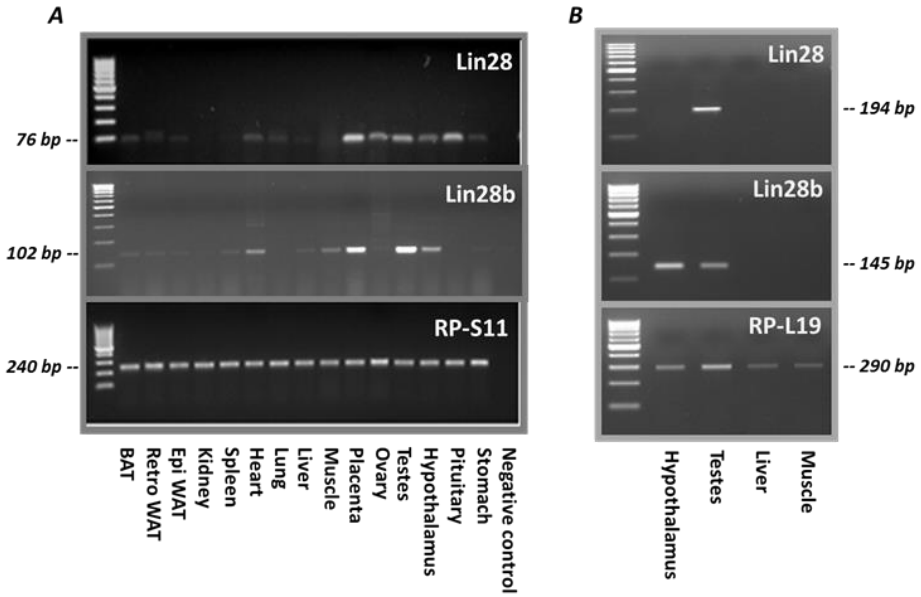

Figure 41. Representative PCR amplification patterns of expression of *Lin28* and *Lin28b* genes in different tissues from adult, male and female, rats (A) and mice (B).

4.2.2. BIOINFORMATIC ANALYSES

It is well established that the *Lin28/let-7* system is subjected to a dual negative feedback regulatory loop. *Lin28* and *Lin28b* repress the synthesis of mature *let-7* miRNAs, which in turn post-transcriptionally suppress *Lin28/Lin28b* expression. Besides the known regulators of this system, *c-Myc* (transcriptional activator of *Lin28*) and *mir-145* (suppressor of *c-Myc*), we searched for additional miRNAs and genes that may be involved in *Lin28/let-7* modulation, using the four bioinformatic algorithms enumerated in Materials and Methods. A summary of the most relevant results obtained from these analyses are shown in **Table 5**.

miRNA/GENE	CONSERVED SITES				POORLY CONSERVED SITES			
	Total	8mer	7mer-m8	7mer-1A	Total	8mer	7mer-m8	7mer-1A
POSSIBLE REGULATORS OF *LIN28B*								
let-7	4	1	3	0	0	0	0	0
mir-208	0	0	0	0	3	0	1	2
mir-144	0	0	0	0	4	1	2	1
mir-9	2	0	2	0	1	0	1	0
mir-499	1	1	0	0	1	0	0	1
mir-132	1	0	0	0	0	0	0	0
POSSIBLE TARGET GENES OF *LET-7* FAMILY								
HMGA2	6	1	3	2	1	0	0	1
LIN28B	4	1	3	0	1	0	0	1
IGF2BP1	3	3	0	0	1	0	1	0
LIN28	2	2	0	0	0	0	0	0
MAP3K1	1	1	0	0	0	0	0	0
ZNF512B	3	0	3	0	0	0	0	0

Table 5. Potential miRNA regulators of *Lin28b* (using TargetScan) and target genes of l*et-7* family in rats. Only the targets identified simultaneously by at least three of the four bioinformatics algorithms, TargetScan, miRanda, PicTar and Diana Lab, used in this Thesis are shown. HMGA2: high mobility group AT-hook 2; IGF2BP1: insulin-like growth factor 2 mRNA binding protein 1; MAP3K1: mitogen-activated protein kinase kinase kinase 1; ZNF512B: zinc finger protein 512B.

Considering the biological profiles of these potential candidates to regulate *Lin28b*, we included *mir-132* in our testicular analyses and both *mir-9* and *mir-132* in our hypothalamic analyses. This selection was further supported by the fact that both are highly conserved across most vertebrates and are highly expressed in the rat

hypothalamus, as compared with other miRNAs, such as *mir-208*, *mir-144* and *mir-499* which are not expressed or at low levels expressed in this tissue. In addition, we also used bioinformatic tools to identify potential target genes of the *let-7* family (**Table 5**).

4.2.3. CHARACTERIZATION OF HYPOTHALAMIC EXPRESSION PROFILES OF THE *c-MYC/LIN28/LET-7* SYSTEM AND RELATED miRNAs IN RATS

4.2.3.1. ANALYSIS OF EXPRESSION OF *c-MYC/LIN28/LET-7* AND RELATED miRNAs IN THE HYPOTHALAMUS DURING POSTNATAL MATURATION

First, the expression profiles of *c-Myc/Lin28/let-7* were analyzed in male and female rats during postnatal maturation in the hypothalamus, cortex and specific hypothalamic areas related with reproductive function, POA and MBH.

In the hypothalamus of both sexes, we observed that *Lin28* and *Lin28b* mRNA showed similar profiles. Thus, both genes displayed very high expression levels during the neonatal period. In the case of *Lin28*, expression levels declined precipitously thereafter, remaining at low, almost imperceptible, values from infantile period until adulthood (**Figure 42 and 43**, upper panels). Regarding *Lin28b* mRNA, its abundance declined less pronouncedly, by about 50% during the infantile period, remaining at these values until early puberty. At puberty, the values declined further remaining low in adulthood (**Figure 42 and 43**, upper panels). In addition, it was detected that expression levels of *c-Myc* mRNA in rat hypothalamus paralleled those of *Lin28b* in both male and female rats (**Figure 42 and 43**).

In clear contrast, *let-7a* and *let-7b*, *mir-132*, and *mir-145* miRNA levels displayed opposite profiles, with minimal neonatal expression levels and progressive increase along postnatal maturation in both male and female rats. However, these trends were not detected for *mir-9*, whose hypothalamic levels showed the highest expression during the neonatal (in males) and late neonatal (in female) periods and declined gradually during postnatal maturation (**Figure 42 and 43**).

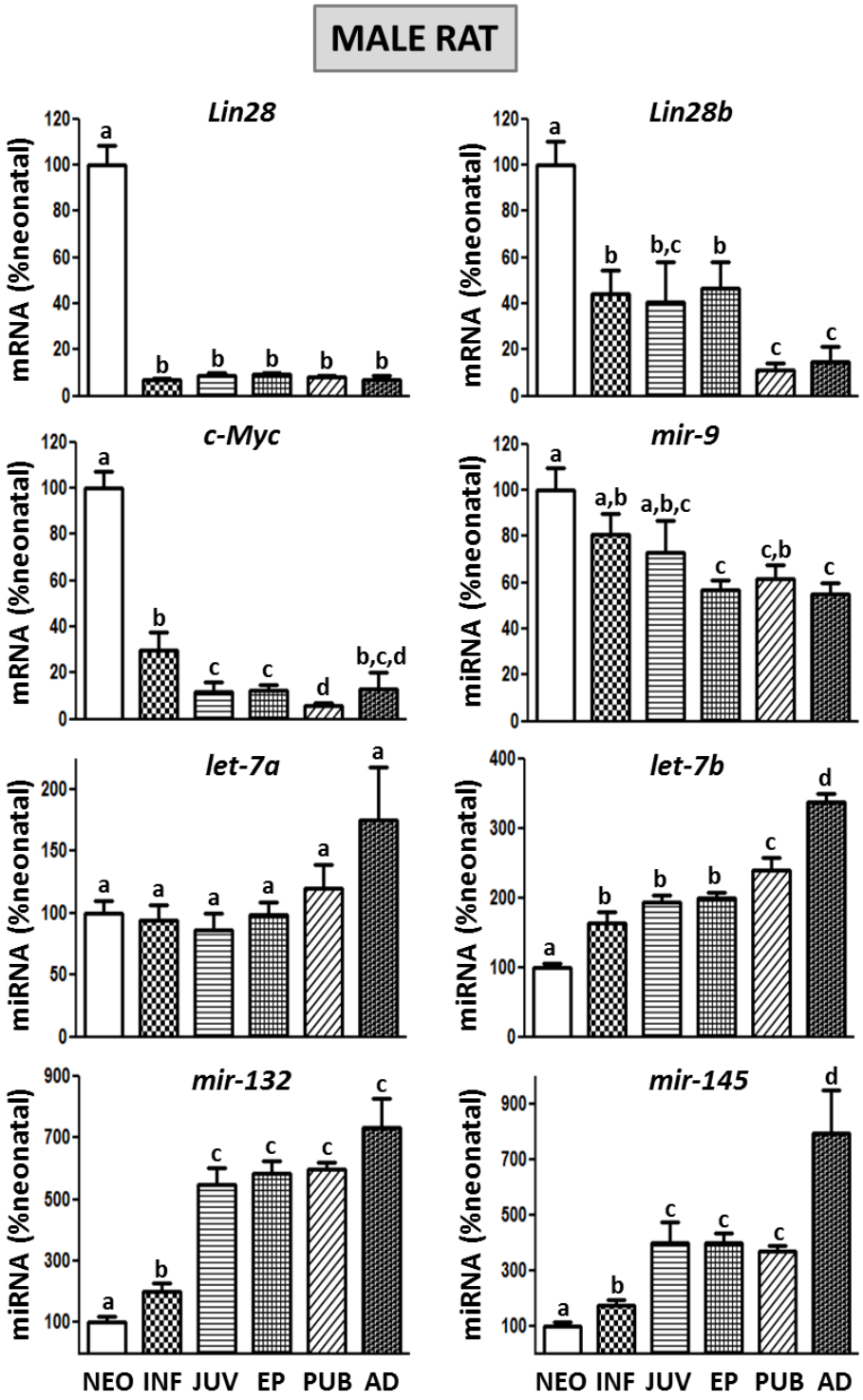

Figure 42. Expression profiles of the components of the *Lin28/let-7* system and related factors in the whole hypothalamus of male rats during postnatal maturation. Expression analyses of *Lin28*, *Lin28b* and *c-Myc* mRNAs, as well as *let-7a, let-7b*, *mir-132, mir-145* and *mir-9* miRNAs. Group sizes: n=8-10. Data are presented as mean ± SEM. Different superscript letters above bars indicate statistical differences, P ≤ 0.05; ANOVA followed by post hoc Tukey test. NEO: neonatal; INF: infantile; JUV: juvenile; EP: early puberty; PUB: pubertal; AD: adult.

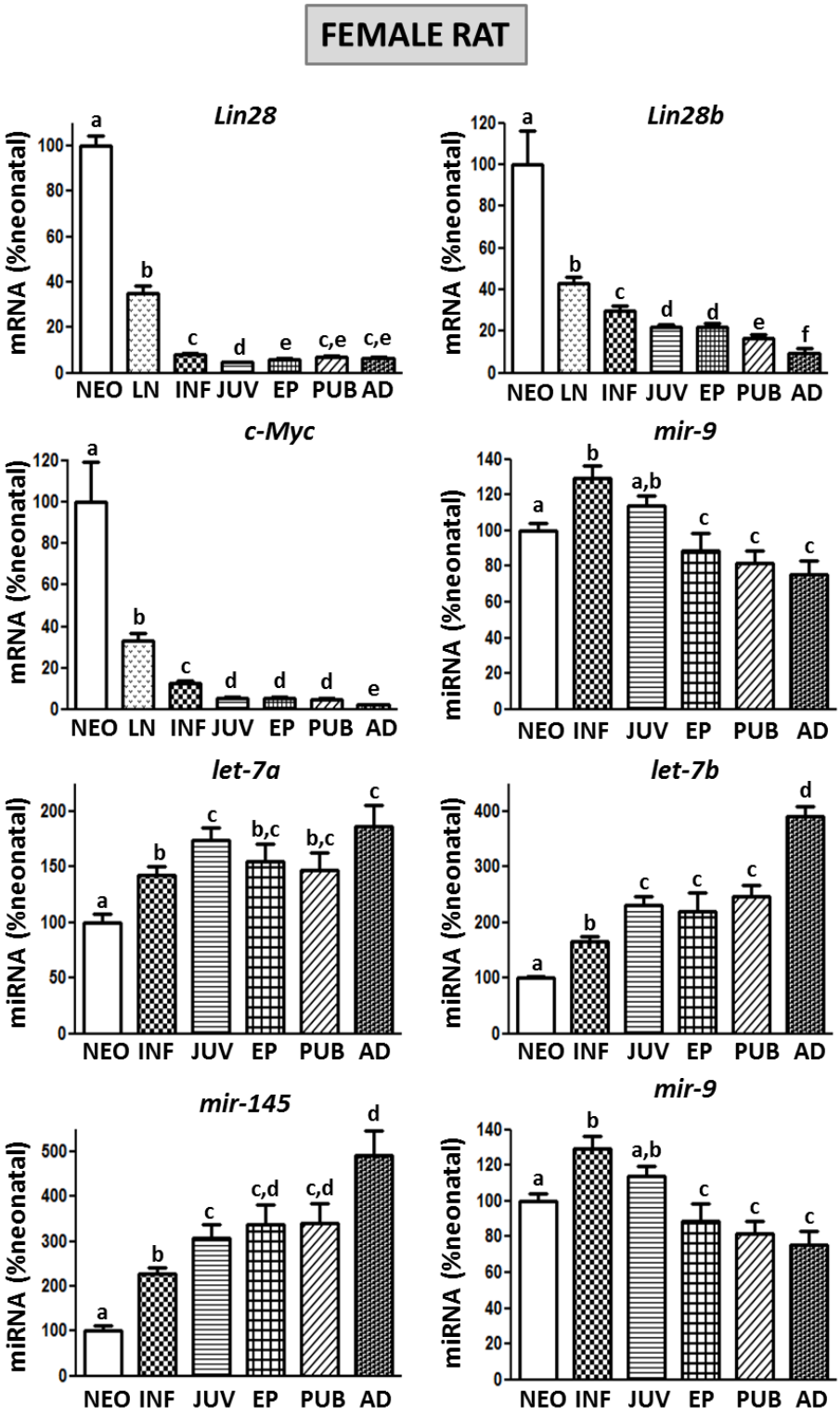

Figure 43. Expression profiles of the components of the *Lin28/let-7* system and related factors in the whole hypothalamus of female rats during postnatal maturation. Expression analyses of *Lin28*, *Lin28b* and *c-Myc* mRNAs, as well as *let-7a, let-7b*, *mir-132, mir-145* and *mir-9* miRNAs. Group sizes: n=8-10. Data are presented as mean ± SEM. Different superscript letters above bars indicate statistical differences, $P \leq 0.05$; ANOVA followed by post hoc Tukey test. NEO: neonatal; LN: late neonatal; INF: infantile; JUV: juvenile; EP: early puberty; PUB: pubertal; AD: adult.

When *Lin28b* expression was analyzed in frontal cortex and hypothalamus from male and female rats during postnatal maturation, opposite patterns were found in both sexes between these two localizations. Thus in male rats, we detected a significant increase of *Lin28b* expression levels between the neonatal and pubertal periods in the frontal cortex, whereas a decrease of expression was observed in the hypothalamus (**Figure 44A**). This trend was observed also in females, but the increase of *Lin28b* expression levels in frontal cortex was not significant (**Figure 44B**).

