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Participación del núcleo supraquiasmático en la regulación de la ovulación en días diferentes al proestro

TESIS

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Index

List of Acronyms

ACTH	Adrenocorticotropic Hormone
AVPv	Anetroventral Periventricular Area
AHA	Anterior Hypothalamic Area
ARC	Arcuate Nucleus
AVP	Arginine Vasopressin
ACSF	Artificial Cerebrospinal Fluid
Bmal1	Brain and Muscle ARNT-like Protein 1
CaMKs	Ca2+/calmodulin-dependent Protein Kinases
CKIε	Casein Kinase Iε
Clock	Circadian Locomotor Output Cycle Kaput
CG´S	Clock-Controlled Genes
Cry	Cryptochrome
DMH	Dorsomedial Nucleus of the Hypothalamus
E2	Estradiol
ER	Estradiol Receptor
ERE	Estrogen Response Element
ERK	Extracellular Signal-Regulated Kinases
FSH	Follicle-Stimulating Hormone
GABA	Gamma Amino Butiric Acid
GPR147	GnIH/RFRP-3 receptor
GnIH	Gonadotropin Inhibitory Hormone
GnRH	Gonadotropin Releasing Hormone
hCG	Human Corionic Gonadotropin
IGL	Inter Geniculate Leaflet
GPR54	Kisspeptin Receptor
LH	Luteinizing Hormone
MS	Medial Septum
ME	Median Eminence
MAPK	Mitogen-Activated Protein Kinases
OVLT	Organum Vasculosum of the Lamina Terminalis
OVX	Ovariectomized
Per	Period
PMSG	Pregnant Mare Serum Gonadotropin
POA	Preoptic Area
RHT	Retino-Hypotalamic Tract
RFRP	RFamide related peptides
RP3V	Rostral Periventricular Region of the Third Ventricle
SCN	Suprachiasmatic Nucleus
TTX	Tetrodotoxin
TSH	Thyroid Stimulant Hormone
VIP	Vasoactive Intestinal Polypeptide

Abstract (Spanish)

La ovulación es un proceso inflamatorio que resulta en la degradación de la pared folicular y, por lo tanto, en la liberación de un ovocito maduro que puede ser fertilizado por el gameto masculino, originando a un nuevo individuo. Esto depende de la exquisita coordinación del eje hipotálamo-hipófisis-ovario. En los roedores, la ovulación ocurre durante la mañana del estro en respuesta a pico pre-ovulatorio de gonadotropinas que se desencadena durante la tarde del proestro. Esto promueve cambios en la vasculatura del folículo, en la actividad esteroidogénica de sus células y en la síntesis de las proteasas que degradarán al folículo. El pico pre-ovulatorio es regulado por diversos sistemas sensoriales que aseguran que suceda en el momento más ventajoso del día o del año. Se ha mostrado que información nerviosa indispensable para la ovulación es generada todos los días entre las 14:00 y las 17:00 horas, el llamado periodo crítico. Se piensa que estas señales desencadenan el pico pre-ovulatorio cuando convergen con una alta concentración de estradiol, lo que indica la presencia de folículos maduros. Según estudios de lesión y desaferentación, estas señales diarias son generadas en el núcleo supraquiasmático (SCN), principal oscilador circadiano. En nuestro primer experimento nos propusimos determinar si las señales nerviosas generadas en días diferentes al proestro participan en la regulación de la ovulación. Ratas hembra fueron implantadas con cánulas guía bilaterales dirigidas al SCN y, después de la recuperación de la cirugía, fueron microinjectadas a las 14:00 horas del estro, metaestro, diestro o proestro con tetrodotoxina (TTX), un inhibidor de los potenciales de acción dependientes de sodio. Encontramos que la inhibición transitoria de la actividad nerviosa del SCN durante el periodo crítico de cada etapa resulta en el bloqueo de la ovulación, pero no en alteraciones del ciclo estral. Esto sugiere que, además de su papel en el desencadenamiento del pico pre-ovulatorio, el SCN participa en la preparación del sistema para responder a señales estimulantes. El núcleo arqueado (ARC) está implicado en la regulación de la secreción tónica de las gonadotropinas que dirige el desarrollo de los folículos ováricos antes del proestro. Contiene neuronas que censan los cambios en la concentración de estradiol y de moléculas relacionadas con el estado metabólico. Además, envía y recibe proyecciones axonales del SCN. Considerando esto, pensamos que participa en la regulación circadiana de procesos peri-ovulatorios que suceden antes del proestro. La actividad nerviosa del ARC fue inhibida a las 14:00 horas siguiendo la metodología descrita para el primer experimento. Encontramos que esta estructura no participa en la regulación de la ovulación durante el estro. Por el contrario, la microinyección de TTX en metaestro o diestro alteró el ciclo estral y bloqueó la ovulación. En proestro, el mismo tratamiento bloqueó la ovulación sin alterar el ciclo estral, pero sí la secreción de estradiol, como demuestra la distención uterina. En conjunto, nuestros resultados indican que las señales nerviosas circadianas que ocurren en el periodo crítico de cada etapa del ciclo estral regulan otros procesos además del desencadenamiento del pico pre-ovulatorio de gonadotropinas. Sugerimos que están involucradas en la preparación del sistema para responder al incremento en la concentración de estradiol.

Abstract

Ovulation is an inflammatory-like process that results in the degradation of the follicular wall and hence in the release of a mature oocyte that can be fertilized by the male gamete to produce a new individual. This is dependent on the exquisitely coordinated activity of the hypothalamic-pituitary-ovarian axis. In rodents, ovulation occurs during the early morning of estrus in response to the pre-ovulatory surge of gonadotropins that is triggered in the afternoon of proestrus. This promotes changes in the vasculature of the follicle, in the steroidogenic activity of their cells and also in the synthesis of the proteases that eventually degrade the follicle. The pre-ovulatory surge is under the control of several sensory systems that ensure its occurrence at the most advantageous time of the day or the year. In this sense, it has been shown that neural information that is pivotal for ovulation is generated every day between 14:00 and 17:00 hours, the so called "critical window". It was hypothesized that such signal triggers the pre-ovulatory surge only if the concentration of estradiol was high, indicative of the presence of mature follicles. According to lesion and deafferentation studies, the daily neural signals were generated in the suprachiasmatic nucleus (SCN), the main circadian oscillator. In our first experiment we sought to determine if the neural signals produced in days other than proestrus also participate in the regulation of ovulation. Female rats were implanted with bilateral guide cannulas aiming to the SCN and, after recovery from the surgery, where microinjected at 14:00 hours of estrus, diestrus, metestrus or proestrus with tetrodotoxin (TTX), an inhibitor of the sodium-dependent action potentials. We found that the transitory inhibition of the SCN´s neural activity at the critical window of each stage results in the blockade of ovulation, but not in alterations of the estrous cycle. This suggest that the SCN participates in the preparation of the system to respond to stimulant signals, in addition to its role triggering the pre-ovulatory surge. The arcuate nucleus (ARC) is implicated in the regulation of the tonic secretion of gonadotropins that drives the development of the ovarian follicles before proestrus. It contains neurons that sense the changes in the concentration of estradiol and molecules related to the metabolic state. In addition, sends and receives axonal projections from the SCN. Considering this, we hypothesized that it participates in the circadian regulation of the peri-ovulatory processes that occurs before proestrus. The neural activity of the ARC was inhibited at 14:00 hours following the methodology described for the previous experiment. We found that this structure does not participate in the regulation of ovulation in estrus. Contrary to this, TTX microinjection in metestrus or diestrus disrupts the estrous cycle and blocks ovulation. In proestrus, the same treatment blocks ovulation without altering the estrous cycle, but the secretion of estradiol was altered, as measured by the uterine distension. Taken together, our results indicate that the neural circadian signals that occurs between 14:00 and 17:00 hours of each stage of the estrous cycle regulate processes other than the triggering of the pre-ovulatory surge of gonadotropins. We suggest that they are involved in the preparation of the system to respond to the increasing levels of estradiol.

Foreword

Reproduction is one of the inherent characteristics of life, just as an internal organization, the capability of undergo metabolism, to grow and develop. From the simplest unicellular organisms to mammals, every lifeform inhabiting our planet has developed reproductive strategies to ensure the continuity of its own species. Evolution by natural selection, which is only possible through reproduction, enhanced such continuity allowing every generation to respond to the pressures of the environment and hence to adapt to an ever-changing world. All animal species, even though many practices asexual multiplication in the wild, can reproduce sexually. This kind of reproduction depends on the production of haploid gametes by meiosis and their interaction during fertilization, which gives origin to the zygote, a diploid cell that contains a mixture of the genetic information from both progenitors and will eventually generate all the somatic and germinal cells of the new organism. As it can be inferred, all the processes involved in sexual reproduction have evolved to be displayed with coherence with the environment in which each species lives, increasing their chances to produce offspring.

In mammals, the production of gametes occurs in the gonads under the regulation of endocrine and neural information arriving by blood vessels and nerves, respectively. The anterior pituitary is responsible for driving the functions of the ovaries and testes by the secretion of stimulant hormones known as gonadotropins. This is in turn regulated by the hypothalamus through the Gonadotropin Releasing Hormone (GnRH), an ancient neuropeptide that can be traced back to basal invertebrates. It is believed that GnRH is the last stimulant pathway that arises in the brain to regulate the activity of the gonads (although this line of thinking does not consider the role of the peripheral nerves that reach the gonads). In order to coordinate reproduction with the external world, the brain integrates information from the environment and orchestrates the occurrence of specific patterns of GnRH secretion at very specific moments of the reproductive cycle. It is not a surprise that early observations pointed towards a close relationship between the reproduction of animals and the time of the day, after all, animals have been exposed to the selective pressures derived from the alternation of days and nights that result from the rotation of the Earth.

In 1949, based on the data from lesion and stimulation studies, John Everett presented a diagram in a local seminar containing the theory that he developed with Charles Sawyer about a 24-hour periodicity governing the secretion of gonadotropins. The diagram contained a putative 24-hour clock that, in Everett´s words, "*Should be close to the optic chiasm, in the anterior hypothalamus*". Twenty-three years later two research groups demonstrated that the small nucleus lying just above the optic chiasm was indeed the biological clock that regulates circadian rhythms in mammals: the SCN. This nucleus was first discovered during the dissection of human brains at the end of the XIX century and then described in the rat in 1927, however, scientists waited almost fifty years to know its role in the temporal regulation of physiology and behavior. The seminal works by Everett and Sawyer, and the "discovery" of the SCN as a circadian oscillator, inspired a great amount of research on the circadian regulation of reproduction that is still in progress today. The importance of such studies was

immortalized in the book "Pioneers in neuroendocrinology" by Joseph Meites, in which the original story and diagram are available. Today it is clear that processes as the secretion of hormones, production of gametes and sexual behaviors are under circadian control, however much is still awaiting to be discovered in order to fully understand the temporal regulation of reproduction. The aim of the theoretical and experimental work contained in this thesis is to contribute to this endeavor.

This document is divided into five chapters, the first and second serve as a theoretical introduction that will give the lector an overview on the relationships between the female reproductive and circadian systems. In the general introduction entitled "*The Gonadotropin Releasing Hormone and the regulation of ovulation*", we explored the regulation of the hypothalamic-pituitary-ovarian axis by classic and novel pathways that leads to the development of the ovarian follicles and the eventual ovulation. A discussion of the adaptive significance of a timely regulated reproduction, as well as the molecular, anatomical and physiological basis that makes it possible is presented in chapter two. This section has a particular focus on the evidences of circadian influences on the regulatory mechanisms of ovulation that occurs during each stage of the estrous cycle and is based on our review article: "*Clock control of mammalian reproductive cycles: looking beyond the pre-ovulatory surge of gonadotropins*" (Reviews in Endocrine and Metabolic Disorders, December 2019, 21(1):149-163). This section ends by setting the question, hypothesis and corresponding objectives that served as an axis for the experimental section of this thesis.

The next two chapters summarizes the experimental approaches and the new findings derived from this study. First, in chapter three, we describe our investigation regarding the involvement of the suprachiasmatic nucleus on the regulation of ovulation during a "critical window" that occurs on the afternoon of each stage of the estrous cycle. This is based on the research article: "*A neural circadian signal essential for ovulation is generated in the suprachiasmatic nucleus during each stage of the estrous cycle*" (Experimental Physiology, November, 2019, 105(2):258-269). Since the arcuate nucleus regulates the tonic secretion of GnRH during the early estrous cycle, and it stablishes a functional crosstalk with the SCN that is necessary for the proper allocation of metabolic resources for reproduction, in chapter four we explored its participation on the regulation of ovulation during the "critical window" of each stage of the estrous cycle. This is based on a manuscript that will be sent for publication briefly: "*The neural activity of the arcuate nucleus is required during the critical window for a proper progression of the rat estrous cycle and ovulation*" (To be published).

Finally, in chapter five we discuss and integrate our findings with the available body of information about the circadian regulation of the estrous cycle, the secretion of gonadotropins and ovulation. We offer a plausible model to explain the participation of the SCN and the ARC in such processes. We hope that the studies contained on this thesis will highlight the complex nature of the regulation of ovulation, dragging attention to the many regulative steps that occur on each stage of the estrous cycle, changing as a function of the time of the day, to prepare the system to ovulate at the right moment, in harmony with the internal milieu and the external world.

Chapter I

Chapter I. To be published:

General introduction

"The Gonadotropin Releasing Hormone and the regulation of ovulation"

Carlos-Camilo Silva

Introduction

Ovulation is a process by which female animals release mature oocytes capable of being fertilized by male gametes and hence it is pivotal for the continuity of all species displaying sexual reproduction. It is regulated by neuroendocrine mechanisms that depends on the proper interaction of the components of the hypothalamus-pituitary-ovary axis. Along with the ovulation, other processes in the ovary as the development of follicles and oocytes and the secretion of hormones are directly regulated by the gonadotropins secreted by the pituitary. The activity of the pituitary is in turn modulated by the GnRH, which is synthesized by hypothalamic neurons. Sex steroids that are secreted by the ovaries exert an inhibitory or stimulant feedback to both the pituitary and the hypothalamus depending on the stage of the reproductive cycle and the time of the day, closing the loop of interactions between the three components (Figure 1, 2A). In addition to this, ovulation also depends on neural information arising at non-GnRH interneurons from hypothalamic and extra-hypothalamic structures in the central nervous system that integrate the information from the environment and from the body to tune the secretion of the GnRH.