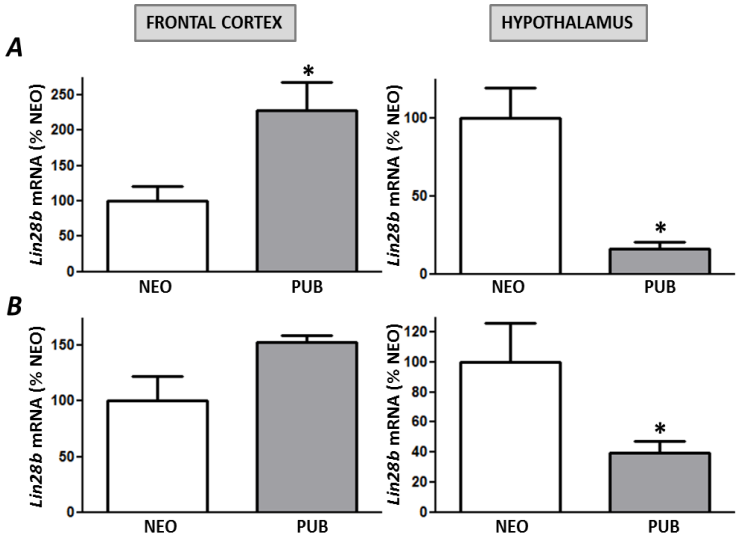

Figure 44. *Lin28b* expression in the frontal cortex and hypothalamus from neonatal and pubertal male (*A*) and female (*B*) rats. Group sizes: n=4-5. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. corresponding neonatal-group, followed by Student t test. NEO: neonatal; PUB: pubertal.

Expression analyses of *Lin28/let-7* system during postnatal development in POA region and MBH of male and female rats showed broadly similar trends to those shown in **Figure 42 and 43**. Thus, we detected high expression of *Lin28* and *Lin28b* in the POA and MBH in both sexes and markedly declined thereafter, with virtually negligible levels of *Lin28* and moderate but detectable levels of *Lin28b* at later stages (infantile, juvenile and pubertal) of postnatal development (**Figure 45 and 46**).

In clear contrast, *let-7a* and *let-7b* expression levels were low in neonatal samples from the POA and MBH, in both male and female rats and progressively increased thereafter, with subtle differences being detected between *let-7a* and *let-7b* profiles depending on the sex and hypothalamic region. Thus, in male rats, we observed that the expression levels of *let-7a* and *let-7b* progressively rose during

postnatal maturation in both the POA and MBH, with a trend for higher expression levels of both targets (at least at some age-points) in the MBH (**Figure 45**).

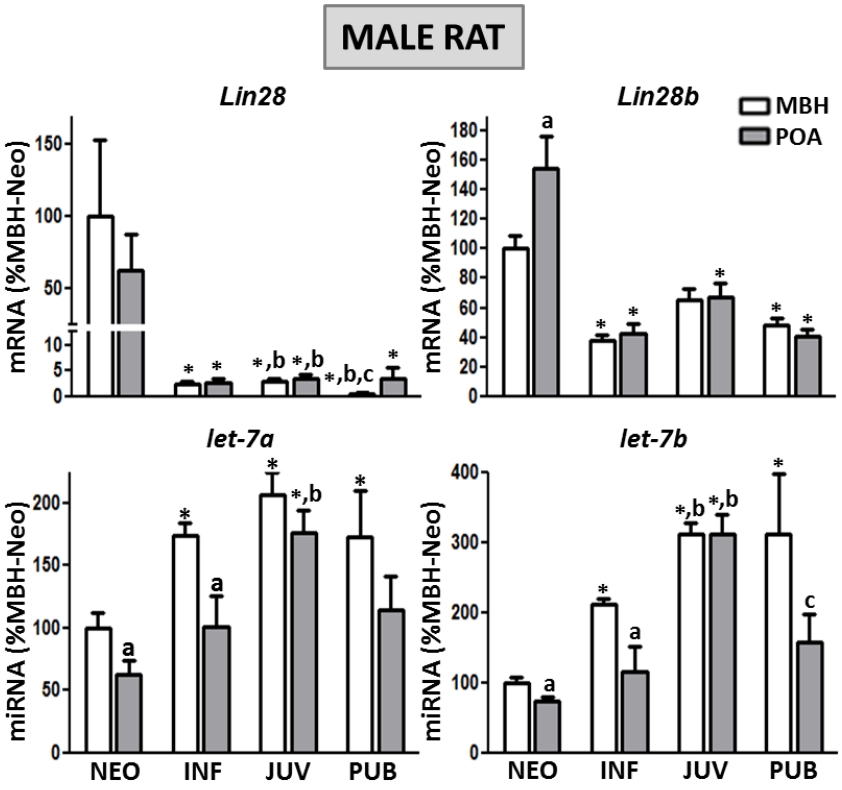

Figure 45. Expression profiles of the components of the *Lin28/let-7* axis in POA and MBH regions from male rats at different stages of postnatal maturation. Group sizes: n=7-9. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. neonatal in each region; a, P ≤ 0.05 vs MBH for each age; b, P ≤ 0.05 vs. infantile in each region; c, P ≤ 0.05 vs. juvenile in each region. Two-way ANOVA followed by post hoc Tukey test. NEO, neonatal; INF, infantile; JUV, juvenile; PUB, pubertal.

In females, we detected that *let-7a* and *let-7b* expression levels were notably higher in the MBH along postnatal maturation, with clear increases during developmental progression. In contrast, in the POA, the rise of expression of *let-7* miRNAs was more modest and only statistically significant for *let-7a* (**Figure 46**).

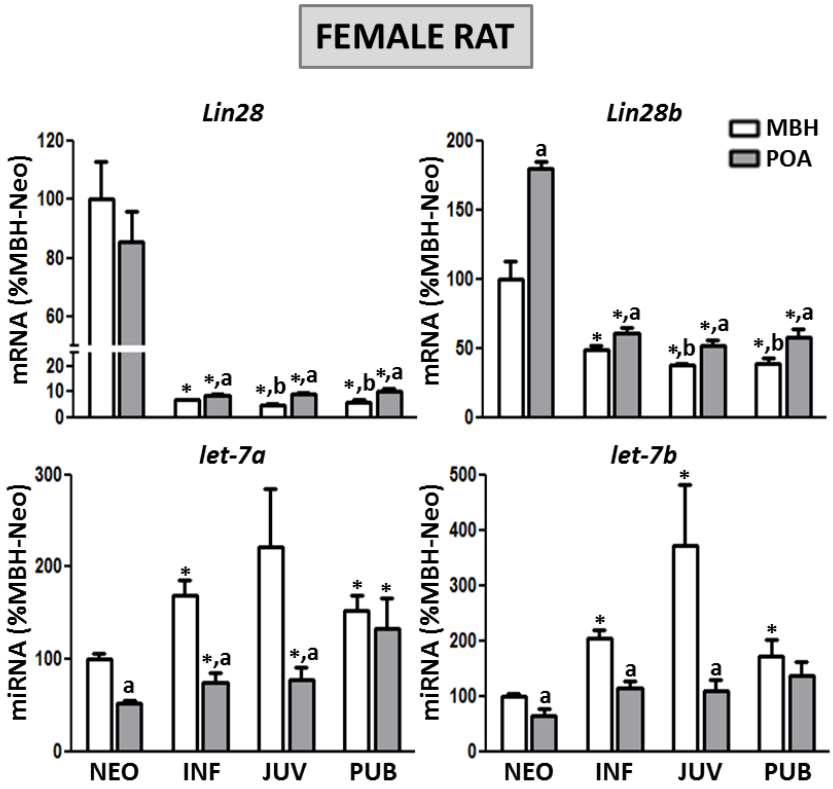

Figure 46. Expression profiles of the components of the *Lin28/let-7* axis in POA and MBH regions from female rats at different stages of postnatal maturation. Group sizes: n=7-9. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. neonatal in each region; a, P ≤ 0.05 vs MBH for each age; b, P ≤ 0.05 vs. infantile in each region; c, P ≤ 0.05 vs. juvenile in each region. Two-way ANOVA followed by post hoc Tukey test. NEO, neonatal; INF, infantile; JUV, juvenile; PUB, pubertal.

4.2.3.2. ANALYSIS OF CHANGES IN THE PROFILE OF HYPOTHALAMIC EXPRESSION OF *c-MYC/LIN28/LET-7* AND RELATED miRNAs IN MODELS OF PERTURBED PUBERTY

In order to provide further evidence for the putative roles of c-Myc/Lin28/let-7 system in the maturational program leading to puberty onset, we analyzed in the hypothalamus the expression patterns of this system, as well as of related miRNAs, in different preclinical models of disturbed puberty.

In the first study, we analyzed in male and female rats the effect of the disruption of pubertal maturation through the alterations of the sex steroid milieu during the critical neonatal period. In agreement with previous data, we observed that male rats treated neonatally with EB showed at PND-45 decreased serum levels of both gonadotropins, absent BPS, diminished TW (data not shown). In these animals, we detected significantly increased hypothalamic *Lin28b* and *c-Myc* mRNA expression levels. A similar profile was detected for *Lin28* expression levels although this increase did not reach statistical significance. In contrast, hypothalamic *let-7a*, *mir-132* and *mir-*

9 miRNA levels were significantly lower than in control animals. In addition, our data demonstrated that in EB-treated male rats the hypothalamic *let-7b* and *mir-145* expression remained unchanged (**Figure 47**).

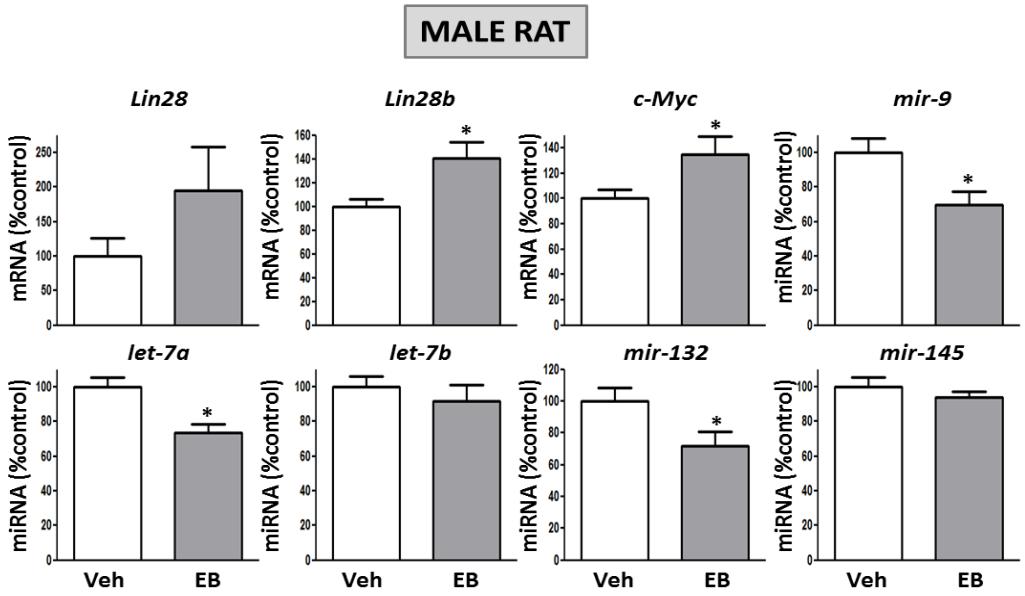

Figure 47. Expression profiles of the components of the *Lin28/let-7* axis and related factors in the hypothalamus of pubertal male rats following neonatal estrogenization. Expression analyses were conducted in whole hypothalamic fragments from PND-45 male rats subjected to a standard protocol of neonatal estrogenization (estradiol benzoate, EB) or androgenization (testosterone propionate, TP). Animals injected with vehicle (olive oil, Veh) served as controls. Group sizes: n=7-9. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. vehicle-injected group, followed by Student t test.

Similarly, sex steroid milieu manipulation during the neonatal period in female rats, by the injection on PND-1 of EB or TP, resulted in hormonal and phenotypic indices of disrupted puberty at PND-35 as evidenced by perturbed gonadotropin levels and altered timing of VO (data not shown). These alterations were associated with detectable changes in the expression patters of the c-Myc/Lin28/let-7 system in the hypothalamus at the expected time of puberty (PND-35). Thus, as in EB-treated males, expression levels of *Lin28b* and *c-Myc* mRNA were significantly increased in neonatally EB- and TP-treated female rats (**Figure 48**). In turn, neonatal treatments with EB or TP decreased the hypothalamic expression levels of *let-7b* and *mir-145*, while there was a trend for a drop of *let-7a* miRNA levels, especially in EB-treated females, which did not reach statistical significance. Hypothalamic expression of *mir-9* and *mir-132* did not change with neonatal EB or TP treatments, except for a modest increase in *mir-132* levels that was observed in androgenized female rats (**Figure 48**).

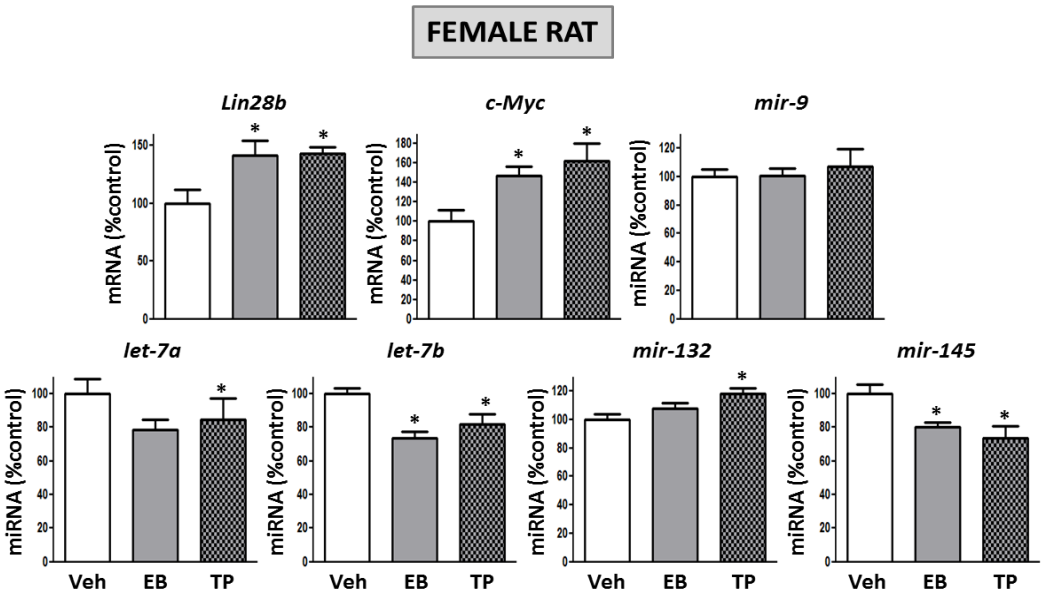

Figure 48. Expression profiles of the components of the *Lin28/let-7* axis and related factors in the hypothalamus of pubertal female rats following neonatal estrogenization or androgenization. Expression analyses were conducted in whole hypothalamic fragments from PND-35 female rats subjected to a standard protocol of neonatal estrogenization (estradiol benzoate, EB) or androgenization (testosterone propionate, TP). Animals injected with vehicle (olive oil, Veh) served as controls. Group sizes: n=7-9. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. vehicle-injected group, followed by Student t test.