GnRH, also known as luteinizing hormone releasing hormone/factor, is a decapeptide first isolated from homogenized samples of pig and sheep hypothalamus in the early 70´s. This neuro-hormone quickly stimulates the secretion of gonadotropins from systemically-injected animals, as well as from whole pituitary explants cultivated *in vitro* (Amoss *et al.*, 1971; Matsuo *et al.*, 1971; Schally *et al.*, 1971). This work led to the Nobel Prize in Physiology or Medicine of 1977 to the leaders of both research groups, Andrew Schalley and Roger Guillemin. The GnRH is synthesized from a larger precursor named pre-pro-GnRH, which contains a signal-peptide sequence followed by the GnRH amino acid chain, a processing sequence and an associated peptide, all encompassed by untranslated 5´ and 3´ regions (Figure 1). Several isoforms of the GnRH have been described in different species and their amino acid sequence is very similar; it has ten amino acids with a highly degree of conservation in the positions 1-4, 9 and 10 (Schneider *et al.*, 2006). In mammals, for example, the GnRH-I isoform has the following composition: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2, which is identical in the pig, sheep, rat, mouse and humans and therefore it was thought that its structure was the same in all animals (King and Millar, 1992).

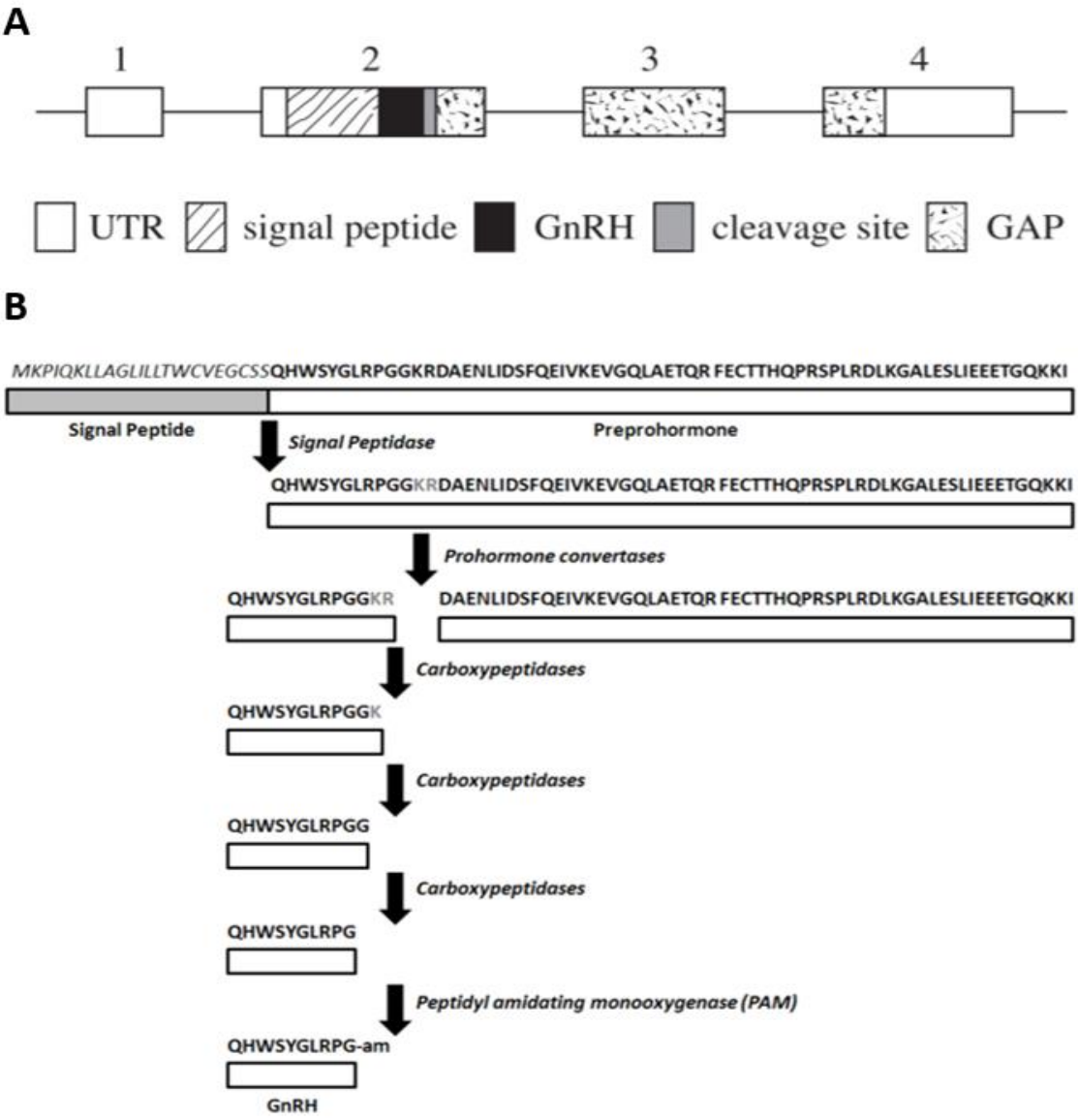

Figure 1. *Structure of the pre-pro-GnRH gene in vertebrates and post-translational modifications. It is composed by four exons (boxes) and three introns (lines connecting boxes). Exon 1 contains an untranslated region (UTR). The signal peptide, GnRH, cleavage site and part of the GnRH-associated peptide (GAP) are encoded in exon 2. The rest of the GAP and an additional UTR region are located in exons 3 and 4 (A). After transcription of the pre-pro-GnRH gene, the mRNA is translated at the ribosomes in the cytoplasm of the cell and then enzymatically processed. The first step involves the removal of the signal peptide by a peptidase, then a convertase cleaves at the dibasic sites encoded by the cleavage site shown in A, separating the GnRH from the GAP. Further processing includes the removal of the C-terminal basic residues by carboxypeptidases, and the amidation of the C-terminus by a peptidyl amidating monooxynease, which results in a mature GnRH decapeptide (B) (Modified from Le et al., 2013; Okubo and Nagahama, 2008).*

The study of the sequence of the GnRH of other vertebrates, however, showed the existence of isoforms. Similar peptides have been also found in other deuterostomes (echinoderms and protochordates), protostomes (nematodes, annelids, arthropods and mollusks), diploblastic animals (cnidarians) and even in some of the most basal extant

animals, the poriferans. Considering the vertebrate an invertebrate GnRH-like peptides, at least 34 isoforms have been described, although it is important to mention that there is no evidence about a reproductive role for these peptides in most of the invertebrate groups (Gorbman and Sower, 2003; Roch *et al.*, 2011). Considering the taxonomic distribution of these peptides, it is suggested that GnRH appeared long before vertebrates, probably with the appearance of the earliest animals and associated to the regulation of other functions (Roch *et al.*, 2011; Roch *et al.*, 2014; Schneider *et al.*, 2006). It is interesting to note that there are fewer GnRH isoforms and their associated receptors in mammals than in other vertebrates and even invertebrates (Morgan and Millar, 2004). An example of this is the presence, in protochordates, of two genes that encode for up to six different isoforms of the GnRH with the capability to stimulate the release of gametes by adult organisms, which in turn demonstrate a direct relationship between these peptides and the reproductive physiology of the species (Adams *et al.*, 2003).

Considering all vertebrates studied, there have been described four genes encoding 25 isoforms of the GnRH that can be grouped in four types, GnRH-I, -II, -III and –IV. GnRH-I and GnRH-II can be found virtually in all extant vertebrates while GnRH-III and GnRH-IV occur only in the most basal groups of fish as lampreys and hagfishes (Kim *et al.*, 2011). In the brain, neurons containing GnRH-I and GnRH-IV are found mainly in the diencephalon while GnRH-II and GnRH-III neurons are located in the mesencephalon and in the telencephalon, respectively (Chen and Fernald, 2008). It has been described that such a position in adults is the result of migratory events driven by chemo-attractive signals occurring during the development of the central nervous system. GnRH-I and GnRH-III neurons originate in the olfactory primordium, on the other hand, GnRH-II and GnRH-IV neurons arise from the ependymal layer in the lining of the ventricular system of the brain (Gorbman and Sower, 2003).

Peripheral and central distribution of the GnRH

Outside the brain, the presence of GnRH-I and GnRH-II immunoreactive cells have been reported in the spinal cord, sympathetic ganglia, retina and pancreas. It is worth to mention that this distribution only applies to a few species of vertebrates and there is little consistence from one group to another (Sherwood *et al.*, 1993). In mammals, GnRH-I can be also found in the oviduct and the placenta, where it appears to act as a paracrine ligand. In the case of the oviduct, it is expressed in the inner epithelium and its concentration increases during the luteal phase of the reproductive cycle. Considering this, the authors speculate about the possibility of a role in fertilization and early development of the embryo (Casañ *et al.*, 2000). In the human placenta, the presence of the gene encoding GnRH-I (Radovic *et al.*, 1990), its mARN (Kelly *et al.*, 1991) and the peptide itself have been found (Tan and Rousseau, 1982). This is an interesting result because it points towards a role for an ancient

molecule that was conserved from early diverging animals in the regulation of a structure that emerged very recently in the evolutionary history of animals.

GnRH is also located in the gonadotroph cells at the pituitary, where it maintains a proper sensitivity to hypothalamic signals, challenging the traditional idea that the sensitivity of the pituitary was only governed by the GnRH of hypothalamic origin and the steroid hormones secreted by the gonads (Krsmanovic *et al*., 2000). Both GnRH-I/GnRH-II and their receptors are synthesized at the ovaries, in particular, in epithelial, granulosa and luteal cells (Kang *et al*., 2001b; McGuire and Bentley, 2010; Peng *et al*., 1994). Exposition of those cells to GnRH results in the activation of the mitogen-activated protein kinases (MAPK) pathway that in turn stimulates the expression of early activation transcription factors as c-Fos, suggesting a relationship in the regulation of the synthesis of hormones (Kang *et al*., 2001a; Leung *et al*., 2003). In addition, exposure of epithelial cells to GnRH results in the inhibition of cell proliferation (Choi *et al*., 2001, Kang *et al*., 2000), which reveals a potential non-reproductive role for this peptide in peripheral organs. In the rest of this thesis GnRH will be used to refer to GnRH-I from hypothalamic origin since it is the one that participates in the regulation of reproductive functions and is also the most studied isoform to date.

In rodents, GnRH neurons arise in the olfactory placode at approximately the 11th day of embryonic development, specifically, near the epithelia of the vomeronasal organ and in the medial wall of the olfactory pit that will eventually originate the nasal canal. These neurons enter the brain following the fibers of the terminal and vomeronasal nerves and by the 16th day of development they can be observed at their final position, mainly in the encephalon (Schwanzel-Fukuda and Pfaff, 1989; Wray *et al*., 1989; 1989b). Such migratory events are regulated by chemical interactions with the mentioned nerves as well as by other molecules that are specific of the different regions of the route (nasal compartment, cribriform placode and anterior brain) and serve to modulate the frequency and direction of the movements of GnRH-neurons (Bless *et al*., 2005; Wierman *et al*., 2011; Wray, 2010).

As a result of this migration, GnRH-neurons are scattered, not grouped in discrete nucleus, along the basal brain of adult mammals. These neurons are distributed as a continuum that extends from the olfactory bulbs to the medial hypothalamus, particularly abundant in the olfactory bulb and tubercle, diagonal band of Broca, medial septum, hypothalamic preoptic and suprachiasmatic areas, anterior and lateral hypothalamus and in the hippocampus (Barry *et al*., 1973; Merchenthaler *et al*., 1984; Silverman *et al*., 1979). Depending on the species, most of these neurons can be located at the medial preoptic area (POA), as is the case of rodents, or at the medial hypothalamus, that is the case of primates (Sternberger and Hoffman, 1978) (Figure 2). This distribution of GnRH-neurons seems to be a conserved trait due to the similarities found in other vertebrates as birds and fish (Barry, 1979; Sternberger and Hoffman, 1978).

GnRH secretion and the regulation of the pituitary gonadotropes

GnRH-neurons send axonal projections to different regions in the brain including the limbic system, olfactory system, organum vasculosum of the lamina terminalis, amygdala, septum, thalamus and different regions inside the hypothalamus (Clarke, 2015; Merchenthaler *et al*., 1984). In addition, neurons containing GnRH receptors have been described in those regions, suggesting a neuromodulator role for the neuropeptide in structures of the central nervous system that relies important information to reproductive processes as olfactory and behavioral cues (Wen *et al*., 2011). GnRH immunohistochemistry combined with the systemic injections of retrograde neural tracers as fluorogold, horseradish peroxidase and lectin wheat germ agglutinin, showed that most of the GnRH-neurons located in the POA and the anterior hypothalamus project to the circumventricular organs, while those located in other regions project to the structures listed above (Jennes and Stumpf, 1986; Rizwan *et al*., 2009; Silverman *et al*., 1990; Witkin, 1990). Similar results have been found after injections placed directly into the median eminence, suggesting that only a subpopulation of GnRH neurons have a neurosecretory role and hence it participates in the regulation of the pituitary (Silverman *et al*., 1987). In support of this idea, only this population of GnRH-neurons is activated in response to estrogen stimulation (Rajendren, 2001). Most of the axons of these neurons incorporate to the hypothalamic-infundibular tract to reach the median eminence, where they stablish close appositions with the capillaries of the hypothalamic-pituitary portal system (Barry *et al*., 1973; Setalo *et al*., 1976). A secondary pathway to the median eminence resides in an axonal tract that travel rostro-caudally below the optic chiasm, lining the lateral walls of the ventral aspect of the third ventricle (Clarke, 2015) (Figure 2B-C).

The hypothalamic-pituitary portal system is constituted by a primary capillary plexus at the median eminence and a secondary one at the anterior pituitary, both interconnected by portal vessels. It was initially described in humans by Popa and Fielding (1930) and thought to mobilize blood from the pituitary to the hypothalamus. This idea was later discarded by the observation of the blood moving in the opposite way, i.e., from the hypothalamus to the pituitary, in anesthetized rats (Green and Harris, 1949). This experiment was conceived to explain the previous observation that electrical stimulation of the hypothalamus was effective to induce ovulation while stimulation of the pituitary consistently failed to do so (Harris, 1948). Nowadays, it is well known that this portal system transports hypothalamic neuropeptides to the anterior lobe of the pituitary where they regulate the synthesis and secretion of the pituitary hormones (Fink, 2015). GnRH, for example, travels along this system to reach the gonadotrophs, where it stimulates the synthesis and release of the gonadotropins: the follicle stimulating hormone (FSH) and luteinizing hormone (LH).

Both gonadotropins are glycoproteins that belong to a family that also includes the human chorionic gonadotropin and the thyroid stimulant hormone. FSH and LH are heterodimers formed by a subunit –α, which is identical for both, and a subunit –β that is

particular for each one (FSH-β y LH-β) (Bousfield and Dias, 2011). The gonadotropin subunits are glycosylated at specific residues and present a high amount of disulfide bounds that increase their stability in the blood. These hormones have been described in all mammalian species studied and similar molecules can be found in the rest of the vertebrates (Pierce and Parsons, 1981). The specificity of the –β subunit determines the biological function of each gonadotropin: FSH stimulates the development of ovarian follicles and the aromatization of androgens into estrogens in the granulosa cells. On the other hand, LH triggers the rupture of the ovarian follicle during ovulation and the posterior luteinization of the remnant cells and is responsible for the synthesis of progesterone and androgens from cholesterol in the theca cells (Marshall and Kelch, 1986).