In the second set of experiments, we analyzed in male and female rats the effect of photoperiodic manipulation during postnatal maturation. In the case of males, the phenotypic indices of disrupted puberty were confirmed by the observation of a delay in the age of BPS in males submitted to constant darkness (CD) between PND-10 and -15. Thus, at PND- 45, only 27.3% of CD males showed complete BPS, as compared with 91.7% of control animals, reared under standard photoperiod conditions (data not shown). At this age, no differences were observed in terms of pubertal BW and TW between control and CD animals. At the end of darkness period (PND-15), the analysis of CD males revealed a significant increase in hypothalamic *Lin28b* and *c-Myc* mRNA levels. In contrast, these animals exhibited a consistent decline in *let-7a*, *let-7b*, *mir-132* and *mir-145* miRNA expression, while *mir-9* remained unchanged (**Figure 49**). Surprisingly, these changes were transient as similar modifications in the hypothalamic expression of the above targets were not detected in CD males when studied at PND-45, i.e., the expected time of puberty (data not shown).

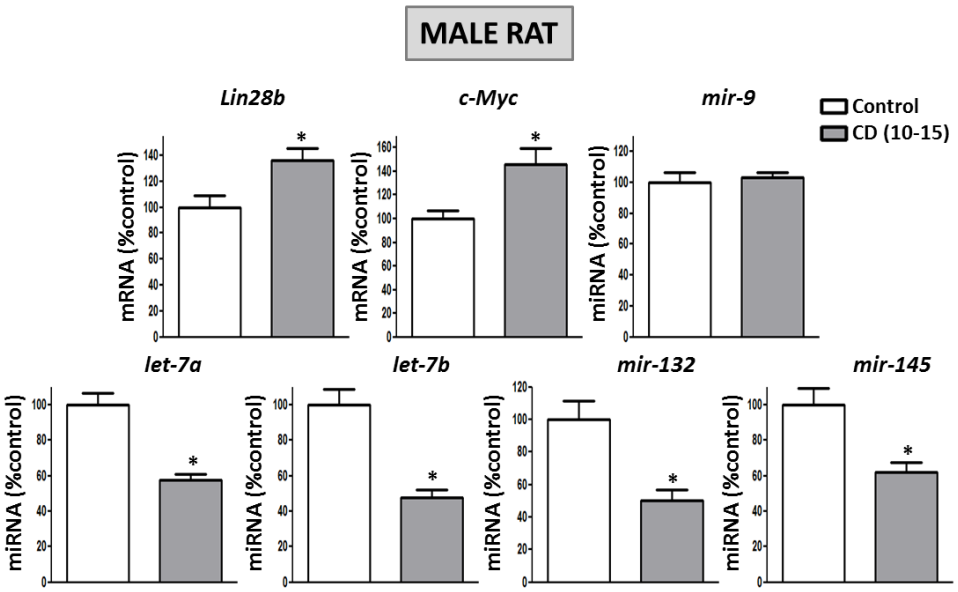

Figure 49. Expression profiles of the components of the *Lin28/let-7* axis and related factors in the hypothalamus of 15 days-old male rats following photoperiod manipulation (CD, constant darkness between PND-10 and -15). Animals reared under standard photoperiod conditions served as controls. Group sizes: n=7-9. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. control group, followed by Student t test.

With respect to females, we detected that at PND-35 only 12.5% of CD females had VO as compared with 90% of control females (data not shown). Yet, no differences were observed at PND-35 on pubertal BW, OW and UW between control and CD animals. Our data indicated that at the end of darkness period (PND-15) animals showed increased expression levels of *Lin28b* and *c-Myc* mRNA levels, decreased expression levels of *mir-145* and no changes in the other targets studied. At PND-35, CD females displayed increased hypothalamic *Lin28b* expression levels, significantly lower levels of *let-7a*, *let-7b*, *mir-132* and *mir-145* miRNA levels and no changes in *mir-9* (**Figure 50**).

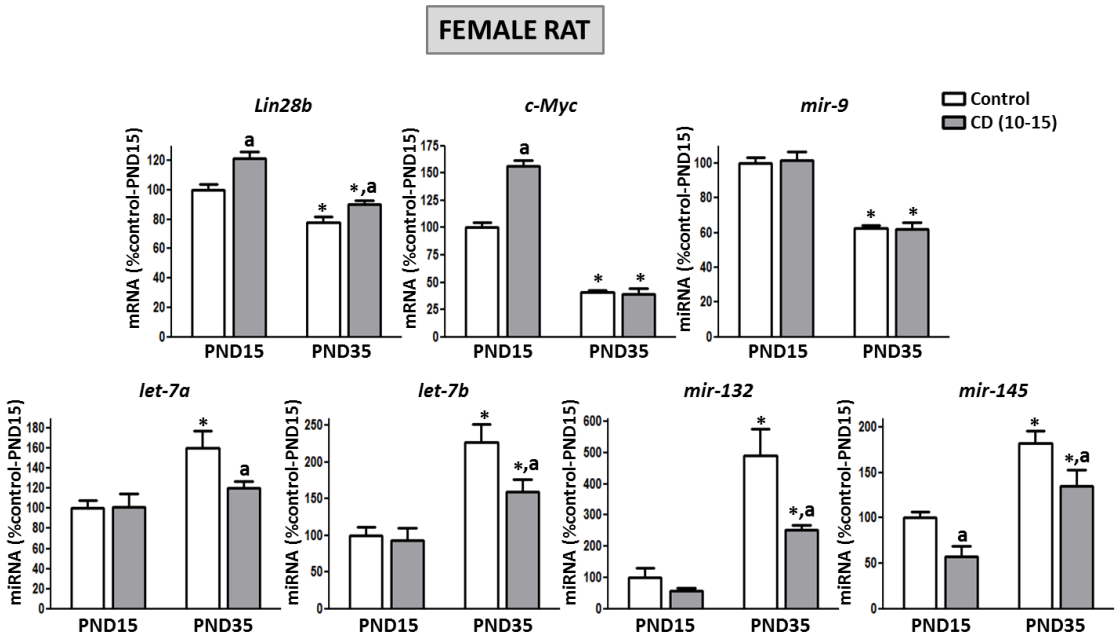

Figure 50. Expression profiles of the components of the *Lin28/let-7* axis and related factors in the hypothalamus of 15- and 35-days-old female rats following photoperiod manipulation (CD, constant darkness between PND-10 and -15). Animals reared under standard photoperiod conditions served as controls. Group sizes: n=7-9. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. PND-15 group for each photoperiodic regimen; a, P ≤ 0.05 vs control group for each age. Two-way ANOVA followed by post hoc Tukey test.

In order to analyze the impact of the disruption of pubertal maturation due to metabolic distress by undernutrition upon the expression profile of *Lin28/let-7* system, two different animal models were generated.

In the first model, male and female rats were submitted to a protocol of subnutrition during lactation, generated by breeding in large litters (20 pups/litter). This manipulation induced a delay in the timing of puberty onset in both sexes. Thus, at PND-45, only 22% of males reared in large litters had BPS as compared with 92.6% of control males, and at PND-35, only 37.5% of females from large litters had complete VO in comparison with 90% of control females (data not show).

Data obtained in this model showed that postnatal underfeeding of males and females resulted in increased *Lin28b* and *c-Myc* mRNA levels during the neonatal (PND-5) and infantile (PND-15) periods (**Figure 51, A and B**). Regarding other miRNAs analyzed, changes in hypothalamic expression induced by postnatal subnutrition were less evident and displayed some degree of sexual dimorphism. Thus, while in males and females *let-7b* miRNA levels remained unchanged, *let-7a* modestly decreased in underfed males during the neonatal period, but increased in infantile females.

Hypothalamic levels of *mir-132* tended to diminish in pubertal females subjected to postnatal undernutrition, but did not significantly change in underfed males. Finally, hypothalamic *mir-145* miRNA showed opposite profiles. Thus, males subjected to postnatal underfeeding showed lower levels during neonatal and infantile periods, while significantly increased expression in underfed infantile females was detected (**Figure 51,A and B**).

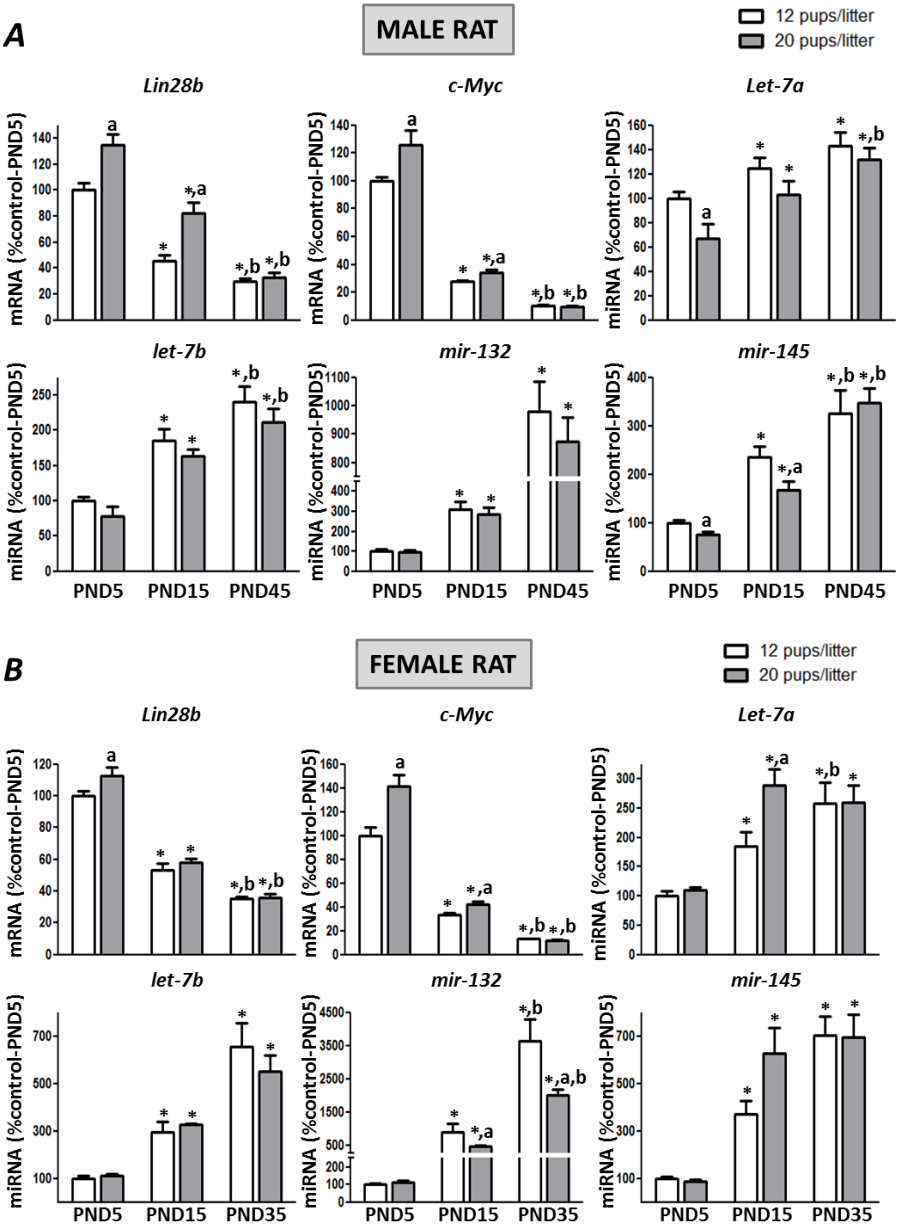

Figure 51. Expression profiles of the components of the *Lin28/let-7* axis and related factors in the hypothalamus of male and female rats following postnatal undernourishment. Expression analyses were conducted in hypothalamic samples obtained at PND-5, -15 and -45 or -35 from male (*A*) and female (*B*) rats, respectively. Group sizes: n=6-8. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. corresponding PND-5 pups litter group; a, P ≤ 0.05 vs. corresponding 12 pups/litter for each age; b, P ≤ 0.05 vs. corresponding PND-15 pups litter group. Two-way ANOVA followed by post hoc Tukey test.

Finally, we analyzed in male and female rats, the impact upon the expression profile of *Lin28/let-7* system of the disruption of pubertal maturation due to persistent caloric restriction (CR) by reduction of 30% daily food intake from weaning to puberty. Our data indicated that CR induces a delay in the timing of puberty onset, especially in females (data not shown). This protocol of late CR caused a modest reduction in hypothalamic expression of *Lin28b* and *let-7b* in males and females at the time of puberty, which was associated with a small reduction in *c-Myc* mRNA levels selectively in females (**Figure 52,A and B**).

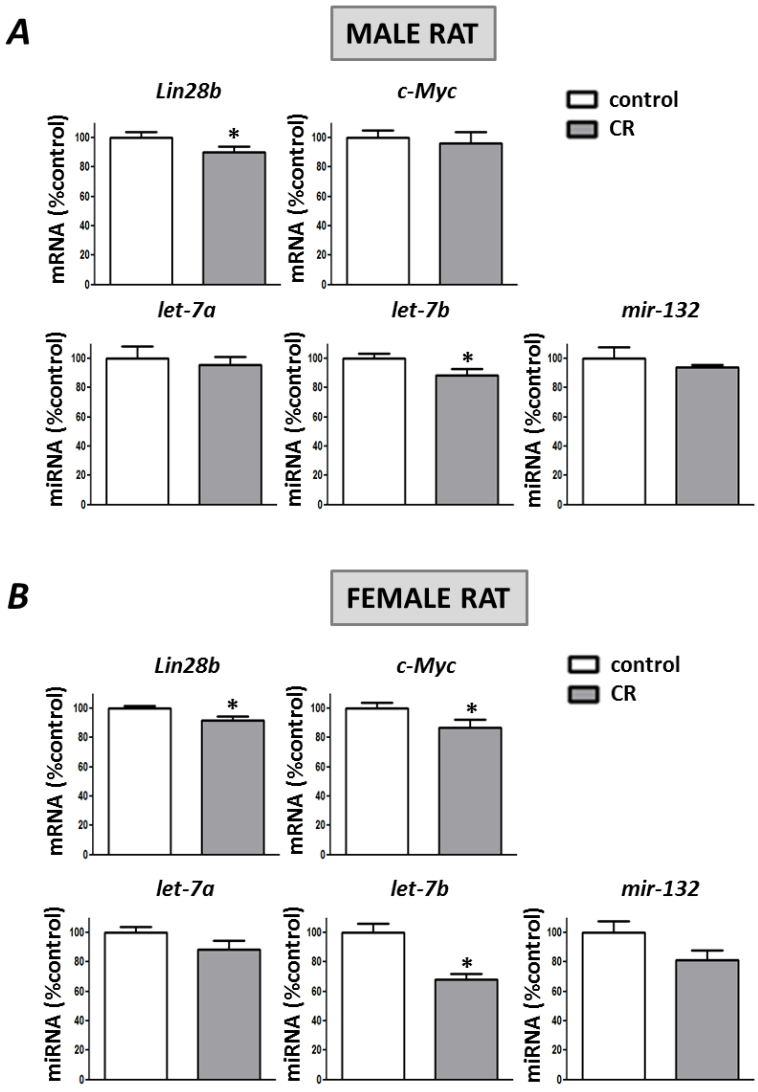

Figure 52. Expression profiles of the elements of the *Lin28/let-7* axis and related factors in the hypothalamus of male (A) and female (*B*) rats subjected to prepubertal caloric restriction (CR). Studies were conducted at PND-45 in males) and PND-35 in females. Group sizes: n=6-8. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. control group, followed by Student t test.