The mechanism by which the GnRH modulates the activity of the gonadotrophs depends on the binding with its metabotropic receptor located in the surface of the membrane of those cells. This stimulates the interaction of the intracellular domain of the receptor with heterotrimeric Gq/11 and Gs proteins, resulting in an increase of the phosphatidylinositol metabolism and also of the intracellular levels of diacylglycerol, Ca^{2+} and Cyclic adenosine monophosphate (Liu *et al*., 2002). These molecules activate several isoforms of the protein kinase C, which in turn start MAPK-depending signaling pathways that stimulate enzymes as the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases and P38, related with the transcription of genes including those that encode the two gonadotropin subunits (Ciccone and Kaiser, 2009; Kanasaki *et al*., 2005; Mulvaney and Roberson, 2000). Another route that is activated by this receptor is the calmodulin pathway, also related with gene expression. These evidences show a direct role for the GnRH in the synthesis of the gonadotropins, in addition to its known role in the rapid secretion of both hormones from gonadotrophs (Haisenleder *et al*., 2003; 2003b).

The intimate relationship between GnRH and gonadotropin synthesis/secretion was shown in experiments in which the communication of the hypothalamus and the pituitary was interrupted. For example, FSH and LH secretion is abolished in primates bearing medial-basal hypothalamic lesions that destroys GnRH-neurons. In these animals, the continuous infusion of the decapeptide does not reestablish gonadotropin secretion while pulsatile infusion in a pattern similar to that occurring naturally (1 µg/min during 6 minutes, each hour) does (Belchetz *et al*., 1978). The probability of reestablishing gonadotropin secretion decreases as the frequency of the pulses increases, contrary to this, as the frequency decreases the secretion of FSH increases while that of LH remains basal. If the frequency of the pulses decreases too much, the probability of ovulation also drops (Pohl *et al*., 1983; Wildt *et al*., 1981). These results suggest an important role for a proper pattern of secretion of the GnRH in the control of the activity of the pituitary. Similar results can be found in experiments using castrated ewes with a section at the level of the pituitary stalk. These animals also display an abolition of gonadotropin secretion, which is explained by a concomitant decrease in the amount of the

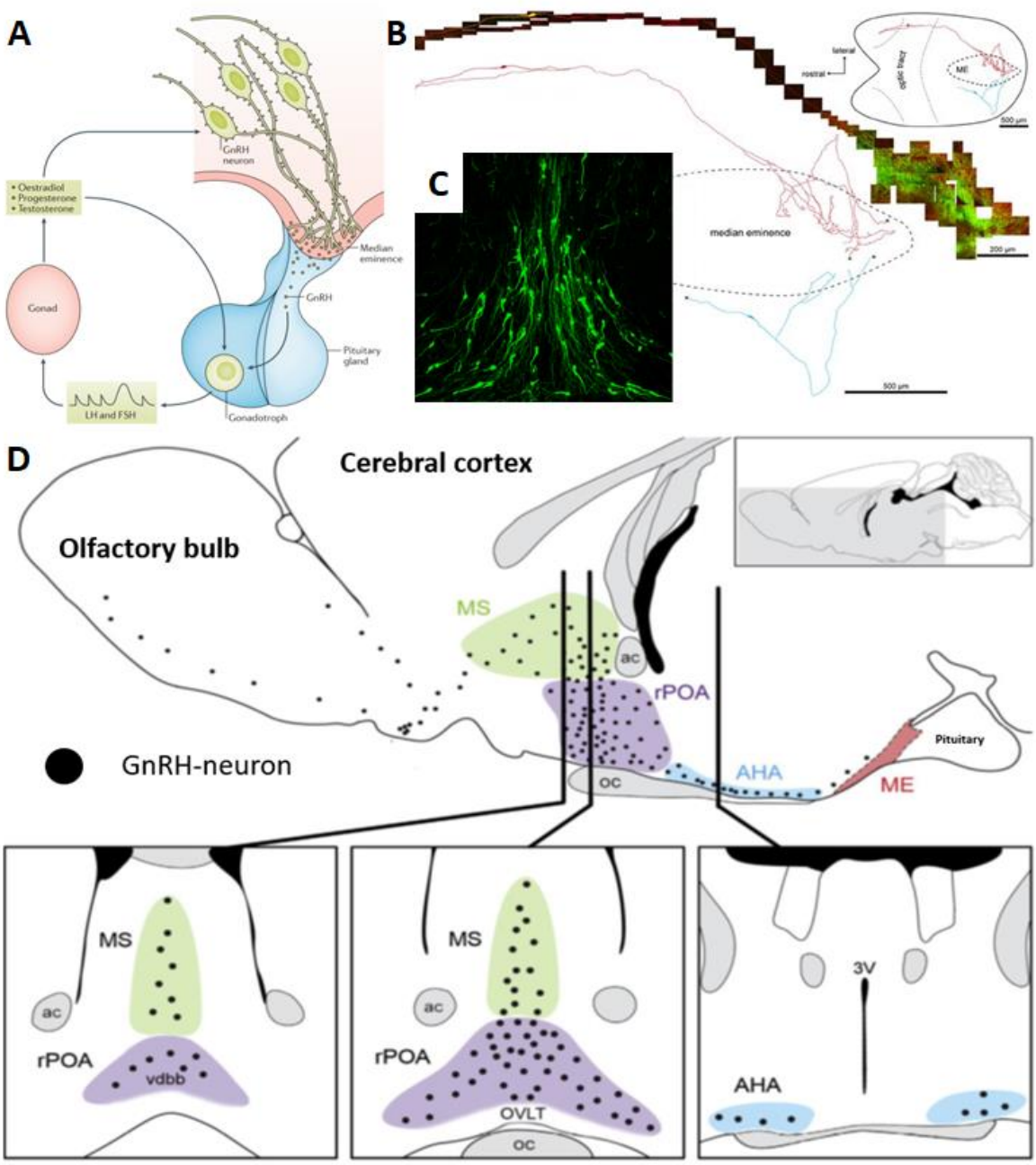

Figure 2. *Overview of the hypothalamic-pituitary-ovarian axis. GnRH-neurons in the hypothalamus project their axons to the median eminence (ME), where GnRH is secreted into the portal system to reach the pituitary. The peptide binds to its receptor in the gonadotrophs, stimulating the release of FSH and LH, which in turn stimulate gamete production and hormone synthesis at the ovaries. Steroid hormones exert a positive and negative feedback at both, the hypothalamus and the pituitary (A). Reconstruction of a GnRH-neuron in the anterior hypothalamus (yellow) and its axon (red) that travels to the ME, where the terminals of several GnRH-neurons overlap (green). Adjacent drawings are representations of the same neuron (red) and another projecting from the mediobasal hypothalamus (blue) (B). Somas of the GnRH-neurons in the anterior hypothalamus are distributed in an inverted "Y" pattern, lining the borders of the third ventricle (3V) (C). GnRH-neurons in the brain of an adult female rodent are scattered from the olfactory bulbs to the ME, near to the anterior commissures (ac); most of them in the medial septum (MS) and structures above Optic chiasm (OC) as the ventral aspect of the diagonal band of Broca (vDBB), rostral preoptic area (rPOA), organum vasculosum of the lamina terminalis (OVLT) and anterior hypothalamic area (AHA). (D) (Modified from Herbison, 2014; 2016; Herde et al., 2013; Xue et al., 2014).*

mRNA of the -α, FSH-β and LH-β chains in the pituitary (Hamernik *et al*., 1986; Hamernik and Nett, 1988). In this model, pulsatile administration of GnRH results in mRNA levels similar than those found in control animals and gonadotropin secretion is also restored (Clarke *et al*., 1984; Hamernik and Nett, 1988).

Despite the differences in the localization of the hypothalamic GnRH-neurons that regulate the pituitary, similar results have also been obtained in laboratory rodents. Systemic injection of a long-acting GnRH-antagonist ([Nal-Glu]-GnRH) to ovariectomized rats suppress the synthesis of the gonadotropin subunits and the secretion of FSH and LH (Wierman *et al*., 1989). Similar to the observed in monkeys and ewes, continuous infusion of GnRH does not restore gonadotropin release while the pulsatile administration does (Shupnik and Fallest, 1994). It is interesting to note that pulsatile administration of GnRH stimulates the synthesis of the –β chains in a selectively manner that depends on the frequency of the infusion and that is regulated by the intracellular mobilization of calcium, dependent on the Ca^{2+}/calmodulin-dependent kinases (CaMK) pathway. A low frequency stimulates FSH–β and a high frequency stimulates LH–β (Dalkin *et al*., 1989; Haisenleder *et al*., 1991; Kanasaki et al., 2005; Papavasiliou *et al*., 1986). Similar results have been also reported in preparations *in vitro* of rat pituitary cells (Shupnik, 1990). These results allow to conclude that the pattern of secretion of GnRH is as important as the presence of the peptide to regulate the synthesis and secretion of the gonadotropins by modulating different signaling pathways inside the gonadotrophs, which in turn regulates the synthesis of the subunits that compose LH and FSH (Ciccone and Kaiser, 2009; Marshall and Kelch, 1986; Ruf *et al*., 2006).

The negative and positive feedback of estrogens on the hypothalamic-pituitary complex

Female rodents display reproductive cycles in the form of estrous cycles, named after the changes in behavior that occur prior to ovulation, indicating sexual receptivity. They are also characterized by the reabsorption of the endometrium instead of its detachment as in animals displaying menstrual cycles. During most of the estrous cycle, encompassing the stages of estrus, metestrus and diestrus, the concentration of FSH and LH is basal. During estrus, their concentration is the lowest of all the stages as a result of a frequency of 1 pulse/120 min in the release of GnRH. In metestrus and diestrus the frequency increases to 1 pulse/60 min and modest increases in the amplitude, from 1.0 to 1.5 pg, are also detectable (Gallo, 1981; Park and Ramírez, 1989). GnRH/gonadotropin pulse frequency and amplitude rise gradually during proestrus morning, doubling those reported for the rest of the cycle and reaching maximal values between 1500 and 1800 hours, which decreases abruptly afterwards. The pre-ovulatory gonadotropin surge, which is the name of this event, is then the result of changes in the frequency and amplitude of GnRH release and not of a sustained secretion. This surge is composed of three phases: ascendant, plateau and descending (Gallo, 1981), and is known to trigger ovulation during the early morning of estrus. Evidently, this process is sexually

dimorphic and it does not occur in males (Butcher *et al*., 1974; Park and Ramírez, 1989; Smith *et al*., 1975).

How is this pattern in the frequency and amplitude of secretion regulated cycle after cycle? Although differences in the frequency of GnRH pulses determine which gonadotropin will predominate, its secretion is closely mimicked by both gonadotropins. The secretion of the GnRH into the bloodstream of the portal system is in turn dependent on the electric activity of the hypothalamic GnRH-neurons, which evidently display a pulsatile pattern with a low frequency during estrus, metestrus and diestrus and an abrupt increase during proestrus, the tonic and phasic patterns of release, respectively (Herbison, 1998). The key to understand this process is its close relationship with the concentration of ovarian hormones, particularly with that of 17β-estradiol. During all the stages when the activity of the hypothalamus-pituitary complex is basal, the concentration of estradiol is also low. On the other hand, during the late diestrus and early proestrus, estradiol levels rise, anteceding the pre-ovulatory gonadotropin surge (Bakker and Baum, 2000; Caraty and Skinner, 2008; Foradori *et al*., 2012; Moenter *et al*., 1990; Nunemaker *et al*., 2002; Pau *et al*., 1991; Van Vught *et al*., 1985; Xia *et al*., 1992; Yagui, 1973; Yoshioda *et al*., 2001).

As it can be inferred from the previous paragraph, in the intact animal, estradiol exerts a feedback action at the hypothalamus that can be either stimulant or inhibitory depending on the concentration, high or low, respectively. An extra level of complexity is that the pituitary is also a target of such feedback actions (Henderson *et al*., 1976). Estradiol exerts their biological actions on cells by binding to estrogen receptors that have been described in the cell surface, cytoplasm and nucleus. There are two isoforms of such receptors encoded by two different genes, the alpha (ERα) and beta (ERβ). The study of knockout mice for one of these receptors led to a general scheme in which the ERα has a major role in the regulation of the mammary gland, uterus, pituitary, adipose tissue, skeletal muscle and bones, while the ERβ plays a minor role. The opposite is true in the case of the prostate, circulatory system, lungs, ovary and central nervous system (Yasar *et al*., 2017). Estrogen receptors act throughout the classic and non-classic pathways. The first one is dependent on the translocation of a homodimer of the receptor-ligand complex to the nucleus, where it binds to estrogen response elements (ERE) located on target genes and gathers cofactors that stimulates or repress gene expression. The non-classic pathways involve the modification of gene expression by pathways that does not rely on the ERE and use direct protein-protein interactions between the receptor-ligand complex and several transcription factors. Additionally, the non-classic pathway includes fast non-genomic signaling that depends on membrane channels or intracellular proteins that activate second messengers (McDevitt *et al*., 2008).

The first evidence supporting the hypothesis that estradiol regulates the pituitary directly came from the observation, in intact rats, that the sensitivity of the gland to GnRH follows a cycle that resembles the secretion of estradiol, low during most of the cycle and

high during proestrus (Aiyer *et al*., 1974b). This pattern is explained by the cyclic changes in the number of receptors for the decapeptide in the gland, whose sensitivity for their ligand does not change during the estrous cycle (Clayton *et al*., 1980; Savoy-Moore *et al*., 1980). The number of cells that express these receptors also increases towards proestrus (Lloyd and Childs, 1988). Another line of evidence comes from ovariectomized rats, in which a lower quote of gonadotropins is released in response to the injection of GnRH if the surgery is performed during diestrus or the morning of proestrus. Similar results have been reported if antiestrogens are injected instead of the extirpation of the ovaries as a tool to remove the priming effects of estradiol (Aiyer and Fink, 1974). Finally, women injected two times with synthetic GnRH during the follicular or luteal phases of the menstrual cycle release more gonadotropins in response to the second treatment (Sollenberger *et al*., 1990).

The evidence presented above shows that the sensitization of the pituitary prior to proestrus day is a pivotal step for the occurrence of the pre-ovulatory surge of gonadotropins, but there is an open debate about if estradiol operates directly in the gland or indirectly by acting on the hypothalamus. It is well known that GnRH itself stimulates the sensitivity of the pituitary (Aiyer *et al*., 1974; Fink *et al*., 1976), in part because it stimulates the expression of its own receptors when it is secreted in pulses of low frequency and amplitude, as during the beginning of the cycle (Clayton, 1982; Hamernik and Nett, 1988). On the other hand, the interruption of the neuro-hormonal communication between the hypothalamus and the pituitary by the section of the pituitary stalk does not inhibits the increase of sensitivity, indicating at least a partial effect of estradiol at the pituitary (Fink and Henderson, 1977; Greeley *et al*., 1975). This idea agrees with the increase of sensitivity in cells of the anterior pituitary that are cultivated in isolation of hypothalamic factors, but in the presence of high levels of estradiol (Menon *et al*., 1985). Despite this evidence, the inhibition of neural activity by sodium pentobarbital injections abolishes the estradiol-dependent sensitization of the pituitary (Henderson *et al*., 1976). This can be due to the inhibition of GnRH release, which clearly contrasts with the previous results. Another explanation is the inhibition of neuro-modulatory signals reaching the gonadotrophs through the innervation of the gland. About this, there is evidence of neural fibers in close apposition with the cells in the anterior pituitary and their electric stimulation results in changes in the secretion of pituitary hormones (Ju, 1997).

At the level of the hypothalamus, the feedback effect of estradiol has been widely described (Herbison, 1998). This was demonstrated empirically in different experimental models, the most popular involving the surgical extirpation of the ovaries to remove the natural feedback of their hormones. For example, in different species, the ovariectomy results in an increase of the amplitude and frequency of the pulses of electric activity in GnRH-neurons (Karsh, 1987; Nunemaker *et al*., 2002; Yagui, 1973). In castrated ewes, injections of estradiol or progesterone decrease the secretion of the gonadotropins. Interestingly, estradiol decreases the amplitude of both the tonic and phasic secretion while progesterone decreases

the frequency of the pulses without any noticeable change in the amplitude. Considering these results, not only an inhibitory role for the steroids was revealed, but also a putative locus for interaction i.e., progesterone may act at the hypothalamus by regulating the frequency of GnRH release, while estradiol can additionally act at the pituitary, modulating the amount of gonadotropins released (Goodman and Karsh, 1980).

According to the physiological evidence, basal levels of estradiol are responsible for an inhibitory effect directly at the hypothalamus that turns into stimulant when the concentration rise. Evidence for this was observed in rats undergoing ovariectomy during the morning of diestrus. In this model, the concentration of GnRH in the portal blood 24 hours after the surgery is lower than in intact animals or ovariectomized rats injected with a high dose of estradiol (Sarkar and Fink, 1979). Even more interesting is the case of the ovariectomized rodents that are chronically treated with high doses of estradiol. These animals present daily increments in GnRH/gonadotropin release that are similar to the proestrus pre-ovulatory surge in amplitude and phase (the time of the day of the event) (Caligaris *et al*., 1971; Henderson *et al*., 1977; Legan and Karsch, 1975; Legan *et al*., 1975; Ramírez and Sawyer, 1974). It is worth to mention that these daily peaks persist for several days after the estrogen treatment is suspended, indicating that high estradiol levels are required to initiate the stimulant feedback but not to maintain it. This makes sense considering that the concentration of estradiol starts to decrease even before the pre-ovulatory surge in the intact animal (Legan *et al*., 1975).

Kisspeptin and the hypothalamic loci for the estrogen feedback

As mentioned before, ERβ mediates the effects of estradiol in some peripheral tissues and in the brain, but most of the experimental evidence supports the idea that it acts through the ERα to mediate both the positive and negative feedback at the hypothalamus. For example, the binding of radiolabeled estradiol in the POA and adjacent structures related with reproductive functions is lower in female $ER\alpha^{-/-}$ homozygous knockout mice than in wildtype conspecifics (Shughrue *et al*., 1997). In general, $ER\alpha^{-/-}$ mutants are infertile and display abnormally high expression of the genes encoding the gonadotropin subunits, resembling those found in ovariectomized rodents (Couse and Korach, 1999). Additionally, the sexual behaviors characteristics of proestrus are abolished in these mutants even after a priming protocol that is well known to induce such behaviors in wild type animals (Rissman *et al*., 1997). Neuron-specific knockout of the ERβ induced in adult mice does not affect the positive nor negative feedback. On the other hand, deletion of the ERα results in the inability of low doses of estradiol to suppress LH release. In ovariectomized animals, the injection of an ERβ-agonist does not have an effect on the secretion of LH, while activation of ERα inhibits the release (Cheong *et al*., 2014). Finally, injection of a selective ERα-antagonist to rats during proestrus afternoon results in the blockade of the pre-ovulatory surge of gonadotropins, which

does not occur if the ERβ is blocked instead. In this experiment, the induction of gonadotropin release by exogenous GnRH was not altered by either antagonist, but the responsiveness of the system to neuropeptides that stimulates GnRH secretion decreased only when ERα signaling was abolished, suggesting that the steroid acts at the hypothalamus by indirectly modulating the sensitivity of GnRH-neurons to stimulant input (Roa *et al*., 2008). The previous report partially disagrees with a study that found that neuron-specific ERβ knockout female mice has reduced sensitivity to stimulant peptides at the GnRH-neurons, leading to a lower tonic and phasic secretion of LH (Novaira *et al*., 2018).

Along with the apparent dependency of the system to ERα, it seems that positive feedback at the hypothalamus is also dependent on the classic genomic-pathway. Mutant mice expressing an ERα that cannot bind to ERE, but in which the non-classic actions are unaltered, does not display estrous cycles, spontaneous ovulation nor LH increases in response to high doses of estradiol. The authors of this study also report that the negative feedback could depend on the non-classic pathways due to the observation that low doses of estradiol decrease the release of LH in ovariectomized individuals (Glidewell-Kenney *et al*., 2007). These results can be explained by the fact that this mutation results in the inhibition of the expression of the LH β-subunit and hence in a lower content of the protein in the pituitary, while the expression of the α-subunit and the FSH β-subunit remained unaltered (Glidewell-Kenney *et al*., 2008). It was later found in the same model that the expression of genes that encode for neuropeptides that stimulate the release of GnRH by POA neurons is dependent on the classic pathway of estrogens while the inhibition of their expression is regulated by non-classic pathways (Gottsch *et al*., 2009).

The first candidate to be the target of the feedback actions of estradiol in the hypothalamus was the POA, which was known to contain a subset of GnRH-neurons that play a critical role in the regulation of the gonadotrophs. Immunohistochemistry studies assessing the co-expression of GnRH and estradiol receptors found positive results in the case of the ERβ, but negative ones for the ERα in rats (Herbison and Theodosius, 1992; Herbison and Pape, 2001; Shughrue *et al.* 1997; Watson *et al*., 1992), mice (Merchenthaler *et al*., 2004; Mitra *et al*., 2003), humans (Hrabovszky *et al*., 2007) and sheep (Skinner and Dufourney, 2005). Considering that this population of GnRH-neurons does not express the ERα, the hypothesis about other ERα-immunoreactive cells in the POA that mediates the feedback actions arose. All the studies cited above found such cells in the region, however, this does not explain the results of the deafferentation studies that found that surgical isolation of the POA leads to the abolition of the characteristic pattern of release of the neuropeptide. For example, the section of neural fibers entering the POA from the caudal brain results in the abolition of the estrous cycle, the secretion of LH and the spontaneous ovulation (Colombo and Phelps, 1981; Hoffman and Gibbs, 1982; Kawakami and Terasawa, 1972; Phelps *et al*., 1976). It is worth to mention that these studies reported the preservation of the infundibular bundle of axons that distribute the GnRH to the median eminence and the periventricular tract

is also intact (Setalo *et al*., 1976). Functional evidence of this statement is observed in rats undergoing the same surgery and displaying normal LH secretion in response to electrical stimulation of the POA (Hoffman and Gibbs; Phelps *et al*., 1976).

Considering the evidence presented above, current models that explain the activity of the hypothalamic-pituitary complex agree in that neural information required for the proper regulation of GnRH release is generated outside the POA and reaches GnRH neurons throughout neural projections. Iontophoretic injections of a retrograde neural tracer into the POA of adult female rats results in the labeling of numerous cells in the telencephalon, diencephalon, mesencephalon and rhombencephalon. Notable hypothalamic examples are the lateral hypothalamus, periventricular region, AHA, suprachiasmatic region, dorsomedial hypothalamus, periformical area, ventromedial hypothalamus, ARC and mammillary region. In the case of the extra-hypothalamic areas, most of the labeled cells are in the lateral septum, amygdala, hippocampus, ventral subiculum, habenula, periacueductal gray, peripeduncular nucleus, raphe nucleus, parabranchial nucleus, locus coeruleus, tegmental area and nucleus insertus. (Berk and Finkelstein, 1981; Chiba and Murata, 1985; Kita and Oomura, 1982).

As revealed by ultrastructure examination by electron microscopy, GnRH neurons receive sparse synaptic contacts in comparison to other neurons in the POA of male and female rats in different stages of the estrous cycle. Less than 15 synapses are usually found per cell (Witkin and Silverman, 1985; Witkin *et al*., 1995), however, several neurotransmitter systems have been reported to contact GnRH-neurons in the POA and their terminals in the median eminence. Among these neurotransmitters are peptides, monoamines and amino acids. Examples of these are vasoactive intestinal peptide, neuropeptide Y, substance P, galanin, corticotropin releasing hormone, neurokinin B, endorphins, enkephalins, dynorphins, melanin concentrating hormone, orexin, kisspeptin, RF-amide related peptides, dopamine, noradrenaline, adrenaline, histamine, γ-aminobutiric acid (GABA) and glutamate. The wide variety of structures innervating the POA, as well as their neurochemical nature, allowed researchers to speculate about the different sensory systems that conveys information about the internal and external media to GnRH-neurons to regulate reproduction in tune with the environment (Dudas and Merchenthaler, 2006; Hrabovszky and Liposits, 2013; Kalló *et al*., 1992).

In the search of the central structures that express the ERα and send afferent fibers to the POA, groups of cells were detected in the rostral periventricular region of the third ventricle (RP3V), medial preoptic nucleus, periventricular preoptic nucleus, ventromedial nucleus, ARC and nucleus of the solitary tract (Chakraborty *et al*., 2003; Simodian *et al*., 1999). The RP3V and the ARC dragged a great amount of attention because, in different species, they also concentrate great populations of neurons that synthesize the major inductors of GnRH release known to date: the kisspeptins (Caraty and Franceschini, 2008; Franceschini *et al*., 2006; Gottsch *et al*., 2004). These are a set of molecules that result of the proteolytic

processing of the pre-pro-kisspeptin, which is a 145 amino acid peptide encoded by the kiss1 gene, resulting in neuropeptides that share the same C-terminus sequence but with a different RF-amide motifs and length of the amino acid chain (Caraty and Franceschini, 2008; Gottsch *et al*., 2004; Roa *et al*., 2011). In addition to the brain, kisspeptins and their G-protein coupled receptor (GPR54) have been described in peripheral tissues as the pituitary, ovary, oviduct, uterus, placenta, pancreas, liver, cardiovascular system and kidney, where they participate in the regulation of local functions as paracrine modulators (Bhattacharya and Babwah, 2015; Hussain *et al*., 2015).

It is now recognized that kisspeptin regulates the reproductive functions in mammals, kiss1 knockout mice resemble the characteristics of the hypogonadism hypogonadotropic since there is an abnormal puberty, no estrous cyclicity, tonic or phasic release of gonadotropins and hence there is no follicular development nor spontaneous ovulation (Yeo *et al*., 2016). It has been shown that kisspeptin regulates the secretion of GnRH by acting directly on the POA GnRH-neurons. First, kisspeptin addition to the culture media of hypothalamic explants induces GnRH secretion, but the same treatment to pituitary explants does not result in the release of gonadotropins (Thompson *et al*., 2004). Second, in ewes, the systemic or intracerebroventricular injection of the peptide result in a robust increase in the secretion of GnRH into the cerebrospinal fluid (Messager *et al*., 2005). In rodents, ventricular administration stimulates c-Fos expression in GnRH-neurons and a sustained secretion of LH, which is abolished if the GnRH-antagonist acyline is injected before the treatment (Irwig *et al*., 2004). Local microinjection of kisspeptin into the POA also stimulates the secretion of LH and this is blocked if a monoclonal anti-kisspeptin antibody is injected, altering the estrous cycle if the treatment continues for several days (Kinoshita *et al*., 2005). In contrast, central administration of peptide 234, a kispeptin antagonist, delays puberty and abolishes the secretion of gonadotropins and the estrous cycle in adult female rats. Systemic injections of this antagonist also abolished the stimulant effects of cerebral injections of kisspeptin on gonadotropins secretion (Pineda *et al*., 2010b).

Binding of kisspeptin to GPR54 results in the activation of a series of second messengers that stimulate the release of intracellular calcium and hence in the depolarization of cells (Franssen and Tena-Sempere, 2018; Tng, 2015). Most of the POA GnRH-neurons contain GPR54 receptors (Higo *et al*., 2016; Irwig *et al*., 2004; Messager *et al*., 2005) and kisspetin-containing appositions have been described on these neurons (Kinoshita *et al*., 2005). It was later demonstrated that kisspeptin depolarizes the GnRH-neuron through the indirect activation of transient receptor potential channels (Zhang *et al*., 2008). The firing rate of GnRH-neurons in brain slices cultivated *in vitro* increases in the presence of kisspeptin and previous priming with estradiol potentiates this effect, demonstrating a sensitizing effect of the steroid to stimulant input (Pielecka-Fortuna *et al*., 2008). The age of the animal also influences the kisspeptin-mediated depolarization since more cells respond in adults than in immature mice. This seems to depend on a delay of kisspeptin synthesis due to the

observation that GPR54 concentration on GnRH-neurons does not change within age groups, but kisspeptin concentration does (Han *et al*., 2005). Finally, it was shown that all the effects of kisspeptin rely exclusively on the GPR54 receptor. GPR54$^{-/-}$ knockout mice show normal expression of the GnRH mRNA and the anatomy of GnRH-neurons and their terminals in the median eminence is identical to wild type mice, however, mutants do not respond to kisspeptin injections (Messager *et al*., 2005). Similarly, hypothalamic explants of these animals do not release GnRH to the culture medium in response to kisspeptin (d´Angelmont *et al*., 2008).