4.2.4. CHARACTERIZATION OF THE PROFILE OF TESTICULAR EXPRESSION OF *LIN28/LET-7* SYSTEM AND RELATED miRNAs IN RATS AND MICE

Our initial analyses showed that *Lin28* and *Lin28b* mRNA are prominently expressed in the testis of rats and mice (see **Figure 41**). Based on these results, we also analyzed the expression profile of key components of the Lin28/let-7 system in the testis of these species during postnatal maturation, as a means to complement our central studies on the roles of this system in the control of pubertal maturation. In addition, the potential changes in their expression patterns in various models of altered puberty and the involvement of different hormonal axes in its regulation were analyzed.

4.2.4.1. ANALYSIS OF TESTICULAR EXPRESSION OF *LIN28/LET-7* AND RELATED miRNAs DURING POSTNATAL TESTICULAR DEVELOPMENT

In rats, data obtained by RT-PCR indicated that *Lin28* and *Lin28b* mRNAs displayed low testicular expression during the neonatal period, increasing markedly during infantile period. However, *Lin28* mRNA levels showed a subsequent decreased to adulthood, when levels were similar to those of the day of birth, while *Lin28b* mRNA levels remained high from the infantile period to adulthood. *Let-7a, let-7b, mir-132* and *mir-145*, showed opposite expression profiles to *Lin-28b* but with minor differences (**Figure 53**). All miRNAs, with the exception of *let-7b*, declined after the neonatal period, but *let-7a* levels gradually decreased until puberty whereas *mir-145* levels remained elevated during the infantile period and dropped during the juvenile transition. Levels of mir-132 decreased during the infantile period and remained low until puberty, increasing again during adulthood, but with levels lower than neonatal period. *Let-7b* levels increased slightly during the infantile period and then decreased to adulthood (**Figure 53**).

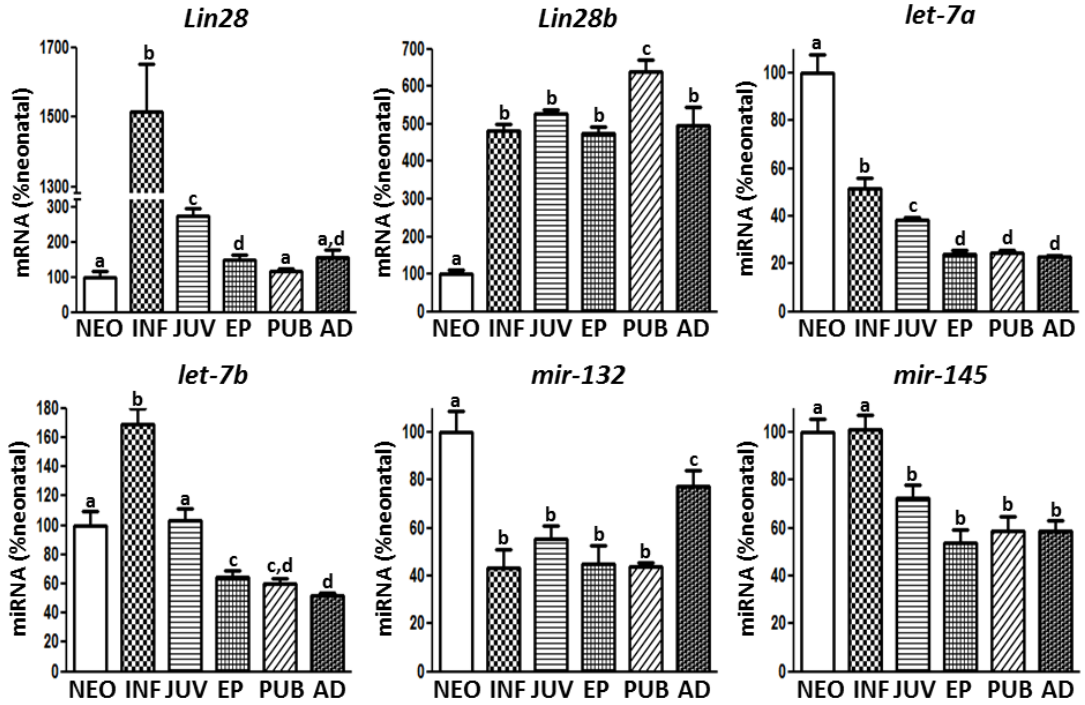

Figure 53. Expression profiles of the components of the *Lin28*/*let-7* axis and related factors in rat testis at different stages of postnatal development. Expression analyses of *Lin28* and *Lin28b* mRNAs, as well as *let-7a*, *let-7b*, *mir-132* and *mir-145* miRNAs were conducted in testis fragments from rats at different stages of postnatal development. Group sizes: n=8-10. Data are presented as mean ± SEM. Different letters above bars indicate statistical differences, $P \leq 0.05$; ANOVA followed by post hoc Tukey test. NEO: neonatal; INF: infantile; JUV: juvenile; EP: early puberty; PUB: pubertal; AD: adult.

In good agreement with results obtained in rats, we also detected expression of *Lin28* and *Lin28b* mRNA in mouse testes at all stages of postnatal maturation selected, corresponding to neonatal (PND-1), infantile (PND-15) and pubertal/early adult (PND-45) ages. In addition, these profiles of expression across the different ages were also different for both signals. Thus, *Lin28* mRNA levels abruptly increased (more than 14-fold increase) between PND-1 and PND-15 and markedly declined (about 60% reduction) between PND-15 and PND-45, whereas *Lin28b* mRNA expression increased between the neonatal and infantile periods (about 7-fold rise) but remained elevated during the pubertal transition, with similar mRNA levels at PND-15 and -45 (**Figure 54**). In clear contrast, relative levels of both *let-7a* and *let-7b* miRNAs were maximal during neonatal period and declined thereafter. During postnatal development, we found differences between both *let-7* miRNAs. Thus, whereas *let-7a* levels gradually declined, with 50% reduction between the PND-1 and PND-15 and further decreased at PND-45,

testicular *let-7b* expression remained elevated at the infantile period and dropped by about 60% during the pubertal transition (**Figure 54**).

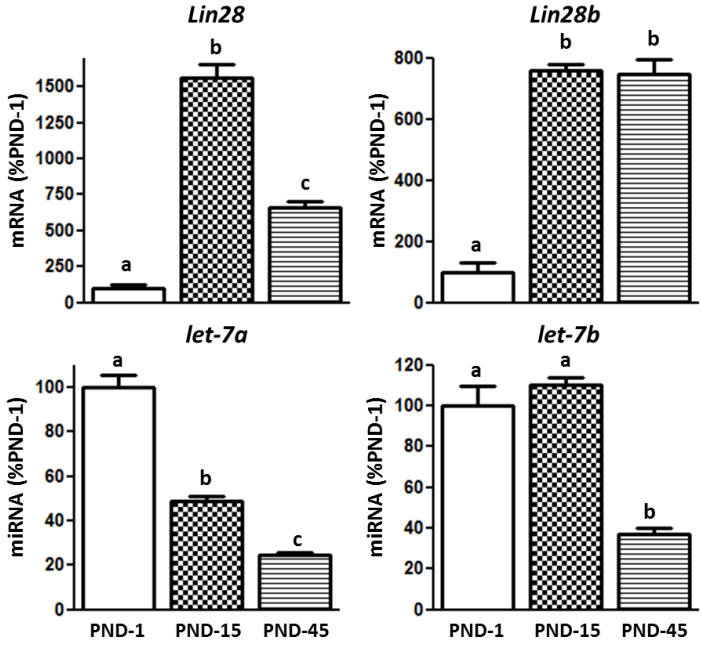

Figure 54. Expression profiles of *Lin28/let-7* system in the testis of mouse during postnatal maturation.. Group sizes: n=4-7. Data are presented as mean ± SEM. Different superscript letters above bars indicate statistical differences, P ≤ 0.05; ANOVA followed by Student-Newman-Keuls multiple range test.

4.2.4.2. HISTOLOGICAL CHARACTERIZATION OF THE TESTICULAR EXPRESSION OF *LIN28/LET-7* AND RELATED miRNAs

In order to further study the testicular expression of *Lin28/let-7* system, we analyzed the distribution patterns of this system and related miRNAs in mice and rats.

Because our initial validation tests in rats were not successful (none of the antibodies tested worked adequately), IHC analyses to study the cellular location of Lin28 and Lin28b was carried out mainly in mice. Such studies confirmed the presence of both Lin28 and Lin28b peptides in testis from adult (>2-month-old) mice from the C57B/6 strain. These assays revealed quite different cellular locations for Lin28 and Lin28b proteins within the testicular tissue. Thus, Lin28-IR was restricted to the seminiferous tubules where the signal was confined to cells at the basal zone of the seminiferous epithelium, in particular corresponding to spermatogonia (**Figure 55, A and C**). However, interstitial Leydig cells were negative (**Figure 55C**). In clear contrast, Lin28b-IR was detected in both spermatids in the seminiferous tubules and Leydig cells in the interstitial compartment (**Figure 55,B and D**), whereas other interstitial cells and

blood vessels were negative. These results set the basis for additional expression analyses during postnatal maturation and in a mouse model of impuberism and hypogonadism.

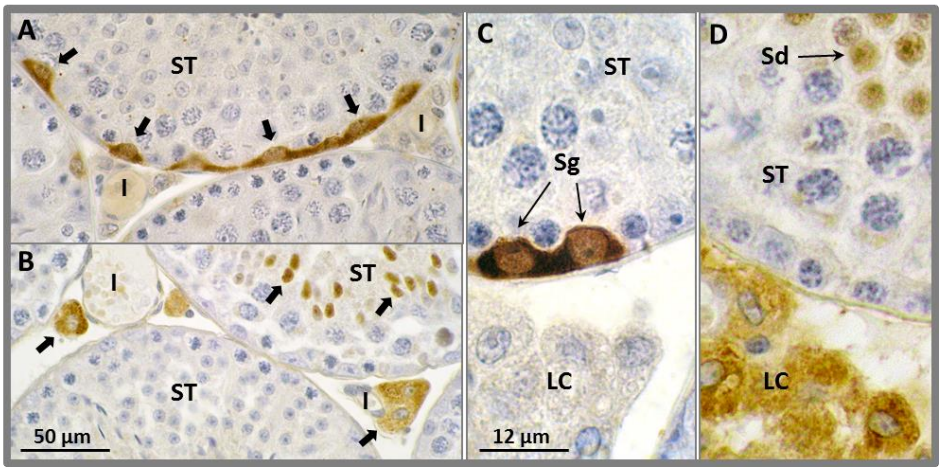

Figure 55. Representative photograph of Lin28 and Lin28b protein distribution in testis of adult mice. A and C, Immunostaining for Lin28; *C*, At higher magnification of Lin28-IR cells in the seminiferous tubules (ST) correspond to spermatogonia (Sg), whereas interstitial Leydig cells (LC) were negative. *B, D*, Immunoreactivity for Lin28b; *D*, At higher magnification of Lin28b-IR cells correspond to spermatids (Sd) in the tubules and to Leydig cells in the interstitium (I).

As a way to complete our initial rat studies of testicular expression of miRNAs linked to the Lin28/let-7 system, detailed ISH assays of the patterns of expression of *let-7b* and the related miRNA, *mir-145*, were conducted in adult male rats. We observed that *let-7b* miRNA expression in adult testis was restricted to germ cells in the seminiferous tubules in a stage-dependent pattern and it was absent in the interstitial areas (**Figure 56A**). The hybridization signal was strong in pachytene spermatocytes (**Figures 56, A-C**), weaker in round spermatids from stage I to stage VII and disappeared in elongating spermatids from stage VIII onwards (**Figures 56, B and C**). The same expression pattern was observed for *mir-145*, with the exception that it was strongly expressed in smooth muscle cells in the interstitial blood vessels (**Figure 56D**).

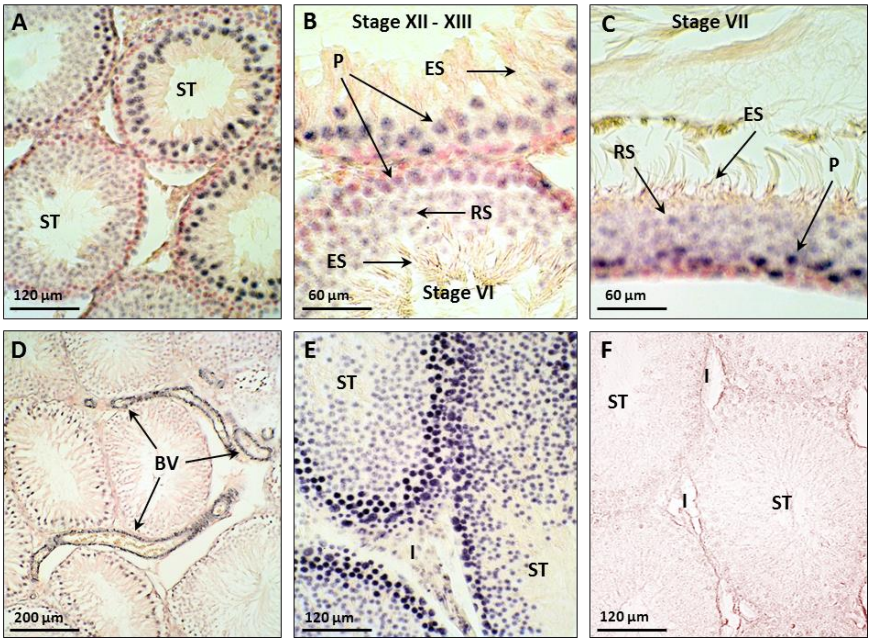

Figure 56. Representative *in situ* hybridization of testicular distribution of *let-7b* and *mir-145* in adult rats. ISH signal (purple color) of *let-7b* (*A-C*) and *mir-145* (*D*) are presented. *E*, Positive control (hybridized for the ubiquitous microRNA U6); F, Negative control (hybridized with sense probes). ST, seminiferous tubules; I, interstitium; P, pachytene spermatocytes; RS, round spermatids; ES, elongating spermatids; BV, interstitial blood vessels.

4.2.4.3. ANALYSIS OF CHANGES OF TESTICULAR EXPRESSION OF LIN28/LET-7 AND RELATED miRNAs IN MODELS OF PERTURBED PUBERTY

With the aim to further characterize changes of the expression of the elements of the *Lin28/let-7* system, we analyzed the testicular expression patterns of this system in different preclinical models of disturbed puberty in both rats and mice.

First, we studied the effect of the disruption of pubertal maturation after neonatal administration of EB to male rats. The hormonal and phenotypic indices of disrupted puberty were mentioned previously (see **Figure 47**). Neonatal estrogenization resulted in evident changes in the *Lin28*/*let-7* axis at the expected time of puberty (PND-45). Thus, neonatally estrogenized males displayed decreased *Lin28* and *Lin28b* mRNA levels. In contrast, *let-7a*, *let-7b* and *mir-145* miRNA levels were significantly higher than in control animals, while *mir-132* expression did not show significant changes (**Figure 57**).

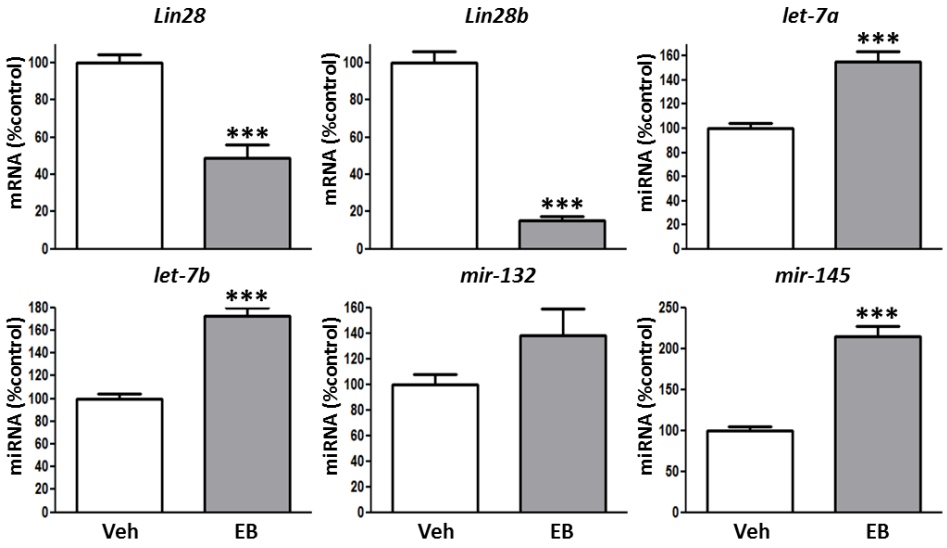

Figure 57. Expression profiles of the components of the *Lin28*/*let-7* axis and related factors in pubertal rat testis from animals neonatally treated with vehicle or estradiol benzoate (EB). Group sizes: n=7-9. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. vehicle-injected group, followed by Student t test.

As previously indicated, male rats submitted to constant darkness (CD) between PND-10 to -15 displayed a delay in the normal timing of puberty onset (see details in **Figure 49**)**.** Testicular analysis at PND-15 and -45 did not show changes in testicular *Lin28* and *Lin28b* mRNAs or *let-7a* miRNA levels, except for *Lin28b* whose expression levels were decreased on PND-45. On the other hand, these animals exhibited a decrease in *let-7b*, *mir-132* and *mir-145* miRNA at PND-15, but these changes in miRNA levels were transient and were not detected at PND-45 (**Figure 58**).

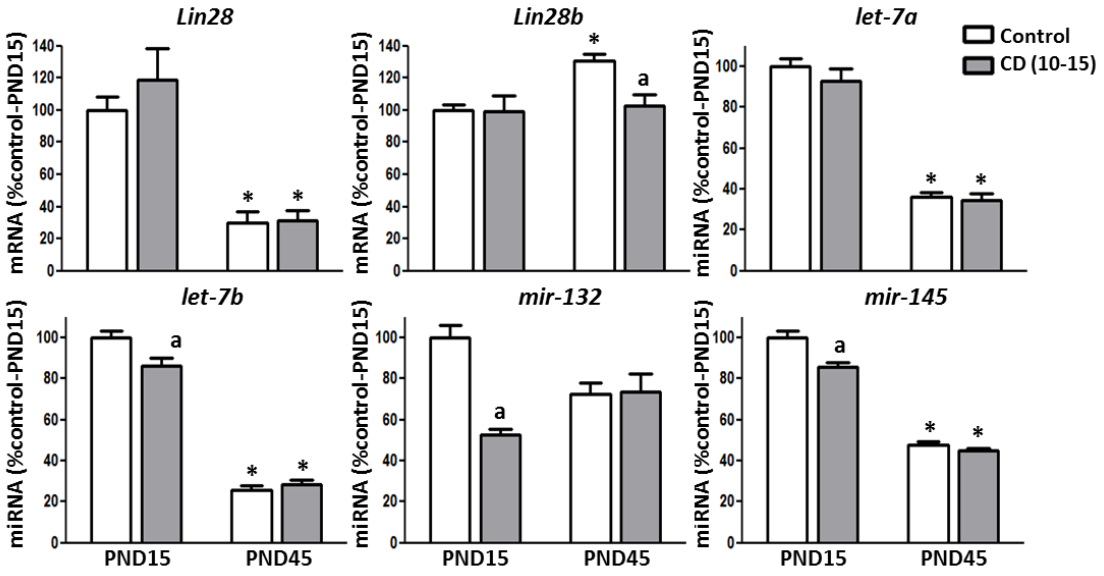

Figure 58. Expression profiles of the components of the Lin28/let-7 axis and related factors in rat testis following photoperiod manipulation (CD, constant darkness between PND-10 and -15). Animals reared under standard photoperiod conditions served as controls. Expression analyses were conducted at PND-15 and -45. Group sizes: n=7-9. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. PND-15 group for each photoperiodic regimen; a, P ≤ 0.05 vs. control group for each age. Two-way ANOVA followed by post hoc Tukey test.

In addition, we analyzed the patterns of testicular expression of the *Lin28/let-7* system in models of perturbed puberty due to metabolic distress associated to subnutrition during lactation in rats. As mentioned above, males bred in large litters (20 pups/litter) presented a delay in the timing of puberty (see details in **Figure 51A**). With regard to *Lin28* and *Lin28b* expression levels, we observed that early postnatal subnutrition resulted in increased expression of both mRNAs at PND-5 and PND-15 (**Figure 59**). However, at PND-45, *Lin28b* mRNA levels showed lower expression compared with male rats bred in normal litters, while *Lin28* mRNA levels remained increased. Changes in testicular miRNA expression were less consistent; thus, while *let-7b* miRNA levels remained unchanged along postnatal maturation, *let-7a* decreased during the neonatal period compared with the control group. Levels of *mir-132* and *mir-145* tended to decrease only at PND-15 in animals from large litters (20 pups/dam)(**Figure 59**).

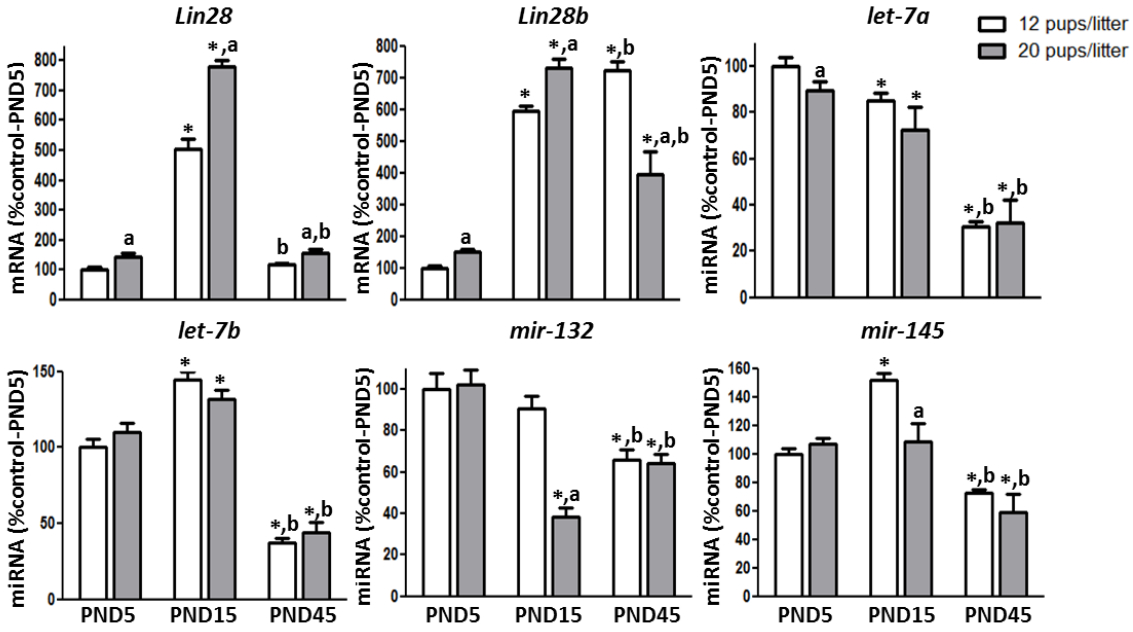

Figure 59. Expression profiles of the components of the *Lin28*/*let-7* axis and related factors in rat testis following postnatal undernourishment. Expression analyses were conducted at PND-5, -15, and -45. Group sizes: n=6-8. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. corresponding PND-5 pups litter group; a, P ≤ 0.05 vs. corresponding 12 pups/litter for each age; b, P ≤ 0.05 vs. corresponding PND-15 pups litter group. Two-way ANOVA followed by post hoc Tukey test.

In order to further analyze gonadotropin regulation of testicular *Lin28/let-7* system, we used HPX male rats with or withput gonadotropin replacement as experimental model. It was observed that long-term HPX resulted in the decrease of testicular *Lin28b* mRNA to virtually negligible levels, a response that was not reverted by the replacement with hCG, FSH or a combination of both (**Figure 60**). Anyhow, relative *Lin28b* levels were partially increased by FSH, either alone or in combination with hCG. In contrast, *Lin28* mRNA expression increased after HPX and was reversed by treatments with hCG or FSH. The administration of both hormones provoked lower *Lin28* levels than those observed in intact rats (**Figure 60**). With regard to miRNAs, HPX did not cause alterations in *let-7a, let-7b* and *mir-145* miRNA levels. However, both hCG and FSH treatments provoked an increase in their expression compared with HPX or intact rats. This increase was more marked when both hormones were administered together (**Figure 60**). On the other hand, levels of *mir-132* miRNA diminished after HXP and significantly increased after treatment with hCG or FSH with higher values than control rats. The combined administration of both hormones caused cumulative effects on *mir-132* miRNA expression (**Figure 60**).

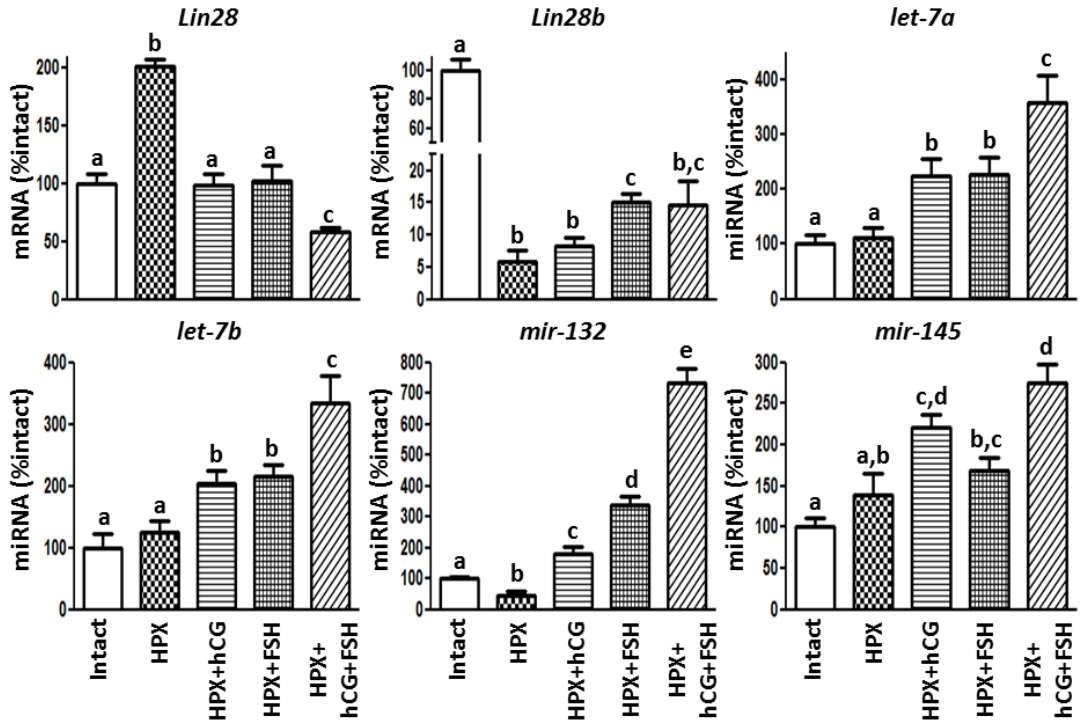

Figure 60. Effects of HPX and gonadotropin replacement on testicular expression of *Lin28/let-7* axis and related factors in adult male rats. Group sizes: n=5-6. Data are presented as mean ± SEM. Different letters above bars indicate statistical differences, P ≤ 0.05; ANOVA followed by post hoc Tukey test.

Finally, we conducted protein and RNAs analyses of the Lin28/Lin28b system using a genetic model of impuberism and hypogonadotropic hypogonadism, as a result of inactivation of Gpr54. As was mentioned previously, Gpr54 KO mice displayed decreased circulating LH and FSH levels, as well as severe reduction in TW in adulthood. WT animals, harboring two alleles for Gpr54, were used as controls.

On one hand, we carried out complementary RNA analyses to identify changes in testicular *Lin28/let-7* levels between WT and Gpr54 KO mice in adult animals. We detected that Gpr54 null mice displayed increased relative levels of *Lin28* mRNA, but not *Lin28b* mRNA in the testis, with about 3-fold increase over WT values (**Figure 61**). In addition, Gpr54 null mice showed enhanced relative levels of *let-7a* (about 2.5-fold increase) and *let7-b* (about 6-fold increase) miRNAs compared with values detected in WT animals. Considering that TW of Gpr54 KO mice was severely decreased compared with WT values (about 14-fold decrease), in addition to relative expression, total expression levels of the above targets per testis were calculated, as estimated by the product of the expression of the specific transcript by the TW. This analysis revealed that total testicular expression of *Lin28* and *let-7* transcripts was markedly reduced in

Gpr54 KO mice, although the degree of suppression varied among the different targets. Thus, total expression levels were 25% for *Lin28*; about 8% for *Lin28b*; 17% for *let-7a*; and 43% for *let-b* of corresponding values in WT testes. Note that this method of calculation of total testicular expression of RNA allows an easy estimation of the absolute expression of a given target per testis and has been previously used by our group to complement relative expression data in models, such as HPX, characterized by dramatic changes in weight and/or cellular composition of the testis.

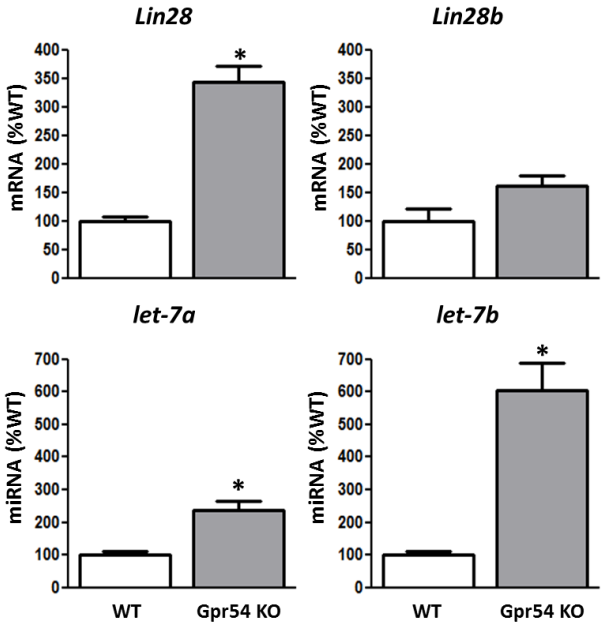

Figure 61. Expression profiles of *Lin28/let-7* system in the testis of adult WT and Gpr54 KO mice. Lin28 and Expression analyses of *Lin28* and *Lin28b* mRNAs, as well as *let-7a* and *let-7b* miRNAs were conducted in WT and Gpr54 KO mie. Group sizes: n=4-6. Data are presented as mean ± SEM. *, P ≤ 0.05 vs. vehicle-injected group, followed by Student t test.