The relationship of the ARC with the secretion of GnRH in rodents was stablished in the early 70´s by the observation that implants of crystalline estradiol in the region abolishes the spontaneous ovulation, suggesting a role in the inhibitory feedback mechanism (Goodman, 1978; Lisk and Ferguson 1973). It was later found that this treatment inhibits ovulation because it downregulates the secretion of gonadotropins (Nagatani *et al*., 1994). Earlier deafferentation studies demonstrated that the anterior hypothalamus was pivotal for the positive feedback, the progression of the estrous cycle and the spontaneous ovulation, however, the tonic secretion of gonadotropins was unaltered in the same rats, suggesting that a different area in the hypothalamus mediates this process. Section of the fibers that link the arcuate region with the rest of the mediobasal hypothalamus disrupts the tonic secretion and hence the ARC is a good candidate to be responsible for the negative feedback (Blake and Sawyer 1974). The ovariectomized rat served as a model to study the effects of estradiol on the hypothalamus. Injections of low doses of the steroid in these rats mimic the inhibitory concentrations that predominate during most of the estrous cycle and these effects are unaltered by a complete deafferentation of the anterior hypothalamus, but not of the ARC (Blake 1977; Blake and Sawyer 1974; Blake *et al*., 1974). Finally, it has been shown that the multi-unit activity of the ARC oscillates in a pattern that is similar in phase and amplitude to the secretion of gonadotropins and this does not occur if the anterior hypothalamus is recorded instead (Kawakami *et al*., 1982; Wakabayashi *et al*., 2010).

In support of the previous results, the kisspeptin system located inside the ARC respond in a negative fashion upon stimulation with estrogens. Kiss1 mRNA concentration is high in the ARC during diestrus and decreases towards proestrus afternoon, opposite to the rising concentration of estradiol. Ovariectomized females show an increase in the number of kiss1-expressing cells and in the amount of the transcript molecules per cell in this region (Smith *et al*., 2005; 2006b) and administration of estradiol to gonadectomized female and male rodents result in the intracellular decrease of kisspeptin mRNA and peptide (Figure 3, Adachi *et al*., 2007; Kinoshita *et al*., 2005; Smith *et al*., 2005). Systemic or intra-ARC injection of an antisense probe for kisspeptin mRNA decreases the concentration of the peptide, leading to a drop in GnRH pulses (Beale *et al*., 2014; Ohkura *et al*., 2009). Another study also found that this treatment result in abnormalities of the estrous cycle, a decrease in the amplitude of an estradiol-elicited LH surge and also in changes of the tonic secretion of

gonadotropins (Hu *et al*., 2015). Similar results are observed if a selective antagonist of kisspeptin is injected directly into the ARC, but not in the POA (Li e*t al*., 2009). Finally, inducible knockdown of the kiss1 gene in the ARC inhibits the pulsatile secretion of gonadotropins in rats (Minabe *et al*., 2020).

Most of the kisspeptin-neurons in the ARC co-express neurokinin B, dynorphin A and their receptors and hence are called KNDy cells. Mutant mice that do not express the dynorphin receptor show an attenuated release of LH in response to the ovariectomy and in wild type mice the injection of a dynorphin agonist inhibit LH secretion (Navarro *et al*., 2009). As mentioned before, multi-unit activity of the ARC follows a pulsatile pattern that mimics that of LH secretion and the observation of neural activity of KNDy cells by GCaMP6 fiber photometry revealed the same pattern in this subpopulation of neurons (Clarkson *et al*., 2017). Central injection of dynorphin A inhibits the pulsatile pattern of activity while neurokinin B stimulates it, suggesting a dual role of this system in the generation of the pulsatility of GnRH release (Wakabayashi *et al*., 2010). Similar results are observed after optogenetic stimulation or inhibition of KNDy cells (Clarkson *et al*., 2017). Finally, specific ablation of these cells by the intra-ARC administration of a dynorphin-receptor agonist conjugated with saporin results in the inhibition of the follicular development and an increase of the incidence of follicular atresia at the ovary. The uterus experiments a reduction of the interstitial glands and the estrous cycle becomes unpredictable. In ovariectomized animals treated with the toxin, an increase in the amplitude of the estradiol-elicited LH surge is observed. It is important to mention that no concomitant activation of the kisspeptin cells in the RP3V nor the GnRH cells at the POA is observed, which may indicate that ARC kisspeptin system regulates the tonic release of the GnRH that is already stored in the median eminence terminals and not the synthesis or transport of the decapeptide from the soma (Mittelman-Smith *et al*., 2016).

Anatomical evidence supporting the idea presented above come from the following studies. Tract-tracing experiments revealed that kisspeptin neurons from the ARC project to the dorsomedial, paraventricular and lateral hypothalamus, as well as to the RP3V and several regions containing GnRH-neurons as the bed nucleus of the stria terminalis, medial septum and POA. A particularly interesting observation is that most of the appositions of these fibers that contact with GnRH-immunoreactive cells are not in the soma, but in the terminals located in the internal layer of the median eminence (Desroziers *et al*., 2010; Lijima *et al*., 2015; True *et al*., 2011; Xu et al., 2012; Yeo 2013; Yeo and Herbison 2011; Yeo *et al*., 2016). Appositions containing GnRH have been also found contacting kisspeptin cells in the ARC, what can be interpreted as the anatomical substrate for a crosstalk between the ARC-kisspeptin system and the GnRH-neurons (Yip *et al*., 2015).

Contrary to what is observed in the ARC, kisspeptin exerts a positive feedback at the RP3V. This structure is located in the basal anterior hypothalamus; caudal to the organum

vasculosum of the lamina terminalis, anterior to the POA and flanked by the third ventricle and the bed nucleus of the estria terminalis (Yeo, 2013). Early lesion studies clearly stated that is pivotal for the continuity of the estrous cycle, phasic gonadotropin secretion, spontaneous and electric-elicited ovulation (Ronnekleiv and Kelly, 1988; Terasawa *et al*., 1980; Wiegand and Terasawa, 1982; Wiegand *et al*., 1980). A role in the stimulant feedback was initially proposed considering that implants of estradiol, but not estradiol benzoate, in this region or in the POA results in the release of LH and this is abolished if the neural connections between both areas are sectioned (Goodman, 1978; Kalra and McCann, 1975). Contrary to this, the implantation of different antiestrogens results in the blockade of the estradiol-induced LH surge in ovariectomized rats, probably explained by the observed reduction in GnRH mRNA in the hypothalamus and GnRH concentration at the median eminence in these rats (Petersen and Barraclough, 1989; Petersen *et al*., 1989). Furthermore, neurons in the RP3V show an activity pattern that is similar to that of GnRH-neurons during the estrous cycle. C-Fos immunoreactivity is absent during metestrus, diestrus and the morning of proestrus, increasing abruptly at the time of the gonadotropin surge (Le *et al*., 1999). In the ovariectomized rat, estradiol priming with high doses stimulates the expression of c-Fos in the RP3V (Insel, 1990) and the same is true in rats treated with a progesterone regime that stimulates LH secretion (Le *et al*., 1997).

The kisspeptin-neurons in the RP3V participate in the regulation of the phasic release of GnRH i.e., mediate the stimulant feedback effect of estradiol. ERα- neurons in this region send afferent projections to GnRH-neurons in the POA (Hahn and Coen, 2006; Le *et al*., 1999; Simodian *et al*., 1999). These estrogen-sensitive neurons contain kisspeptin, galanin and met-enkephalin and innervate not only the POA but also other areas with GnRH neurons as the septum, organum vasculosum of the lamina terminalis and the bed nucleus of the stria terminalis (Gu and Simerly, 1997; Porteous *et al*., 2011Yeo, 2013; Yeo and Herbison, 2011). Close appositions of kisspeptin-immunoreactive fibers arising at the RP3V have been detected at the ARC kisspeptin somas and also in contact with GnRH terminals in the median eminence (Yip *et al*., 2015). The kisspeptin-neurons in the RP3V are activated along with GnRH-neurons during the pre-ovulatory surge of gonadotropins, when estradiol levels are maximal, and also in response to estradiol treatment in ovariectomized females (Adachi *et al*., 2007; Smith *et al*., 2006; 2006b). The electric activity of this neurons is basal during most of the estrous cycle, increasing shortly before the pre-ovulatory surge of gonadotropins and remains high during the afternoon of proestrus (de Croft *et al*., 2012). An increase in kisspeptin mRNA in the region is observed during proestrus afternoon with respect to the content in the morning and even in diestrus. A decrease in the messenger is observed after ovariectomy and this is reversed by estradiol administration (Figure 3, Adachi *et al*., 2007; Smith *et al*., 2005; 2006b). Injection of an antisense probe against kiss1 mRNA into the RP3V result in a delay of the vaginal opening, alterations of the estrous cycle and in the

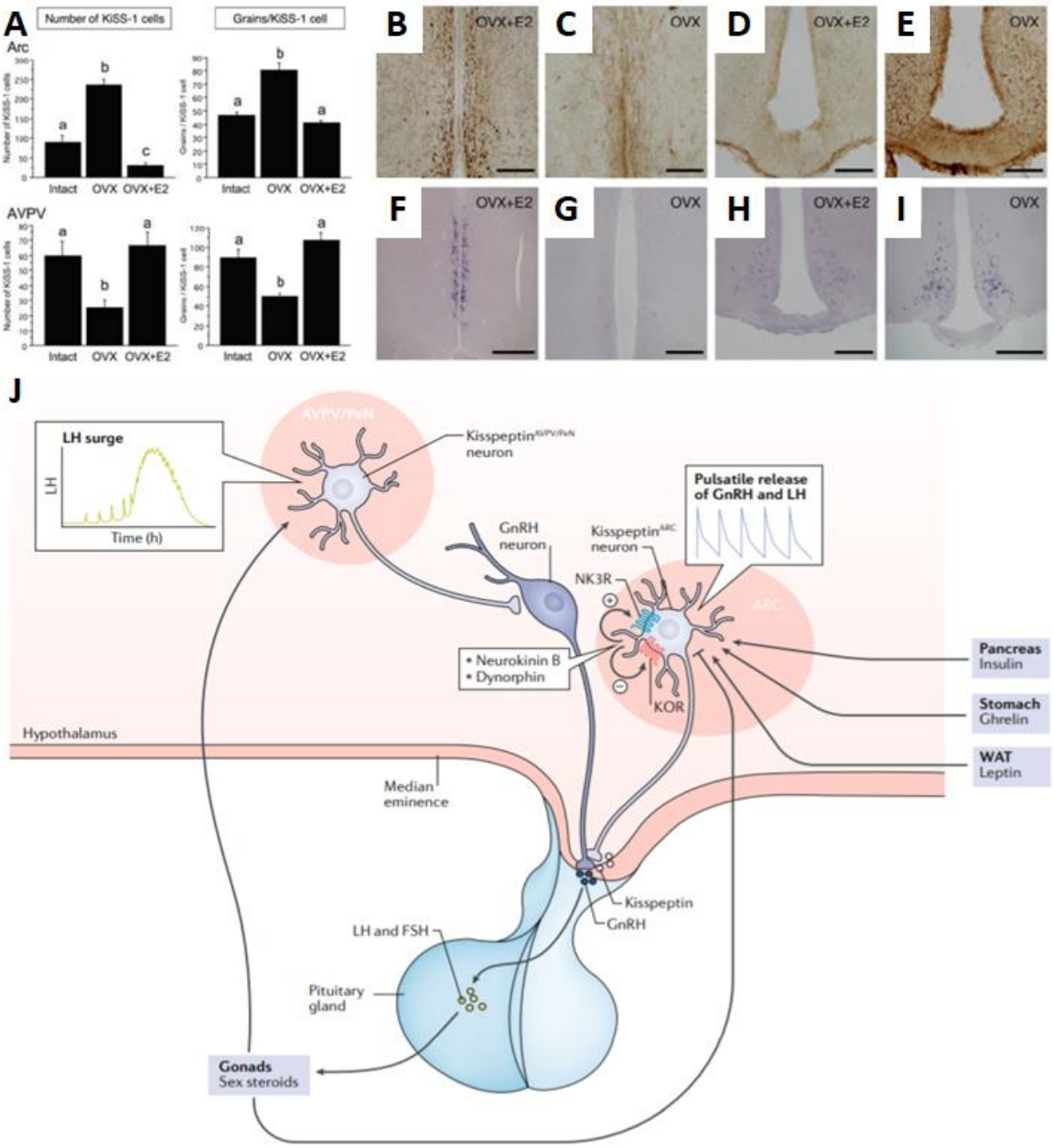

Figure 3. *Neural basis of the negative and positive feedback of estrogens at the hypothalamus through kisspeptin-neurons. Estrogens inhibits the synthesis of kisspeptin in the arcuate nucleus (ARC) since ovariectomy (OVX) increases the number of its kisspeptin-neurons and mRNA molecules per cell. Estradiol replacement (OVX+E2) decreases both parameters. The opposite occurs in the anteroventral periventricular area and periventricular preoptic nucleus (AVPV/PeN) i.e., ovariectomy decreases and estradiol enhances (A). Kisspeptin peptide, as revealed by immunohistochemistry, in the ARC and AVPV of OVX and OVX+E2 rodents (B-E). Kisspeptin mRNA, as revealed by in situ hybridization, in the ARC and AVPV of OVX and OVX+E2 rodents (F-I). According to recent models, ARC kisspeptin-neurons are active in the presence of low concentrations of estradiol. They receive autocrine input through neurokinin B (NK3R) and dynorphin (KOR) receptors (and also synaptic input from other neurons that inform about the metabolic state to regulate the pulsatile release of GnRH that drives the tonic secretion of the follicle stimulating hormone (FSH) and luteinizing hormone (LH), leading to the development of ovarian follicles. On the other hand, AVPV kisspeptin-neurons are activated in the presence of high concentrations of estradiol, indicative of the maturation of ovarian follicles, and stimulate the phasic release of GnRH that drives ovulation. White adipose tissue (WAT) (J). (Modified from Navarro, 2020; Smith et al., 2005; Xu et al., 2012).*

phasic release of LH (Hu *et al*., 2015). Similarly, injection of a monoclonal antibody against kisspeptin into the area results in alterations of the estrous cycle, phasic secretion of LH and inhibition of spontaneous ovulation (Adachi *et al*., 2007; Kinoshita *et al*., 2005).

It is worth to mention that the effects of estradiol in the ARC and RP3V seems to be dependent on the ERα since $ER\alpha^{-/-}$ knockout mice does not release LH in response to estradiol while $ER\beta^{-/-}$ does (Smith *et al*., 2005). It was also observed that in ovariectomized rodents implanted with estradiol capsules the amount of kiss1 mARN decreases in the ARC and increases in the RP3V and this is inhibited after the selective elimination of the ERα from kisspeptin-neurons. These animals also fail to secret LH in response to estradiol injections (Dubois *et al*., 2015). Li and colleagues (2007) demonstrated that estradiol- ERα complexes are transported to the cell nucleus, where they bind to SP1/SP3 transcription factors and then the heterodimer binds to the GC-rich motifs in the kiss1 gene, promoting its transcription, suggesting a molecular mechanism that explains some of the effects of estradiol in kisspeptin neurons explained above.