Finally, we carried out morphometric analyses in adult WT and Gpr54 KO mice. The results obtained from WT mice showed normal seminiferous tubules with complete spermatogenesis and prominent eosinophilic Leydig cells in the interstitial areas (**Figure 62A**). In contrast, adult Gpr54 KO mice showed small seminiferous tubules with arrested spermatogenesis; the most advanced germ cells corresponded to leptotene (or occasional pachytene) spermatocytes (**Figure 62C**). Analyses of testicular sections, stained with hematoxylin and eosin, from WT and Gpr54 null mice, documented the important alterations of testicular histology present in this model of impuberism. Regarding the characterization of Lin28 and Lin28b, we observed that Lin28-IR was detected at the base of the seminiferous epithelium (**Figure 62B**),

confirming our previous results (see **Figure 55**). In Gpr54 KO mice, Lin28-IR was also present in analogous spermatogonial populations, but irregular distribution access testicular sections was observed (**Figure 62D**). With respect to Lin28b, IHC analyses in Gpr54 KO mouse testes failed to detect any specific signal (data not shown).

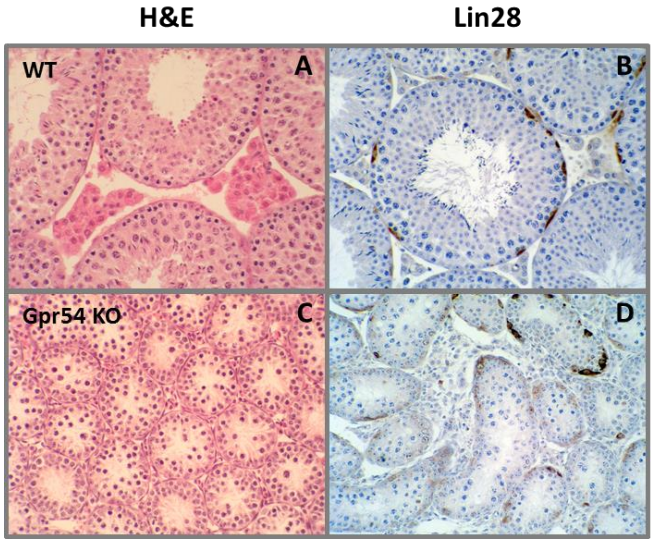

Figure 62. Ultrastructural features and cellular distribution of Lin28 in the testis of adult WT and Gpr54 KO mice. *A* and *C,* Representative testicular sections stained with hematoxylin and eosin (H&E) from WT and Gpr54 null mice are shown. *B* and *D,* Representative IHC analyses of Lin28 in WT and Gpr54 null mice are presented.

In order to further understand the hormonal regulation of the testicular expression patterns of Lin28 and Lin28b, IHC analyses were also implemented in Gpr54 null mice subjected, or not, to protocols of gonadotropin replacement with recombinant hCG or FSH, as previously described. Our analyses indicated that treatment with hCG for 7 days resulted in a marked increase in the seminiferous tubule diameter with progression of spermatogenesis up to elongating spermatids and enlargement of interstitial Leydig cells (**Figure 63C**). In turn, FSH administration resulted in a detectable, although less prominent, increase in tubular diameter and progression of spermatogenesis up to the round spermatid stage, without any discernible effect on the morphology of Leydig cells (**Figure 63D**).

Considering that Lin28-IR was persistently detectable in testes of Gpr54 KO mice, but Lin28b was absent, and that testicular Lin28-IR did not change substantially after gonadotropin treatment of Gpr54 null mice (data not shown), only the analyses of changes in the expression profiles of the Lin28b following effective gonadotropin stimulation are present below.

In agreement with our initial analyses in C57B/6 adult mice (see **Figure 55**), we detected intensive Lin28b-IR in both Leydig cells in the interstitial areas and elongating spermatids within the seminiferous tubules in WT mice (**Figure 63A**). In contrast, Gpr54 null mice did not show detectable Lin28b immunostaining because of the lack of elongating spermatids and fully differentiated Leydig cells (**Figure 63B**). It was observed that gonadotropin administration rescued Lin28b expression with different effects between hCG and FSH treatments. Thus, hCG administration induced very intense Lin28b-IR in Leydig cells and elongating spermatids (**Figure 63C**), whereas FSH treatment caused more modest effects, with Lin28b-IR detected in round spermatids, but not in Leydig cells (**Figure 63D**).

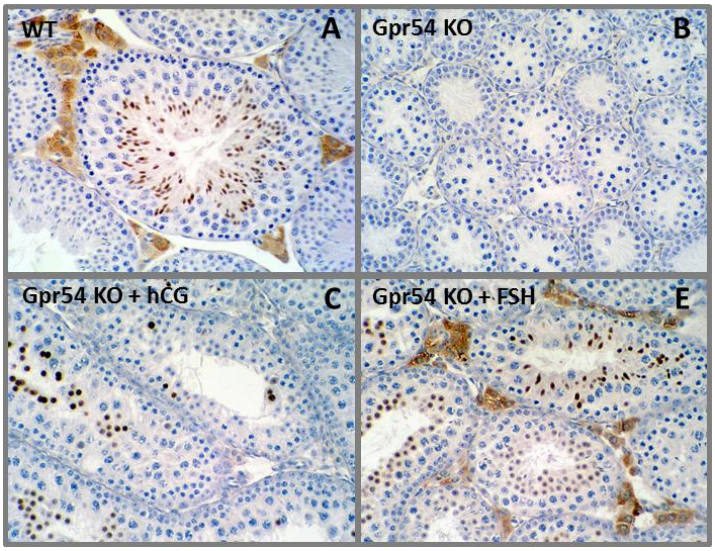

Figure 63. Representative testicular section of Lin28b-IR of WT and Gpr54 KO mice after gonadotropin treatment. IHC analyses of Lin28b in the testis of adult WT (A) and Gpr54 KO mice treated with vehicle (*B*) or an effective doses of recombinant hCG (*C*) or FSH (*D*) are presented.

DISCUSSION

5. DISCUSSION

Puberty is defined as the transitional period from the juvenile stage to adulthood during which the reproductive capacity is acquired. From a neurobiological perspective, the onset of puberty and so, the full activation of reproductive axis, requires an increase in the pulsatile neurosecretion of GnRH [12]; this induces full gonadotropin stimulation at the pituitary, which subsequently increases gonadal hormone secretion and funtion.

During the last years, substantial efforts have been made to identify the ultimately mechanisms responsible for the control of puberty onset. In this sense, it is know that the components of the HPG axis are controlled, among others, by peripheral factors that relay the nutritional information to the CNS, where they interact with central regulators involved in the control of the onset of puberty [396, 397]. GnRH neurons are considered the main hierarchical element in the neuroendocrine control of reproductive function and act as the final integrator of central and peripheral regulators of the HPG axis. However, several lines of evidence have demonstrated that these neurons may not be targeted directly by metabolic signals, such as leptin [252], a key factor involved in the integral control of energy balance and reproduction. Despite the progress made during the last few years in the identification of the potential candidate afferents responsible for transmitting metabolic information to GnRH neurons, our knowledge about this area remains incomplete.

Additionally, different studies have described that, rather than the result of a single trigger, the pubertal increase in GnRH secretion depends on the coordinate changes of neuronal excitatory and inhibitory inputs to GnRH neurons [149], in conjunction with signals from non-neuronal cells, such as glia cells [147, 148, 154]. In this context, it has been proposed the hypothesis that puberty is controlled by a set of genes hierarchically arranged within functionally connected networks where GnRH is the final effector [159]. Several studies suggested that miRNAs could modulate gene expression at multiple hierarchical levels [162, 163]; hence, miRNA pathways are well suited to act as dynamic regulatory elements able to integrate the different networks involved in the control of puberty. Indeed, recent GWAS have demonstrated an

association of early menarche with a sequence variation in *Lin28b* [164-167], which is involved in the blockade of the processing into mature miRNAs of the *let-7* family of miRNAs [168]. However, the actual roles of miRNAs in general, and of the Lin28/let-7 system in particular, in the control of the events that lead to the puberty onset remained unexplored until recently.

Based on the above, and in order to advance in our current knowledge about the systmes involved in the control of the pubertal activation of the HPG axis, this Thesis had the following main objectives: I) to characterize the potential function of central α-MSH signaling in the control of puberty onset and its putative interaction with leptin and kisspeptin signaling; and II) to define the profiles of expression of the Lin28/let-7 system, and several associated factors, during pubertal maturation, and study the changes in their expression patterns in preclinical models of altered puberty at different sites of the HPG axis, including the hypothalamus and testis. These expression studies would pave the way for future studies addressing the functional dissection of the regulatory roles, molecular targets and neuronal pathways involved in such miRNA modulation of puberty onset.

In order to facilitate the discussion of the results, this section has been divided in two major subsections corresponding to each of the two major objectives listed above.

5.1. MELANOCORTIN SIGNALING AND PUBERTY: INTERACTIONS WITH LEPTIN AND KISSPEPTIN

As indicated in previous sections of this Thesis, the fine tuning of the tempo of puberty depends on the delicate balance between peripheral and central regulators, which interplay to ultimately modulate the GnRH output from the hypothalamus to drive full activation of the downstream elements of HPG axis at puberty. Among the central transmitters involved in such function, kisspeptins have received considerable attention in recent years, given their prominent roles in the direct (and possibily indirect) regulation of GnRH neurons and the overt phenotype of humans and rodents harboring inactivating mutations in the elements of this system (see Section 1.4.). In this context, specific analyses have addressed whether Kiss1 neurons participate in

conveying the regulatory actions of different peripheral metabolic signals, such as leptin, insulin and ghrelin, with prominent roles in the control of puberty[175]. Likewise, the putative interplay between kisspeptins and other central neuropeptide pathways with relevant roles in energy and body weight homeostasis has been explored[175]; yet, these analyses have been fragmentary and our knowledge in this area remains incomplete.

In this work, we aimed to provide further insight into this phenomenon by exploring the specific roles of central melanocortin signaling, mediated by MC3 and MC4 receptors, in the regulation of puberty, and its interplay with key peripheral (i.e., leptin) and central (i.e., kisspeptins) modulators of puberty. The interest in α-MSH signaling derives from its prominent role in the central control of energy homeostasis, and the proposed functional interplay between Kiss1 and POMC neurons; the latter, however, was suggested solely on the basis of electrophysiological studies in adult mice, and the physiological relavence of this phenomenon remains unexplored. In fact, some experimental studies had pharmacologically addressed the roles of α-MSH in the control of gonadotropin secretion in adulthood, with predominant stimulatory actions, and more recently the potential interplay between melanocortin and kisspeptin pathways had been studied, mainly in the sheep (also in adulthood). Also recently, direct effects of α-MSH on GnRH neurons, as measured by electrical responses, have been characterized in adult mice [305]. Despite this compelling evidence, the actual role of melanocortin signaling in the regulation of puberty remained largely unexplored.

To assess the putative role of central α-MSH signaling in the control of puberty, we implemented a series of studies addressing three major aspects of such role. First, we studied gonadotropic responses to central melanocortin receptor activation during the infantile-pubertal transition and assessed the effects of central blockade of melanocortin signaling on the timing of puberty. Second, we explored the role of melanocortin signaling in mediating the permissive effects of leptin, as major metabolic regulator, on puberty onset. Finally, we studied the putative bidirectional interplay between α-MSH and kisspeptin signaling in the control of the HPG axis at puberty. Altogether, our studies have surfaced a prominent physiological role of α-

MSH signaling in the fine control of puberty onset, by playing an essential role as transmitter of leptin effects and modulator of the hypothalamic Kiss1 system.

Using LH responses as surrogate marker of GnRH activation, the present data are, to our knowledge, the first to document the precise program of developmental acquisition of the stimulatory actions of melanocortin (MC3/MC4) receptor signaling on the GnRH/gonadotropic axis, which takes place during the infantile-juvenile transition in female rats, as documented by the unambiguous LH responses to the MC3/MC4 agonist, MT-II, in juvenile, but not in infatile rats. The physiological relevance of melanocorting signaling in the control of gonadotropin secretion at this age is further supported by the finding that acute bloackade of MC3/MC4 receptors resulted in a significant lowering of basal LH levels. Likewise, chronic inhition of central melanocortin signaling during the pubertal transition resulted in a dramatic suppression/ delay of puberty onset, as monitored by a combination of external and internal indices of pubertal maturation, such as delayed vaginal opening, reduced uterus and ovarian weight, and a clear trend for a decrease in circulating LH levels. Altogether, our pharmacological gain- and loss-of-function experiments are clearly indicative of a stimulatory role of α-MSH signaling in the regulation of puberty in rats.

The lack of stimulatory effects of the agonist MT-II in infantile rats is in clear contrast with the potent gonadotropin-releasing effects of kisspeptin-10 detected in male and female rats at the same age [207]. Assuming that kisspeptins might operate as downstream effectors of α-MSH (*see below*), one tempting possibility is that, while the capacity of kisspeptins to activate GnRH neurons is achieved at very early developmental stages (e.g., the neonatal/infantile period), upstream activators of endogenous kisspeptin pathways, which become operational at alter maturational periods, at are needed to actually set in motion afferent kisspeptin signals responsible for the full activation of GnRH neurons at puberty. Interestingly, the switch between null (at PND-15) and detectable (at PND-25) LH responses to MT-II coincides with the pattern of developmental maturation of Kiss1 neurons reported in mice and rats during postnatal maturation. This observation indirectly supports the hypothesis that

Kiss1 neurons might actually mediate the GnRH/gonadotropin releasing effects of α-MSH.

Our pharmacological studies also revelaed that robust stimulatory responses, in terms of LH secretion, were only observed after central administration of high doses of the selective MC4-R agonist, while the MC3-R agonist was ineffective at such high dose. However, moderate, but significant LH-releasing effects of the MC3-R agonist were detected at low doses. These observations, together with the fact that responses to MT-II were similar in magnitude to those evoked by the MC4-R agonist, suggest that this melanocortin receptor subtype is responsible for mediating the major stimulatory actions of α-MSH on the GnRH/gonadotropic axis. It must be noted, however, that maximal responses to the MC4-R agonist were obtained at doses 5-times higher than those of MT-II; this might be explained by the fact that MT-II activates also MC3-R and, as mentioned above, the agonist of this receptor was also capable to induce modest stimulatory responses in the low dose range. Also notably, our data unveil that the stimulatory effect of melanocortin signaling on the GnRH/goadotropic axis persists even in face of severe conditions of negative energy balance that significantly lower basal gonadotropin levels. Again, this is reminiscent of the situation with the Kiss1 system, as kisspeptins can also revert the potent inhibitory effects of conditions of energy deficit on gonadotropin secretion, therefore suggesting the convergence of α-MSH and kisspeptin signaling in the central control of the HPG axis.