An avian neuropeptide in the regulation of the reproductive function in mammals

Since the discovery of GnRH in the early 70´s, it was thought that the decapeptide was the single terminal output from the brain to regulate reproductive functions in vertebrates and no antagonistic peptides were found. In 2000, however, a research team reported the existence of a novel dodecapeptide (Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH (2)) in birds that is present in neurons of the paraventricular hypothalamic nucleus and that projects to the median eminence. This peptide inhibits the release of gonadotropins from anterior pituitary explants cultured *in vitro* in a dose-dependent manner and it was named gonadotropin inhibitory hormone (GnIH) (Tsutsui *et al*., 2000). Further experiments demonstrated that GnIH fibers are in close apposition with GnRH-immunoreactive somas and fibers in the POA and median eminence, respectively. These results suggest that the GnIH modulates the secretion of gonadotropins by acting directly at the pituitary but also indirectly by modulating the release of GnRH (Bentley *et al*., 2003). Opposite to the discovery of GnRH, which was first isolated from the mammalian hypothalamus and then reported in a wide variety of vertebrates and invertebrates, this novel peptide was traced first in invertebrates, where it was found to have a role in the regulation of the cardiovascular system, and then inferred to occur in vertebrates. This research further highlighted the importance of the studies in comparative biology, demonstrating that a great amount of knowledge in vertebrate physiology can be gained by studying basal groups. To date, peptides similar to the avian GnIH have been reported in fish, amphibians, reptiles and mammals (Tsutsui and Ubuka, 2018; Tsutsui *et al*., 2010).

A search in a gene database of several mammalian species revealed a subset of cDNA sequences encoding RFamide related peptides (RFRP) similar to the avian GnIH.

Intracerebroventricular of a synthetic version of a RFRP modulates the secretion of prolactin in rats. This effect is mediated by a G protein-coupled receptor for which a natural ligand was not identified before; the GPR147 (Hinuma *et al*., 2000). GPR147 coupled to an inhibitory $G\alpha_i$ is expressed in a gonadotroph cell line and addition of RFRP to the culture medium inhibits the GnRH-stimulated activation of the adenylate cyclase, reducing the intracellular levels of cAMP. This result in the inhibition of the protein kinase-A pathway, downregulating ERK activation and hence reducing the expression of the genes that encode for the gonadotropin subunits. Activation of GPR147 also inhibits the release of intracellular calcium, blocking LH and FSH exocytosis (Son *et al*., 2012). The RFRP receptor is also expressed in GnRH-neurons, where it operates through the same signaling pathway described above, inhibiting GnRH release. Activation of this pathway on GnRH-neurons also inhibits the stimulant effects of kisspeptin and vasoactive intestinal peptide (Son *et al*., 2016).

Immunohistochemistry and *in situ* hybridization studies demonstrated that RFRP neurons are clustered in the hypothalamic dorsomedial nucleus (DMH) of several species of rodents including the rat, mouse and hamster (Gospodarska *et al*., 2019; Henningsen *et al*., 2016; *et al*., 2007; Johnson *et al*., 2007; Kriegsfeld *et al*. 2006; Legagneux *et al*., 2009; Ubuka *et al*., 2012; Yano *et al*., 2003). A different distribution was shown for other species of mammals, for example, in the sheep these cells are located in the DMH but also in the paraventricular nucleus (Clarke *et al*., 2008; Qi *et al*., 2009). In primates, the periventricular nucleus is the main locus for RFRP-neurons (Ubuka *et al*., 2009). As well as the distribution of the cell bodies, the studies cited above found that the pattern of innervation of the external layer of the median eminence by their cell processes is different in rodents than in large mammals. Primates and sheep possess a dense net of fibers in the region, suggesting a role in the regulation of the anterior pituitary (Qi *et al*., 2009; Ubuka *et al*., 2009), while rodents only show sparse fibers and does not uptake fluorogold from the portal circulation so it is hypothesized that RFRP in these species has a role in the regulation of GnRH-neurons instead (Kriegsfeld *et al*. 2006; Rizwan *et al*., 2009; Ubuka *et al*., 2012; Yano *et al*., 2003). A similar pattern of innervation of several hypothalamic and extra-hypothalamic areas was found in all species studied, of particular importance for this study are the limbic system, RP3V, POA, suprachiasmatic nucleus, paraventricular nucleus and ARC.

Close appositions of RFRP-immunoreactive fibers with GnRH-neurons in the POA, which also express the GPR147, have been reported in mammals (Rizwan *et al*., 2012; Ubuka *et al*., 2009; 2012). A functional interaction between these systems can be observed in a cell line of GnRH neurons in which exposure to RFRP results in the acute inhibition of the transcription of GnRH mRNA, even in the presence of kisspeptin in the medium (Gojska *et al*., 2014). Additionally, a direct inhibitory effect of the peptide in the firing activity of GnRH neurons in brain slices from male and female mice in diestrus and proestrus was described. In this study no differences were found depending on sex of the animal or stage of the estrous cycle (Ducret *et al*., 2009). A different research team observed a similar inhibition and

reported that RFRP acts selectively in GnRH-neurons since GABAergic and cholinergic neurons are not sensitive to the treatment. It seems that the dodecapeptide acts by hyperpolarizing GnRH-neurons, making them irresponsive to kisspeptin and other stimulant neurotransmitters (Wu *et al*., 2009). A direct role of the RFRP in the brain is also supported considering the pattern of innervation described above and the fact that it modulates processes that does not depend on the regulation of the pituitary. For example, Johnson and colleagues (2007) found that intracerebroventricular administration in male rats inhibits sexual behaviors and decreases the ingestion of food. An interesting observation is that a small population of kisspeptin-neurons in the RP3V and ARC express the GPR147 and receive direct apposition of RFRP fibers, suggesting an indirect pathway for the regulation of GnRH secretion through the kisspeptinergic system. It is worth to mention that RFRP-neurons in the DMH do not express the GPR54 and no kisspeptin fibers were reported to contact these cells (Poling *et al*., 2013).

As in the case of birds, RFRP has a role in the regulation of the secretion of gonadotropins in mammals (Ubuka and Tsutsui, 2019). In the case of the sheep, fibers immunoreactive to the peptide have been found in the median eminence and a pulsatile secretion was observed in the portal blood, with a higher amplitude and frequency in anestrus animals compared to females in the follicular or luteal phase of the cycle. In addition, it was observed that there is no relationship between the pulses of secretion of RFRP and those of LH in the peripheral blood, but the peptide can inhibit the GnRH-stimulated release of gonadotropins (Smith *et al*., 2012). These effects can be explained by the observation, in ovine pituitary explants cultured *in vitro*, that treatment with the RFRP inhibits the expression of the genes encoding the gonadotropin subunits, not affecting the expression of other mRNAs. It also interferes with the intracellular mobilization of calcium, blocking the exocytosis of hormones (Clarke *et al*., 2008; Sari *et al*., 2009). Similar effects *in vivo* and *in vitro* have been also found in cows (Kadokawa *et al*., 2009) and at least one study detected the suppressive effects of the peptide in women (George *et al*., 2017).

In rodents, the predominant isoform of the RFRP is known as the RFRP-3 and central administration of the peptide results in the inhibition of LH release in male (Johnson *et al*., 2007) and female rats (Kriegsfeld *et al*. 2006; Xiang *et al*., 2015). The study by Xiang and colleagues (2015) reported an inhibition of GnRH mRNA and peptide at the hypothalamus after treatments, suggesting a role directly at the brain. Two previous studies found a reduced suppression of LH and FSH release after central administration but a potent inhibitory effect after intravenous injections and also in cultures of anterior pituitary cells, indicating that the peptide mainly acts at the pituitary level (Murakami *et al*., 2008; Pineda *et al*., 2010). Estradiol modulates the activity of the RFRP-neurons in a negative fashion throughout the estrous cycle. A considerable population of these cells express the ERα (Kriegsfeld *et al*. 2006) but not the ERβ (Molnár *et al*., 2011) and their activity, as measured by the c-Fos co-expression and the RFRP mRNA synthesis, is lower in ovariectomized animals implanted

with estradiol capsules than in animals implanted with oil (Kriegsfeld *et al*., 2006; Molnár *et al*., 2011). Similar results can be observed in intact rodents, the expression of c-Fos and RFRP is higher in diestrus and drops dramatically during proestrus afternoon (Gibson *et al*., 2008; Jorgensen *et al*., 2014; Poling *et al*., 2017). Finally, RFRP seems to be an important regulative element of the seasonal reproduction exhibited by some rodents as the hamster. The number of RFRP-neurons in the DMH and the amount of the mRNA per cell is lower during the non-reproductive season (short days) and increases in response to long days in the mating season. These effects are abolished in pinealectomized hamsters and restored by the injection of melatonin, indicating a direct role of this hormone on the RFRP-mediated reduction in reproductive function of seasonal mammals (Henningsen *et al*., 2016; Revel *et al*., 2007; Ubuka *et al*., 2012).

Conclusion

This chapter reviewed historic and current knowledge about the regulation of the hypothalamic-pituitary-ovarian axis, with an emphasis in the interplay between the classic actors: GnRH, gonadotropins and estradiol. Additionally, the participation of novel peptides that act as regulators of reproductive functions, kisspeptin and RF-amide related peptide, was discussed. However, it is important to note that the neuroendocrine reproductive axis is rich in interactions with other neurotransmitter systems arising in different regions of the central nervous system. In the past years our laboratory and other research teams around the world have shown the important role of acetylcholine, catecholamines, tryptamines, peptides and traditional neurotransmitters in the regulation of the ovarian functions. This make sense considering that reproduction is a complex process that involves the activity of sensory systems that collect information about the external and internal environment in order to signal the brain to regulate the physiology and behavior that is finally evident after activation of effectors. Having said that, the regulation of ovulation does not depend only in the information arising at the brain, the intrinsic nervous system of the gonad plays a pivotal role on the synthesis of hormones and the development of ovarian follicles. Also, intra-ovarian autocrine and paracrine signaling is pivotal for ovulation, for example, in this chapter we described the presence of an intra-ovary GnRH- and kisspeptin-system and in recent years it was also discovered that RFRP-3 and its receptor are present in granulosa cells during proestrus and estrus and in luteal cells in metestrus and diestrus (Singh *et al*., 2011). Exposure to the peptide results in alterations in the development of the follicles, steroidogenesis and a reduction of LH receptor expression in ovaries cultured *in vitro* (Singh *et al*., 2011b). These results highlight the complexity of the system and the long path that remains ahead to fully disclose all of the mechanisms governing ovulation in mammals. In the next chapter, an extra layer of complexity is added to this research problem: the relationship of the reproductive and the circadian system that occurs in order to reproduce in harmony with the environment, increasing the chances of reproductive success.

Chapter II

Chapter II. Based on:

“Clock control of mammalian reproductive cycles: looking beyond the pre-ovulatory surge of gonadotropins”

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Abstract

Several aspects of the physiology and behavior of organisms are expressed rhythmically with a 24-hour periodicity and hence called circadian rhythms. Such rhythms are thought to be an adaptive response that allows to anticipate cyclic events in the environment. In mammals, the circadian system is a hierarchically organized net of endogenous oscillators driven by the SCN. This system is synchronized by the environment throughout afferent pathways and in turn it organizes the activity of tissues by means of humoral secretions and neuronal projections. It has been shown that reproductive cycles are regulated by the circadian system. In rodents, the lesion of the SCN results on alterations of the estrous cycle, sexual behavior, tonic and phasic secretion of the GnRH/gonadotropins and in the failure of ovulation. Most of the studies regarding the circadian control of reproduction, in particular of ovulation, have only focused on the participation of the SCN in the triggering of the proestrus surge of gonadotropins. Here we review aspects of the evolution and organization of the circadian system with particular focus on its relationship with the reproductive cycle of laboratory rodents. Experimental evidence of circadian control of neuroendocrine events indispensable for ovulation that occur prior to proestrus are discussed. In order to offer a working model of the circadian regulation of reproduction, its participation on aspects ranging from gamete production, neuroendocrine regulation, sexual behavior, mating coordination, pregnancy and deliver of the product should be assessed experimentally.

Adaptive significance of biological rhythms

Life oscillates because it evolved in an oscillating world. Since the beginning of their existence, living systems have been exposed to geophysical cycles as the rotation of the moon around the Earth and the movement of the latter around its own axis and the sun. The tides, days and seasons resulting from these cycles, are accompanied by variations in aspects that impact on the physiology and behavior of organisms as the illumination, temperature, humidity, prey-predator and conspecific interactions. In response, every lifeform, from prokaryotes to eukaryotes, show oscillations on some of its own functions. The occurrence of biological rhythmicity is then a property inherent to life (Bhadra *et al*., 20017; Nikhil and

Sharma, 2017) and it has been hypothesized that it evolved from simpler systems that oscillated only as a response to external stimuli (Roennenberg and Merrow, 2002).

The rotation of the Earth is the geophysical cycle that has left the most profound mark on living systems as suggested by the wide variety of biological rhythms exhibited in the circadian (~24 h) frequency. Circadian rhythms share three main characteristics: they are generated by endogenous pacemakers, can be synchronized by environmental time cues and their periods are temperature compensated i.e., their periods are stable under a wide range of physiological temperatures (Refintetti, 2016). At the molecular level, the circadian system of all species studied so far share a common model based on a loop of transcription and translation of clock genes (CG´s). Positive elements in the loop stimulate the transcription of negative elements, which eventually interact with the first ones inhibiting their own transcription (Bhadra *et al*., 2017; Edgar *et al*., 2012; Young y Kay, 2001). Differences in the genes that compose the core components of such feedback loops support the hypothesis that circadian rhythmicity evolved at least twice, first in bacteria and then in the ancestor of eukaryotic organisms (Rosbash, 2009; Young and Kay, 2001). Nonetheless, the possibility that circadian programs evolved once and were then inherited by each emerging group has not been ruled out (Edgar *et al*., 2012).

Circadian rhythmicity confers intrinsic and extrinsic adaptive value to organisms. In the first case, it allows temporal organization of the different processes occurring inside cells and tissues optimizing space and resources. This is particularly interesting in the case of prokaryotes, which does not have internal compartmentalization. On the other hand, extrinsic adaptive value refers to the ability of circadian programs to synchronize the internal environment of organisms with the external environment. This allows them to anticipate changes around that occur with regular periodicity and hence to exhibit certain aspects of their physiology and behavior at optimal phases of the outside cycles (Nikhil and Sharma, 2017; Sharma, 2003; Vaze and Sharma, 2013; Vaze *et al*., 2014).