The fact that central melanocortin (MC3/MC4) receptor activation could bypass the suppression of basal LH levels in pubertal animals is compatible a role α-MSH as transmitter for the reproductive effects of peripheral metabolic regulators of the HPG axis, such as leptin, whose levels are suppressed in conditions of negative energy balance linked to inhibition of the reproductive system. Indeed, this was further confirmed by our physiological experiments in female rats that revealed that the permissive effects of leptin on puberty onset, as monitored by the standard phenotypic index (namely, vaginal opening), could be prevented by the concomitant blockade of central α-MSH signaling. Of note, assessment of the effects of leptin on puberty onset, and its interplay with melanocorting signaling, was conducted in a model of chronic subnutrition, in which moderate caloric restriction during the

pubertal transition induces the lowering of endogenous leptin levels and prevents normal pubertal maturation. This model has been previously used by our group in experimental studies on the metabolic control of puberty, as this background permits to uncover the permissive/stimulatory effects of leptin. Of note, our previous studies have revelaed that the permissive effects of leptin on puberty onset are also blocked after inhibition of central mTOR signaling [398]. While the magnitude of the reported effects of the mTOR inhibitor on the HPG axis probably exceeds that of the melanocortin receptor antagonist used in the present study, the fact that blockade of these two signaling systems equally prevents the permissive action of leptin is strongly suggestive of some degree of convergence. In fact, previous studies have revealed that mTOR signaling is controlled by energy status and leptin in specific regions of the hypothalamus and colocalizes with POMC neurons in the ARC, thereby playing a major role in body weight homeostasis. Altogether, our previous and current data are compatible with a leptin-mTOR-MSH pathway, coverging on POMC neurons, as key element for the metabolic regulation of puberty onset.

Our study also surfaces a relevant α-MSH$\rightarrow$kisspeptin regulatory pathway with a putativelty prominent role in the regulation of puberty. Considering previous electrophysiological and expression data in adulthood, which fragmentarily pointed out a potential bidirectional interplay between these two systems, hormonal and expression studies were implemented in this Thesis to test the feasibility and physiological relevance of such bidirectional interaction. Our data strongly suggest that kisspeptins are very unlikely to operate, at least for their LH-releasing effects, via modulation of the α-MSH output to GnRH (or other intermediary) neurons, as illustrated by the fact that the potent LH responses to (even) submaximal doses of kisspeptin-10 were fully preserved in spite of effective blockade of MC3/MC4 receptors. These results are not compatible with the previous proposal of a major regulatory role of kisspeptins on POMC neurons; yet, it must be stressed that such hypothesis was solely based on electrophysiological recordins in adult mice. Hence, our current experiments cast doubts on a physiological translation of such electrical responses into GnRH regulation in vivo. This is in good agreement with unpublished data from our group showing that LH responses to kisspeptin-10 are fully preserved in

mice engineered to lack Gpr54 selectively in POMC neurons. Admittedly, however, it is possible that direct effects on POMC neurons might serve additional functions of kisspeptins, as their proposed anorectic effects, although the physiological relevance of such feeding-suppressing actions of kisspeptins is yet to be demonstrated.

In contrast, Kiss1 neurons appear to play a central role in transmitting the stimulatory effects of melanocortin signaling onto the reproductive centers. This is nicely documented by (i) the severe attenuation of LH responses to MT-II in mice with congenital inactivation of the kisspeptin receptor, Gpr54; and (ii) the significant suppression of *Kiss1* mRNA expression in the ARC of pubertal females subjected to chronic blockade of MC3/MC4 receptors; i.e., a protocol that was previously shown to inhibit/delay puberty onset.

Of note, blunted, but detectable responses to the MC3/MC4 receptor agonist were observed in Gpr54 null mice. This finding suggests that part of the stimulatory effects of α-MSH on GnRH secretion is independent on kisspeptin signaling. Indeed, as mentioned earlier in this Thesis, direct electrical responses to α-MSH and MT-II have been recorded in GnRH neurons [305]; our findings do not refute and are fully compatible with such direct effects, as part of the stimulatory actions of α-MSH on the gonadotropic axis. Yet, our data clearly illustrate the striking impact of the lack of kisspeptin signaling upon GnRH/gonadotropin responses to α-MSH activation. In the same vein, our ISH results illustrate a rather dramatic drop of *Kiss1* mRNA expression in the ARC following inhibition of central α-MSH signaling. Previous studies in the sheep had illustrated divergent, nucleus-specific *Kiss1* expression responses to activation with MT-II. Intriguingly, however, those studies suggested that while melanocortins stimulate *Kiss1* expression in the preoptic area, they actually down-regulated *Kiss1* mRNA levels in the ARC; the latter response to the agonist is at odds with the decrease in ARC *Kiss1* expression following antagonist administration, as reported here. It must be stressed, though, that previous studies were conducted in the adult sheep and involved the pharmacological activation of MC3/MC4 with an exogenous compound, rather than the blockade of the endogenous receptors. Thus, we are persuaded that, beyond obvious differences in experimental conditions, our present results are endowed with a notable physiological interest and, together with

our hormonal data, are strongly suggestive of an unidirectional ARC α-MSH→Kiss1/ kisspeptin pathway, which might integrate upstream signals (e.g., leptin) and would play a central role in the metabolic regulation of puberty onset.

As discussed earlier in this Thesis (see Section 1.6), mice with genetic inactivation of MR3 and MC4 receptors, while not being infertile, display a variable degree of fertility problems. Thus, MC3-R KO female mice are subfertile, while MC4-R null mice, although fertile, are poor breeders and show decreased ovulatory rates [310]. Altogether, these observations in mouse models of congenital deficiency of MC3 or MC4 receptors suggest that although α-MSH signaling might not be indispesanble to achieve reproductive competence, this system actually plays a discernible role in the control of some functional aspects of the HPG axis. In any event, it must be stressed that in the above null mouse lines detailed characterization of puberty was not conducted, and it must be remembered that congential KO models may suffer some developmental compensation of the nullified pathway; a possibility that is specially tenable in partially redundant systems, such as MC receptors, when only one element of the family (MC3-R or MC4-R) is congenitally ablated. Notably, mice with functional blockade of both MC3-R and MC4-R, by overexpression of the endogenous antagonist, AgRP, have been shown to be infertile [309], whereas ablation of AgRP rescues fertility in leptin receptor deficient, *db/db* mice [315]. As a whole, the above data are suggestive of a relevant role of melanocortin signaling in the control of the adult HPG axis. Our data expands these previous observations and documents for the first time the physiological relevance of a leptin→α-MSH→Kiss1 pathway for the metabolic control of puberty.

5.2. CHARACTERIZATION OF PATTERNS OF EXPRESSION OF miRNAs AND RELATED FACTORS DURING PUBERTAL MATURATION

As mentioned above, it seems clear that the increase in secretion of GnRH, required for the initiation of puberty, is driven by the coordinated changes of neuronal inputs to GnRH neurons of excitatory and inhibitory nature, in conjunction with glial stimulatory signals [149]. Recent studies have led to the hypothesis that puberty is

brought about by the concerted activation of central (hypothalamic) gene networks, in which central regulatory hubs and many subordinate genes interplay for the fine control of puberty onset [159]. According to this concept, these coordinated changes in network activity would ensure proper timing of the pubertal process [399, 400]. The existence of regulatory systems capable to integrate at different levels the functional output of such networks appears mandatory for the precise control of puberty, but these systems remain to be identified.

In this context, miRNAs might be especially a good candidate to exert these functions, because of their ability to modulate gene expression at multiple hierarchical levels [162, 163]. In this Thesis, we tried to address this question by providing an initial characterization of the potential roles that miRNAs may have in the central control of puberty. Considering recent GWAS and functional genomic data [164-167, 401], we focused our work on the Lin28/let-7 system and, in addition, included in our analyses additional related factors of this regulatory pathway, such as c-Myc, mir-145, mir-9 and mir-132; the latter two were identified in our group as putative regulators of the hub using several bioformatic algorithms (see **Figure 17**).

Consistent with a role of the Lin28/let-7 system as one of the factors involved in the control of puberty, *Lin28* and *Lin28b* expression was detected in key reproductive tissues in both rat and mouse. Our data showed that *Lin28* mRNA is prominently expressed only in placenta, testis, ovary and pituitary of adult rats, with modest expression in the hypothalamus. Likewise, *Lin28b* mRNA is also expressed in adult rat testis and placenta, however, contrasting with *Lin28,* substantial hypothalamic *Lin28b* mRNA levels were observed in adult rats, whereas no expression was detected in the ovary. Our data in adult mouse showed that *Lin28* is also prominently expressed in the testis and weak expression was detected in pituitary and ovary. Similarly, *Lin28b* mRNA was abundantly expressed in adult mouse testes and hypothalamus, whereas modest expression was observed at the pituitary and weak expression in the ovary. Taking into account the putative relationship, as predicted by different GWAS, between *Lin28b* and the age at menarche [164-167] and recent works suggested a role for Lin28/let-7 axis in fertility and spermatogenesis [380, 381], along with the lack of expression of *Lin28b* in rat ovary and the weak expression of *Lin28* and *Lin28b* in mouse ovary, we

focused our expression analyses on the hypothalamus and the testis of rats and testis of mice.

5.2.1. HYPOTHALAMIC STUDIES

Our study documented for the first time that *Lin28* and *Lin28b*, as well as *c-Myc*, mRNAs are abundant in the rat hypothalamus of both sexes during the neonatal period, declining strikingly during the infantile-to-juvenile transition. The fact that a similar trend was not observed in other brain areas, such as the cortex, where *Lin28b* mRNA levels increased between the neonatal and the pubertal period, suggests that postnatal loss of *Lin28b* expression is specific to the hypothalamus. Recently, it has been demonstrated that hypothalamic *Lin28b* mRNA levels show a similar profile in female rhesus monkeys during postnatal development [376], suggesting that this maturational change is evolutionary conserved.

In contrast to the decline observed in the hypothalamic expression of *Lin28/Lin28b*, *let-7* (mainly, *let-7b*), *mir-132* and *mir-145* miRNAs levels displayed opposite profiles, with minimal neonatal expression levels and progressive increase along postnatal maturation in both male and female rats. These results are coherent with previous studies showing that *mir-145* suppresses *c-Myc* expression [375], resulting in reduced *Lin28/Lin28b* transcription and derepression of *let-7* family maturation. Moreover, a recent study showed that seven of the eight miRNAs of the *let-7* family are highly expressed in ARC and PVN of adult rats [402]. The above developmental profile is reminiscent of that found in *C. elegans*, in which high levels of *Lin28* at the beginning of first larval stage (L1) are followed by an abrupt drop at the end of the L1 phase to allow progression to late larval stage, while *let-7* levels increase progressively to reach maximum values at the adult stage [347]. Moreover, overexpression of *let-7* at early stages of worm development causes premature adoption of adult fates, while *let-7* underexpression results in failure to terminally differentiate at the larval to adult transition. Altogether, these data suggest that the level and timing of mature *let-7* miRNA expression, in a reciprocal equilibrium with *Lin28*, plays a vital role in defining key aspects of development in C. elegans [347]. An inverse relationship between the expression levels of *Lin28* and *let-7* had been also

reported in mammals, suggesting that Lin28 is an important regulator of *let-7* biogenesis across species [348, 365, 369, 370, 403].

We also analyzed the changes in hypothalamic levels of *mir-132* and *mir-9* miRNAs, two putative negative regulators of *Lin28b.* We observed that *mir-132* levels increased gradually during postnatal/prepubertal maturation, in keeping with a potential role of this miRNA in the negative regulation of Lin28b. In contrast, *mir-9* miRNA levels were the highest during the neonatal period, declining gradually thereafter. This expression pattern suggests that *mir-9* does not play a major role in repressing hypothalamic *Lin28/Lin28b* expression during postnatal maturation. Instead, *mir-9* may be involved in the regulation of neurogenesis and maturational events at earlier fetal stages of brain development [404, 405].

In order to provide further evidence for a role of the Lin28/let-7 system in the development of the hypothalamic systems leading to the initiation of puberty, we conducted a series of expression analyses using different preclinical models of disturbed puberty.

As first model, we used rats in which sexual differentiation of the brain had been altered by neonatal exposure to high doses of estrogen or androgens. Consistent with previous studies [388], these treatments induced persistently decreased LH and FSH levels and prevented normal puberty onset. We observed that hypothalamic levels of *c-Myc* and *Lin28b* mRNAs were enhanced in these animals, while *let-7* and *mir-145* miRNA levels were decreased at the expected time of puberty, similar to the high *Lin28* and low *let-7* levels detected in the hypothalamus of immature animals. The association between enhanced *Lin28* expression and delayed puberty observed in rats exposed to high sex steroid levels during neonatal period is in agreement with the delay of puberty onset reported in *Lin28a* Tg mice [27], thus supporting the idea that decreasing hypothalamic *Lin28* expression is permissive for the correct timing of puberty. A discordant finding is the sex difference in *mir-9* and *mir-132* miRNA levels after neonatal estrogenization. Whereas in males both *mir-9* and *mir-132* miRNA levels diminished with estrogenization, no such changes occurred in females rats treated with EB. In fact, *mir-132* levels increased in females treated with TP. The physiological importance of these differences remains to be established.

We also observed a trend for an increased ratio of *Lin28/let-7* expression in male and female rats with delayed puberty caused by early manipulation of the photoperiodic conditions. Thus, animals subjected to CD during between PND-10 and -15 not only displayed delayed puberty onset, but also disturbed expression levels of *Lin28* transcripts and several miRNAs in the hypothalamus at different stages of postnatal development. Like the immature rats and neonatally estrogenized/ androgenized animals, *Lin28b* and *c-Myc* mRNA levels were significantly increased in the hypothalamus of CD male and female rats, whereas *let-7a* and *let-7b* miRNA levels were decreased. However, a sex dimorphism was detected for the time course of these changes, so that altered *Lin28/let-7* ratios were detected in infantile, but not peripubertal males, whereas in females such alteration was only detectable around the time of puberty. As observed in rats exposed to high doses of sex steroids during neonatal period, *mir-132* and *mir-145* miRNA levels, but not those of *mir-9*, were decreased in male and female rats exposed to CD. These data suggesting that delayed puberty caused by either altering neonatal sex differentiation of the brain or disrupting photoperiod signals during infantile development is associated with alterations of the *Lin28/let-7* regulatory system, a finding that indirectly supports a tentative inhibitory role of this system on the timing of puberty. The functional relevance of these changes, and of their temporal differences, for the alteration of puberty observed after photoperiodic manipulation requires further investigation.

In clear contrast, altered puberty due to manipulation of the metabolic/feeding status did not cause comparable changes. Thus, *let-7a* levels decreased in males during the neonatal period, but increased in infantile females reared in large litters, whereas hypothalamic *let-7b* miRNA levels remained unchanged in male and female rats subjected to early subnutrition. Likewise, while *mir-132* levels were lower in pubertal female rats subjected to postweaning undernutrition, *mir-145* miRNA levels in the hypothalamus displayed opposite profiles between males and females subjected to postweaning underfeeding. Therefore, neither early subnutrition, through rearing large litters, nor a condition of negative energy balance around puberty, which had caused delayed puberty onset, altered the hypothalamic *Lin28/let-7* system as did early androgenization, estrogenization and CD conditions. However, these

observations strongly suggest that, in spite of conditions of early metabolic stress causing pubertal alterations can perturb the expression of some of the elements of the *Lin28/let-7* system in the hypothalamus, the underlying mechanisms and cellular substrate for such alterations are likely different from those caused by early manipulations of brain sex differentiation or photoperiod.