There is a general agreement in that circadian rhythms represent an evolutionary adaptation that increment fitness in the wild. Several studies analyzed this possibility in different organisms, for instance, it has been shown that cyanobacteria with free running periods that closely matches the period of the environment can overgrowth competitors with diverging periods in a few generations (Hellweger, 2010; Ma *et al*., 2013; Ouyang *et al*., 1998; Woelfle *et al*., 2004). In eukaryotes there is evidence to suggest that the circadian system of plants synchronizes the expression of photosynthesis–related genes with the photophase, increasing their chlorophyll production, carbon fixation and growing rates (Dodd *et al*., 2005). It also facilitates its adaptation to different latitudes throughout the ability to measure day length, which is pivotal for the determination of the proper floration time (Green *et al*., 2002; Michael *et al*., 2003). Circadian systems also mediate plant interaction with other organisms as herbivores and pollinators increasing their survival and reproductive chances, respectively (Atamian *et al*., 2016; Goodspeed *et al*., 2012).

In animals, the loss of proper circadian regulation has a detrimental effect on fitness in both invertebrates and vertebrates. It has been shown that clock gene-mutants of *Drosophila melanogaster* have shorter life spans and lower survival rates than wild type individuals (Klarsfeld and Rouyer, 1998). Mutant flies also show a significant decrease in reproduction, which is associated with a lower number of eggs laid and fewer spermatozoa released (Beaver *et al*., 2002). For vertebrates, a study in artic Gillemots showed that fledglings display a circadian rhythm of jumping activity in order to reach the ocean from the cliffs where they nest. The acrophase of this rhythm avoids the time when their common predators feed and it has been observed that birds jumping outside the acrophase of this rhythm are prone to be killed (Daan and Timbergen, 1979). In a series of lesion studies, DeCoursey and colleagues shown evidence about the role that circadian clocks play on the survival chances of mammals. Rodents with bilateral lesions of the clock tend to live less than sham operated and intact controls in the lab (DeCoursey and Krulas, 1998). In the wild, the circadian timing system seems to allow them to organize their daily foraging behavior avoiding the time when predators are active. In this sense, diurnal animals with disrupted circadian systems spend more time outside their burrows at night and vice versa, thus increasing their chances to be depredated (DeCoursey et al., 1997; 2000; DeCoursey, 2014).

The studies summarized above depict the important role that the circadian system plays on the ability of organisms to survive and reproduce and are in general agreement with the early ideas by Cloudsey-Thompson (1960). According to him, biological rhythmicity represents an advantage in the wild because it coordinates both physiology and behaviors related to reproduction, feeding, competence for resources and escaping from predators. In the particular case of reproduction, timing seems to be pivotal. Sexual reproduction, for instance, is a widespread process that can be found in all vertebrate groups. As any other highly conserved trait, it must offer an important advantage over other plausible mechanisms that could have evolved under similar constraints.

The exact value of adopting this model is still not clear and there is an active debate around the subject, particularly considering that sexual reproduction involves overwhelming costs that are no present in asexual reproduction (de Visser and Elena, 2007; Otto and Lenormand, 2002). An example of the increasing costs of sexual reproduction is the fact that most vertebrates have separate sexes and hence the production of offspring relies not only on the proper regulation and coordination of male and female reproductive processes as individuals, but also on the coordination of both sexes. Virtually all species must also coordinate their reproduction with the environment in order to mate and produce offspring at the most advantageous phase of the year (Alder, 1978). The following sections of this chapter are focused on the molecular and anatomical organization of the circadian timing system in mammals and its relationship with central and peripheral structures involved on the regulation of reproduction.

Clock and clock-controlled genes

The circadian system of mammals, and virtually of all other organisms, operates at the cellular level. Its core components are the proteins encoded by the so called "CG's". As mentioned before, CG´s are different from group to group, even between invertebrates and vertebrates, but the basic program works in a similar way. In the case of mammals, Circadian Locomotor Output Cycle Kaput (Clock) and Brain and Muscle ARNT-like Protein 1 (Bmal1, also known as Mop3), encode the positive elements of the feedback loop. Both proteins are transcription factors of the basic helix-loop-helix-PAS family (Bunger *et al*., 2000; Gekakis *et al*., 1998; Hogenesch *et al*., 1998; King *et al*., 1997b). Mutations of these genes impact directly on the expression of circadian rhythms. The recording of the daily locomotor activity pattern is a non-invasive tool that allowed researchers to demonstrate alterations on the circadian system after spontaneous, mutagen-induced or directed mutations in mammals. Clock homozygous knockout mice showed to be rhythmic when entrained to a light/dark cycle but their periods lengthen gradually in constant conditions and then become completely arrhythmic (Antoch *et al*., 1997; King *et al*., 1997; Vitaterna *et al*., 1994; Wilsbacher *et al*., 2000). In contrast, Bmal1 mutants are immediately arrhythmic in constant conditions and show abnormal onsets of wheel running activity, poor synchronization and a decrease of the total activity when entrained to a light/dark cycle (Bunger *et al*., 2000).

Period and Chryptochrome paralogues (Per1, Per2, Per3 and Cry1, Cry2; respectively) encode the negative elements of the circadian machinery. Per proteins belong to the PAS family while Cry proteins belong to a family of blue-light photoreceptors based on vitamin B2 (Kume *et al*., 1999; Lee *et al*., 2001; Shearman *et al*., 2000b). As showed for the positive elements, mutant mice lacking Per1 or Per2 genes display disrupted rhythms of activity with less stability and shorter periods followed by arrhythmicity in constant darkness (Bae *et al*., 2001; Zheng *et al*., 1999; Zheng et al., 2001). In addition, Per2 mutants also show reduced expression of other clock proteins (Bae *et al*., 2001). Per3 seems to be redundant or not having a central role in circadian regulation since homozygous mutants show only slight decreases in the length of the period in constant conditions (Shearman *et al*., 2000). Mutants lacking both Per3 and any of the other two Per genes resemble the phenotype of the single Per1/Per2 mutants (Bae *et al*., 2001). Cry1 and Cry2 mutants are rhythmic in constant conditions but show a shortening and a lengthening in the free running period, respectively, in contrast, a mutant lacking both genes is completely arrhythmic (Thresher *et al*., 1998; van der Horst *et al*., 1999; Vitaterna *et al*., 1999). Besides the behavioral analyses, Per1 and Per2 expression in these mutants did not follow a circadian pattern and acute light-induction was abolished for Per1 (Vitaterna *et al*., 1999).

As depicted in the insert in Figure 4, circadian rhythms result from the following cycle of expression and repression: Clock is expressed constitutively throughout the day while Bmal1 expression follows a circadian rhythm peaking at the middle of the circadian night (Maywood *et al*., 2003). In the cytoplasm, both proteins bind throughout their PAS domains

and form a heterodimeric complex that is then translocated into the nucleus, where it stimulates the transcription of genes containing E-box cis-regulatory sequences (Gekakis *et al*., 1998; Hogenesch *et al*., 1998). Among such genes are all the negative elements of the loop, Per and Cry paralogues, which are expressed peaking during the circadian day in antiphase to the peak of expression of Bmal1. Per and Cry proteins dimerize in the cytoplasm and are translocated into the nucleus where they interact with the CLOCK:BMAL1 heterodimer inhibiting its activity and hence its own transcription (Etchegaray *et al*., 2003; Jin *et al*., 1999; Kume *et al*., 1999; Lee *et al*., 2001).

Another gene that is a transcriptional target of the CLOCK:BMAL1 heterodimer is Rev-erbα (Preitner *et al*., 2002; Ueda *et al*., 2002), which encodes an orphan nuclear receptor (Lazar *et al*., 1989). REV-ERBα inhibits the transcription of Bmal1, Clock and Cry by binding retinoic acid-related orphan receptor response elements (ROREs) on their promoters. It can be inferred that the concentration of BMAL1 decreases as it participates on the transcription of its repressor, at the same time, REV-ERBα levels gradually decrease as a result of its direct inhibition of the transcription of Bmal1. REV-ERBα is usually described as a link between the positive and negative loops of the circadian molecular clock (Onishi *et al*., 2002; Preitner *et al*., 2002; Ueda *et al*., 2002).

The cycle of expression/repression described above lasts around 24 hours and its length is determined by the time that transcription, translation and degradation of clock proteins takes. It is also influenced by several posttranslational modifications that depend on other proteins, for instance, the turnover and nuclear translocation of Per proteins is dependent on phosphorylation by the casein kinase I ε (CKIε) (Akashi *et al*., 2002; Keesler *et al*., 2000; Lee *et al*., 2001; Vielhaber *et al*., 2000). It has been shown in the hamster that a single mutation in the CKIε gene, resulting in the synthesis of an enzyme with a reduced capacity to phosphorylate PER proteins, leads to a shortening of the free running period (Lowrey *et al*., 2000). These animals are called *tau* mutants and display a locomotor activity rhythm with a period of about 22 hours when heterozygous while it is of 20 hours in homozygous (Ralph and Menaker, 1988).

The concentration of ARNm of many other genes that are not part of the core clock mechanism oscillate in a circadian fashion. Several of these genes present E-box cis-regulatory sequences in their promoters and are hence regulated by CLOCK:BMAL1 heterodimers (Duffield, 2003; Lowrey and Takahashi, 2004). Tissues from Clock and Cry mutants fail to show rhythmic expression of such genes, indicating that those transcripts are indeed under the control of the core clock components and are then called "clock-controlled genes" (Oishi *et al*., 2003). Microarray technologies permitted to show that at least 150 genes are expressed in a circadian fashion in a rat hypothalamic line of immortalized cells and that more than 30% of these genes are also rhythmic *in vivo* (Menger *et al*., 2005). A study demonstrated that around 10% of all the genes expressed in the liver and hearth of the mouse also follow a circadian pattern of expression. It is interesting to notice that there is little

overlap on these clock-controlled genes between tissues and also on the phases of expression, even considering that a similar set of genes are expressed in both tissues (Storch *et al*., 2008). This tissue-specific expression of clock-controlled genes is also true in the brain (Panda *et al*., 2003) and muscle (Duffield *et al*., 2002). There is evidence to suggest that these genes are related with the specific circadian regulation of the physiology of the organ where they are expressed rhythmically (Duffield *et al*., 2002; Panda *et al*., 2003).

An early study by Tosini and Menaker (1996) in which melatonin release by cultured hamster retinas was analyzed raised the hypothesis that the core clock genes were expressed in most of the cells of the body as is the case of the clock-controlled genes. They found a circadian rhythm of melatonin secretion that could be synchronized by light/dark cycles. This rhythm is controlled by the clock genes since its period is about 21 hours in cultures from homozygous *tau* mutant hamsters. It was later confirmed that the core clock genes are indeed expressed rhythmically in this tissue (Sherman *et al*., 1997; Zylka *et al*., 1998; Yoo *et al*., 2004). Experiments assessing the expression of clock genes in cells from different tissues brought definitive evidence in support of a multi-oscillatory model in which each tissue act as an independent oscillator. An *in vitro* study where the bioluminescence from explants of several structures in the central nervous system of PER1:LUC rats was measured demonstrated that the olfactory bulb, organum vasculosum of the lamina terminalis, retrochiasmatic area, paraventricular nucleus, lateral hypothalamus, supraoptic nucleus, ventrolateral preoptic nucleus, arcuate nucleus, paraventricular thalamic nucleus and median eminence display rhythmic expression of PER1 (Abe *et al*., 2002). Similar paradigms using PER1:LUC and PER2:LUC rats revealed that circadian expression of clock genes also occur in peripheral tissues as skeletal muscle, liver, testes, kidney, lung, skin pituitary gland, pineal gland and cornea (Yamazaki *et al*., 2000; Yoo *et al*., 2004; Zylka *et al*., 1998). It is also important to note that cultures of rat-1 fibroblasts still show a circadian pattern of clock gene expression, even after the dissociation and immortalization processes (Balsalobre *et al*., 1998; Welsh *et al*., 2004).

Organization of the mammalian circadian system

Despite the ubiquity in the functional expression of the molecular machinery that drives circadian rhythms, mammals possess a hierarchically organized circadian system which includes a set of peripheral oscillators that are coordinated by a central oscillator. This makes sense considering that part of the utility of a circadian system resides in its capacity to synchronize processes inside the organism so they can anticipate and respond accurately to the demands of the environment. According to this model peripheral oscillators regulate rhythmic functions inside tissues and organs after being entrained by signals coming from the output pathways of the central oscillator in order to offer the right response at the right time, the central oscillator is in turn entrained by the environment throughout input pathways. As shown in Figure 4, this model includes three basic components: self-sustained endogenous

oscillators, input and output pathways (Albretch, 2012; Dibner *et al*., 2010). This model of organization is ancient in the evolutionary history of vertebrates since it was already present in the earliest extant vertebrates, lampreys and hagfishes (Menaker *et al*., 1997).

Central oscillator

Light is the main entraining signal for the circadian system of vertebrates and photoreception is the linking process between it and the environment. It can be then inferred that the central oscillator must have a direct connection with the structures that perceive light and it was indeed the search for the anatomical pathways that convey light information from the eyes to the hypothalamus what led to the discovery of the central oscillator in mammals. Moore and Lenn (1972) injected radiolabeled amino acids into the eye of male and female albino rats and then analyzed their distribution in the brain by autoradiography. It was shown that the amino acids were present in all the visual-associated areas and, in addition, in the ventral aspect of a small nucleus in the anterior hypothalamus, the SCN.

The SCN is a paired structure laying above the optic chiasm at each side of the third ventricle. Each side is about 350 µm high, 300 µm wide and 600 µm depth, occupying a total volume of 0.068 mm^3 (Abrahamson and Moore, 2001). It tightly encapsulates between 8000 and 10 000 small neurons with few dendritic branches (Van Den Pol, 1980). This nucleus can be divided in two regions according to the architecture of neurons, neurotransmitter identity, the origin of input pathways and the destiny of efferent fibers. The *core* represents the ventrolateral portion, its neurons are larger and more dispersed than the neurons in the dorsomedial portion or *shell* (Van Den Pol, 1980). Neurons in the *core* synthetize the vasoactive intestinal polypeptide (VIP), GABA, calretinin, calbindin, gastrin releasing peptide and neurotensin while *shell* neurons are immunoreactive to arginine vasopressin (AVP), GABA, calbindin, angiotensin II and met-enkephalin (Abrahamson and Moore, 2001). Projecting axons from VIP-containing neurons in the *core* make close appositions on the AVP-synthetizing *shell* neurons (Moore, 1982). As it will be described in further sections, the core receives most of the entraining fibers coming from the retina, the thalamic geniculate nuclei and the raphe, which contains glutamate, neuropeptide Y and serotonin, respectively. The shell receives input from other regions in the brain including the thalamus, hypothalamus and limbic system, which are all generally immunoreactive to tyrosine hydroxylase, galanin and VIP, among others (Abrahamson and Moore, 2001). Different patterning in output pathways will also be described.