System biology approaches have allowed identification of a putative gene network operating within the female hypothalamus to control the initiation of puberty. Among the elements of this network, a set of tumor suppressor genes [406], including enhanced at puberty protein1 (*EAP1*) [399, 407], the *POU* gene, octamer transcription factor 2 (*OCT2*) [400], was shown to play a major role in the timing of puberty. Hypothalamic TSG networks are hierarchically controlled by specific regulatory centers. One of the predicted main hubs is p53, whose expression has been shown to increase in the hypothalamus during the pubertal transition in female rhesus monkeys [159]. Importantly, p53 is a tumor suppressor that negatively regulates *c-Myc*, while it induces the transcription of *mir-145*, which in turn represses a number of oncogenes, including *c-Myc* itself [375, 385]. Given the positive regulatory loop between *c-Myc* and *Lin28/Lin28b* expression [372, 373], it is possible that the decline in *c-Myc/Lin28* expression along postnatal maturation observed in our analyses can be caused by the increase in p53 and hence, *mir- 145*, that takes place during postnatal development. In turn, decreased levels of *Lin28* might cause the observed increase in *let-7* miRNA levels. Because bioinformatic analysis predicts that let-7 or related miRNAs regulate *OCT-2* and *EAP1*, the latter only is regulated by *let-7e* and not by several members of the let-7 family, these genes might serve as portals conveying the regulatory actions of the *Lin28/let-7* system to the neuroendocrine complex that controls the timing of puberty. Since *let-7* and other miRNAs are considered to be tumor suppressors, it is tempting to assume that they represent an additional component of the regulatory TSG network postulated to be involved in the physiological regulation of puberty.

5.2.2. TESTICULAR STUDIES

In mammals, the spermatogenesis is a complex event involving numerous cell types and a large range of specific regulatory factors, which act at the transcriptional

or posttranscriptional levels and are essential for the adequate control of male gametogenesis. Among these factors, miRNAs, which mainly exert post-transcriptional control the stability or translation of their target messenger RNAs [162], have emerged with force in last years [408].

Recent studies have reported the expression of *let-7* family in rodent testis [409, 410] and have suggested a role for lin28/*let-7* system in fertility and spermatogenesis. Thus, it has been demonstrated that *Lin28* KO mice compromises the size of the germ cell pool in mice both males and females by affecting primordial germ cell proliferation during embryogenesis leading to reduced fertility in adults [381]. Moreover, *Lin28* KO males have altered levels of FSH and testosterone [381]. In the same line, the embryonic overexpression of *let-7* provoked a reduction of the germ cell pool [381] and *Lin28* transgenic mice showed delayed puberty [378]. Moreover, it has been reported that conditional knockout models of Lin28 in adult germline stem cells showed reduced testis weight, sperm number and impaired spermatogonial cell proliferation without compromising their differentiation capacity [382].

At the initiation of this Thesis, little was known on the testicular roles of the Lin28/let-7 system as it relates to pubertal maturation and no studies existed about Lin28/Lin28b and let-7 distribution in rodent testis. Our results demonstrate that *Lin28* and *Lin28b* mRNAs are abundant in the rat and mouse testis and their profiles of expression along male gonadal maturation are roughly similar in both species. Thus, *Lin28* and *Lin28b* mRNAs displayed low testicular expression during the neonatal period, and both markedly increased from the birth to the infantile period. However, while *Lin28* mRNA levels showed a subsequent decreased from the infantile period to adulthood where levels were similar to the newborn, *Lin28b* mRNA levels remained high from infantile to adulthood.

In order to extend our previous observations, IHC analyses to determine protein distribution of Lin28 and Lin28b in adult mouse testis were carried out. Our data demonstrated that Lin28 and Lin28b proteins, as evaluated by IHC in mouse testes, display totally different distribution expression protein patterns. Thus, they did not show overlapping in terms of tissue compartments (germ cells vs. interstitial cells), stages and cell types within the seminiferous epithelium (spermatogonia vs

spermatids), and even preferential intracellular location (cytoplasmic vs nuclear). Notably, regarding Lin28, our analyses detected this factor in all type-A spermatogonia, which is in partial contrast with previous findings that suggested that Lin28 is restricted to undifferentiated (As-Aal) spermatogonia and absent in differentiating (with the exeption of A1) spermatogonia [379].

Overall, our findings reinforce the view that Lin28 and Lin28b are likely to conduct different roles in the regulation of male gametogenesis and gonadal function during postnatal maturation in rodents. Admittedly, due to technical limitations with the available antibodies, our IHC data are restricted to the mouse testes. Nonetheless, considering that *Lin28* and *Lin28b* mRNA expression profile along postnatal maturation in rats and mice is identical, we consider tenable that Lin28 and Lin28b protein distribution in the rat testis is the same that in mouse. Hence, in adult rat testis Lin28 protein would be expressed in undifferentiated and type-A spermatogonia, whereas Lin28b protein would be expressed in spermatids and Leydig cells.

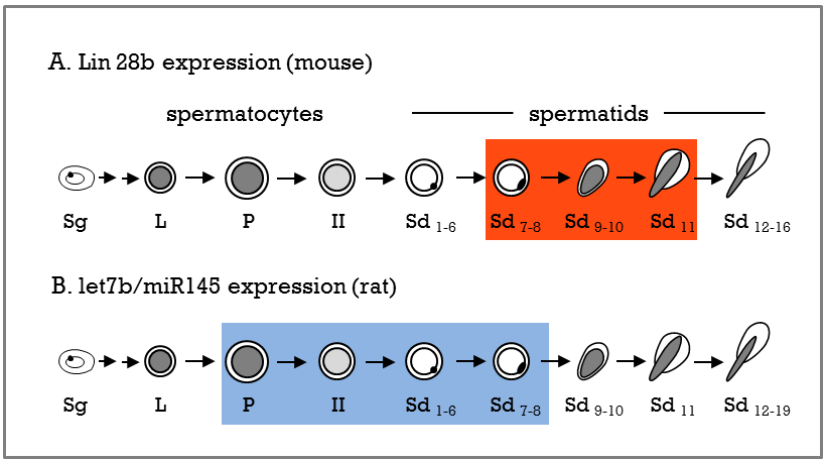

Figure 64. Schematic representation comparing the expression of Lin28b protein in mouse germ cells [410] and let-7b and miR-145 microRNAs in rat germ cells.

In addition to IHC studies in mouse, we analysed by ISH analyses the expression patterns of one member of *let-7* miRNA family, *let-7b,* and *mir-145* in adult rat testis. Our data showed that the expression of *let-7b* was strong in pachytene spermatocytes and weaker in round spermatids from stage I to stage VII, whereas it disappeared in elongating spermatids. In addition, *let-7b* was absent in the interstitial areas. The same expression pattern was detected for *mir-145*, with the exception that it was

strongly expressed also in smooth muscle cells in the interstitial blood vessels, in accordance with previous works [411, 412].

As was mentioned in the introduction of this Thesis, numerous studies have demonstrated that Lin28/Lin28b represses the synthesis of mature *let-7* miRNAs, which in turn are able to suppress Lin28 levels, therefore creating a double-negative feedback loop (see **Figure 17**). Taking into account the patterns of cellular distribution of *let-7b* observed and, if we assume that the cellular distribution of Lin28/Lin28b in testis is the same in the rat and mouse, the differences in this distribution could explain the partial lack of inverse correlation between some of the elements of this system studied in this Thesis. Nonetheless, it is significant that during neonatal period, when *Lin28/Lin28b* mRNA expression showed minimal levels*, let-7, mir-132* and *mir-145* miRNAs expression was maximum, decreasing progressively thereafter. These results are coherent with previous studies showing that *mir-145* suppresses *c-Myc* expression, resulting in reduced *Lin28/Lin28b* transcription and derepression of *let-7* family maturation. Moreover, in germ cells of the adult rat testis we could expect that expression of Lin28 is restricted to spermatogonias, where Lin28b and *let-7b* and *mir-145* expression is absent. When Lin28 expression disappears in spermatocytes, strong expression of both *let-7b* and *mir-145* was detected. Thus, both signals showed weak expression in round spermatids from stage I to stage VII and no expression was detected in elongating spermatids from stage VIII onwards when Lin28b expression appear.

In order to provide further evidence for a role of the Lin28/let-7 system in the testis development and sexual maturation, we conducted a series of expression analyses using various models of perturbed puberty in rats and mice.

Neonatally estrogenized rats, in which their puberty was altered, showed decreased testicular Lin28 and Lin28b mRNA levels, while let-7a, let-7b and mir-145 miRNAs expression levels were enhanced. It is known that these rats present alterations in cellular composition of testes, i.e. spermatogenic arrest with degenerating germ cells and delayed maturation of Sertoli and Leydig cells. Based on this, we can suggest that the latter expression changes could be attributable, at least partially, to impaired development of testicular cell types expressing these genes after

neonatal estrogenization. Likewise the decrease observed in Lin28b mRNA expression levels in rats with delayed puberty caused by early undernutrition and manipulation of the photoperiodic conditions, also would be a consequence of alterations in cellular composition, probably by a decrease in the number of spermatides.

In order to further analyse gonadotropin regulation of testicular *Lin28/let-7* system, HPX rats with or without gonadotropin replacement were used. Our data showed that deprivation of pituitary hormones enhanced *Lin28* mRNA expression levels but replacement with human hCG or FSH, completely restored the *Lin28* mRNA expression and the combined use of FSH and hCG had synergic effects. However *Lin28b* mRNA expression levels diminished considerably by HPX but hormonal replacement did not restore this levels. Anyhow, a minor increase after treatment with FSH alone or in combination with hG was deteted. Regarding the differet miRNA expression levels, it was detected that *let-7a*, *let-7b* and *mir-145* remained unchange by HPX. However, expression levels increased after the FSH and hCG replacement and, this increased was marked, after the combined use of both hormones. On the other hand, *mir-132* miRNA levels diminished in animals HXP compared with intact rats, and their expression levels increased significantly, with higher values to those of control rats, after treatment with FSH or hCG. The combined administration of both hormones caused additive effects on *mir-132* miRNA expression.

In the normal adult rat testis there is a complex interaction between Leydig Sertoli and germ cells. Leydig cells drive spermatogenesis since LH stimulates the Leydig cells to secrete testosterone, which acts on the Sertoli cells to produce an environment which enables normal growth and division of the testicular germinal cells. In turn, FSH stimulates Sertoli cells; without this stimulation, the conversion of the spermatids to sperm would not occur. In rats, long-term HPX provokes atrophy of all testicular compartments [413, 414]. The alterations in Leydig cells and germ cells together with the partial recovery of these cells after treatment with hCG and/or FSH, as well as the differential pattern of cellular expression of *Lin28*, *Lin28b* and miRNAs studied would explain the results observed for *Lin28/let-7* system in HPX rats.

Our analyses in Gpr54 KO mice, as model of impuberism and hypogonadotropic hypogonadism, due to the lack of kisspeptin signaling and, hence, low gonadotropin

levels, further supported our findings in control mouse testes. In line with the obvious hypogonadotropism of Gpr54 null mice, these animals failed to show the prototypical increase in tubular diameter during pubertal maturation, suffered spermatogenic arrest and lacked mature Leydig cells in the testicular interstitium, as was previously described by our group [65].

Regarding Lin28 mRNA and protein expression, it was clearly detectable in the testes of Gpr54 KO mice, where Lin28-IR was observed in undifferentiated and type A spermatogonia, whose appearance and maintenance seem to be independent of gonadotropic stimulation. This latter was further supported by the fact that the pattern of cellular expression of Lin28 in Gpr54 KO testes did not change substantially after the FSH or hCG replacement for one week. In clear contrast, Lin28b-IR was undetectable, and its absolute mRNA levels dropped markedly in Gpr54 KO mouse testes, likely reflecting the lack of mature Leydig cells and spermatids in this model, hence suggesting that testicular expression of Lin28b is gonadotropin-dependent. In fact, gonadotropin administration to Gpr54 null mice rescued such defective expression. Thus, hCG treatment induced the appearance of strong Lin28b immunostaining, coincident with the appearance of hyperthropic Leydig cells and elongating spermatids, whereas the effects of FSH replacement were less robust, with Lin28b-IR being detected only in round spermatids.

The comparison between the testicular expression profiles of *Lin28* and *let-7* transcripts in WT and Gpr54 KO (see **Figure 61**) reveals clear similarities with changes associated to the maturational transition between the infantile and young adult periods (see **Figure 54**), as both Gpr54 KO and immature testes displayed markedly elevated *Lin28*, *let-7b* and, to a lesser extent, *let-7a* levels than control adult values, whereas the relative *Lin28b* mRNA levels were not significantly different. These observations provide a molecular mark for the state of testicular immaturity of Gpr54 null mice and help to dissect out the gonadotropin-dependent vs independent regulation of Lin28 and let-7 expression in the testis.

CONCLUSIONS

6. CONCLUSIONS

In the present Thesis we set out: *1)* to investigate the role α-MSH signaling in the control of puberty onset, as well as to characterize its potential interaction with leptin and kisspeptin pathway; and *2)* to characterize the hypothalamic and testicular profiles of expression of the Lin28/let-7 system during pubertal maturation and to study the changes in their expression patterns in preclinical models of altered puberty. The main conclusions of our studies are the following:

- Central activation of α-MSH signaling stimulates LH responses (as surrogate marker of GnRH secretion) in peripubertal, but not in infantile rats; this stimulatory action is conducted preferently via MC4-R.
- Central inactivation of melanocortin signaling during the pubertal transition disrupts the normal timing of puberty, suppresses *Kiss1* expression in the ARC and blocks the permissive effects of leptin on puberty onset.
- The stimulatory actions of α-MSH on LH secretion at puberty are, to a large extent, dependent on preserved kisspeptin signaling, while kisspeptin effects on LH are independent of central melanocortin signaling. These findings, together with the expression and pharmacological data summarized above, are suggestive of a leptin→α-MSH→Kiss1 pathway with a relevant role in the (metabolic) control of puberty
- During prepubertal maturation, there is marked decline in hypothalamic *c-Myc*/*Lin28/Lin28b* expression coupled to significant increases in *let-7*, *mir-145* and *mir-132* miRNA levels; these changes do not occur at other brain sites, such as the cortex. The above trends in hypothalamic expression are perturbed in situations of delayed/disrupted puberty, due to either neonatal exposure to sex steroids or infantile exposure to constant darkness, suggesting that the inverse changes in hypothalamic expression of the above elements of the Lin28/let-7 system may be permissive for and/or drive the onset of puberty.
- Lin28 and Lin28b display clearly distinct patterns of RNA expression and cellular distribution in rodent testes, suggesting specific, non-redundant functions of each Lin28 RNA-binding protein in the control of testicular development and/or function.

- Inverse changes in *Lin28b* and *let-7a/let7-b* expression are detected in rodent testes along postnatal maturation, but these are opposite to those observed in the hypothalamus, with increases in *Lin28b* mRNA and decreases in *let-7a/let7-b* miRNA levels. The testicular expression levels of these (and related) targets are perturbed in rat and mouse models of altered puberty and under the regulation of pituitary gonadotropins.

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