Evidence about the SCN as the central oscillator of mammals came from different lines of research: lesion studies, experiments assessing its intrinsic oscillatory properties and transplant surgeries. Bilateral lesions of the SCN result in the permanent loss of the circadian rhythms of locomotor activity (Stephan and Zucker, 1972), water and food intake (Stephan and Nunez, 1977; Van Den Pol and Powley, 1979), N-acetyltransferase/melatonin synthesis (Moore and Klein, 1974), corticosterone secretion (Moore and Eichler, 1972), luteinizing

hormone phasic release (Coen and MacKinnon, 1976; 1977), heart rate (Saleh and Winget, 1977), central temperature (Eastman *et al*., 1984; Refinetti *et al*., 1994; Ruis *et al*., 1987; Stephan and Nunez, 1977) and sleep (Coindet *et al*., 1975; Ibuka and Kawamura, 1975; Refinetti *et al*., 1994; Stephan and Nunez, 1977), among others physiological variables that are expressed on daily basis. These studies alone, however, were not satisfactory because a plausible explanation for the loss of circadian rhythmicity after the lesions is that the SCN is just an important output structure.

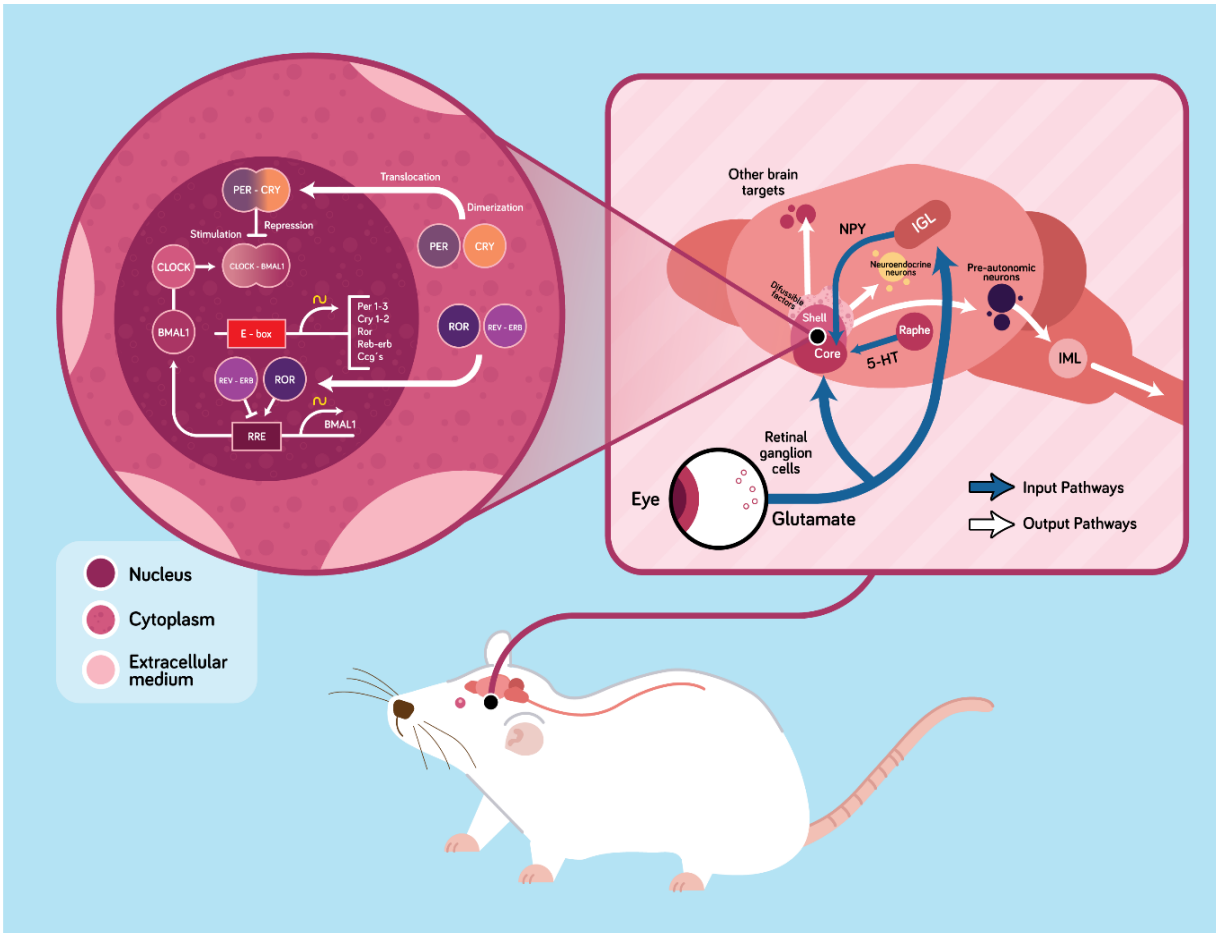

Figure 4. *Organization of the mammalian circadian system. The circadian system works at the cells of several tissues in the body by means of transcription and translation feedback loops. The core elements of the positive limb of the loop are Clock and Bmal1 genes, whose proteins dimerize and stimulate the transcription of the negative elements: Period and Cry paralogues (Per1-3 and Cry 1-2) as well as other clock-controlled genes as Rev-erb and Ror. ROR and REV-ERB stimulate and inhibit the transcription of Bmal1, respectively. On the other hand, the PER-CRY heterodimer blocks the activity of CLOCK-BMAL1, inhibiting their own transcription. Despite the ubiquity of this molecular mechanism, the main oscillator resides at the SCN and receives entraining temporal cues by retinal ganglion cells, the intergeniculate leaflet (IGL) and the raphe, using glutamate, neuropeptide Y (NPY) and serotonin (5-HT), respectively (blue arrows). The SCN organizes the rhythmic activity of other tissues by neural pathways reaching neuroendocrine neurons that regulates the secretory functions of the pituitary, pre-autonomic neurons that project to the intermediolateral column (IML) and are part of multi-synaptic pathways that innervates the organs and also by the secretion of diffusible signals that act on neighbor cells. Neural projections to other brain targets involved in behavior and cognition have been also described (white arrows).*

Considering that circadian rhythms are endogenous by nature and that they do not need entraining signals to be expressed under a free running period, the central oscillator must produce intrinsic oscillations. Several studies shown an *in vivo* circadian rhythm in 2-deoxy-D-[1-^{14}C]-glucose uptake by the SCN that was not present in other areas of the rodent brain (Fuchs and Moore, 1980; Schwartz and Gainer, 1977; Schwartz *et al*., 1980; 1983). This rhythm, which peaks during the subjective day, was also demonstrated *in vitro* (Newman and Hospod, 1986; Newman *et al*., 1992). Metabolic rhythms in the SCN correlate with those in the firing activity of its neurons. *In vivo* recordings revealed a rhythm of electric activity in the intact brain that persist even when the SCN is isolated from the rest of the brain by means of deafferentation (Groos and Hendricks, 1979; Inouye and Kawamura, 1979; Inouye and Kawamura, 1982). These results are supported by *in vitro* studies that shown that circadian rhythmicity is retained in cultures of whole slices of rat brain (Gillette and Reppert, 1987; Green and Gillette, 1982; Groos and Hendricks, 1979; Groos and Hendricks, 1982; Kow and Pfaff, 1984; Shibata and Moore, 1993; Shibata *et al*., 1982).

Cultures with small portions of the hypothalamus containing only the SCN and the optic chiasm, one side of the SCN (Gillette, 1991) or the *core*/*shell* portion are also rhythmic (Yamaguchi *et al*., 2003). There is also evidence indicating that individual neurons of the SCN display firing rhythms (Honma *et al*., 1998; Honma *et al*., 2000; Honma et al., 2004; Shirakawa *et al*., 2000; Welsh *et al*., 1995). The rhythms expressed after dissociation revealed that synaptic communication between neurons is not necessary for circadian timing and that each neuron operates as a single unit with its own phase and period. Despite this, their activity lacks the robustness, resilience and precision that characterizes the SCN *in vivo* and even in the slice cultures, meaning that synaptic contacts between neurons is important for internal synchronization of the nucleus. As discussed for other tissues, SCN neurons also display circadian rhythms in the expression of the core clock genes (Quintero *et al*., 2003; Shearman *et al*., 1997; Yamazaki *et al*., 2000; Yoo *et al*., 2004) and clock homozygous mutants do not display circadian rhythms in their firing rate (Herzog *et al*., 1998).

Circadian rhythms in the release of the main peptide neurotransmitters synthetized by SCN neurons have been also observed. An AVP rhythm was first demonstrated in the cerebrospinal fluid of freely moving rats (Reppert *et al*., 1981; Schwartz and Reppert, 1985; Schwartz *et al*., 1983b) and then in organotypic and cultures of neurons dissociated from the SCN (Earnest and Sladeck, 1986; 1987; Earnest *et al*., 1991; Gillette and Reppert, 1987; Honma *et al*., 1998b; Murakami *et al*., 1991; Shinohara *et al*., 1995; Watanabe *et al*., 1993). This rhythm is coordinated by clock genes, AVP locus is upregulated by the CLOCK:BMAL1 heterodimer and downregulated by Per, explaining the absence of AVP secretion rhythms in Clock mutants (Jin *et al*., 1999). The circadian rhythm in VIP release was first inferred from immunohistochemical studies. Rats were sacrificed at different times of the day and the content of VIP at the SCN was measured, showing a diurnal variation peaking early in the morning (Morin *et al*., 1991; Takahashi *et al*., 1989). The rhythm was then confirmed *in vitro* (Honma *et al*., 1998b; Shinohara *et al*., 1995), even in cultures of SCN immortalized cells

(Earnest *et al*., 1999). These results are important considering that periodic release of neurotransmitters could represent an output pathway conveying temporal information from the SCN to brain targets related to the regulation of behavior and physiology.

The definitive evidence regarding the SCN as the central oscillator in mammals came from the transplant studies. As mentioned before, animals bearing bilateral lesions of the SCN loss circadian organization. It was shown that transplantation of grafts and even of dispersed naïve or immortalized cells from the fetal SCN into the third ventricle of lesioned hosts restored their rhythm of locomotor activity. Most of the grafts that restored rhythmicity sent projections into the host brain and the projecting neurons exhibited synthesis of either AVP or VIP (Druker-Colin *et al*., 1984; Lehman *et al*., 1987; Sawaki *et al*., 1984; Silver *et al*., 1990) while transplantation of other brain areas or tissues did not restored any rhythmic function (DeCoursey and Buggy, 1989; Earnest *et al*., 1989; 1999b). The experiments involving heterotransplants between animals from different species (Saitoh *et al*., 1991; Sollars and Pickard, 1998; Sollars *et al*., 1995), grafts from a *tau* mutant implanted into a wild type hamster (Kaufman and Menaker, 1993; Ralph *et al*., 1990; Vogelbaum and Menaker, 1992; Vogelbaum *et al*., 1993) or from a wild type animal into a clock knockput host (Sujino *et al*., 2003) were more conclusive since the restored rhythms always presented the free running period of the donor.

SCN grafts encapsulated in a semipermeable polymer can restore the locomotor activity rhythm, showing that the SCN can control some of its output targets throughout diffusible signals (Lehman *et al*., 1995). On the other hand, functional grafts that restored activity rhythms consistently failed to restore endocrine rhythms as the phasic secretion of luteinizing hormone, melatonin, corticosterone and cortisol (Meyer-Bernstein *et al*., 1999). It also failed in restoring rhythmic functions that depend on the circadian measuring of day length as the estrous cycle in females (Meyer-Bernstein *et al*., 1999) and the testicular regression in male photoperiodic rodents (Lehman *et al*., 1987; Silver *et al*., 1990). These results imply that the SCN regulates different effectors by means of particular efferent pathways. Endocrine rhythms seem to depend on very specific neural pathways and not in diffusible factors that could be released into the brain.

Input pathways

It has been shown that non-mammalian groups have photoreceptive cells that perform irradiance detection in the pineal, parapineal organ, parietal eye and even deep in the brain, in addition to those in the eyes (Nakane *et al*., 2014; Zordan *et al*., 2001). These structures participate in the expression of several aspects of their physiology and behavior, including circadian entrainment and photoperiodic responses related to reproduction and metabolism (Doyle and Menaker, 2007; Fernandes *et al*., 2013; Menaker, 2014). In mammals, a study reported the expression of an opsin in several regions of the central nervous system of rodents. This opsin is similar than those found in the retina and pineal of other vertebrates but no evidence of extra-retinal photoreception was found (Blackshaw and Snyder, 1999). There

are reports in humans claiming that certain regions of the skin could mediate circadian photoreception. According to this study, light exposure of the popliteal region could induce phase shifts in the temperature and melatonin rhythms. The authors concluded that humans and possibly other mammals are capable of sensing light with extra-retinal photoreceptors (Campbell and Murphy, 1998).

Despite this confounding evidence, it has been shown that melatonin suppression by light is not present in humans that lack both eyes and that those patients display non-synchronized circadian rhythms (Hull *et al*., 2018; Lockley *et al*., 1997). Blind patients that conserve the eyes are usually capable of entrain to the photoperiod and show melatonin suppression during the light phase (Czeisler *et al*., 1995; Hull *et al*., 2018). This data strongly suggests that humans do not possess extra-retinal photoreceptors and hence they use the eyes for both visual and non-visual photoreception. Enucleation studies in rodents also showed that no responses to light can be elicited by light exposure (Foster and Hankins, 2002; Nelson and Zucker, 1981; Yamazaki *et al*., 1999). According to these results, mammals do not possess extra-retinal photoreceptors. It has been hypothesized that their loss can be explained by the passage for the "nocturnal bottleneck". This theory states that early ancestors of mammals spent daytime in burrows to avoid predation from diurnal reptiles and that these receptors were not sensitive enough to be useful for a nocturnal animal (Gerkema *et al*., 2013; Zordan *et al*., 2001).

The fact that blind people could still entrain their activity rhythms allowed researchers to speculate that circadian photoreception is independent from the classic visual photoreceptors. Mice bearing a mutation that results on the complete degeneration of rods and cones still show melatonin suppression and entrainment of activity rhythms (Foster *et al*., 1991; Freedman *et al*., 1999; Lucas *et al*., 1999; Provencio *et al*., 1994). In these animals, light exposure stimulates c-Fos expression in the SCN (Foster *et al*., 1993), indicating that information is traveling to the central oscillator, where it stimulates gene expression. In agreement with these studies, administration of a toxin that destroys most of the cells in the retina failed to prevent circadian entrainment in all the animals that retained projections from ganglion cells into the SCN (Pickard *et al*., 1982). These results implied that novel photoreceptors in the retina mediate visual-independent light responses. A subset of retinal ganglion cells, denominated "type III" or "W cells", was found to express melanopsin, an opsin first described in the eyes, brain and melanophores of amphibians (Provencio *et al*., 1998b). These cells depolarize in the presence of light even when rods and cones are silent and their photic kinetics match with those reported for circadian entrainment (Berson *et al*., 2002; Card *et al*., 1991; Provencio *et al*., 1998). It was recently demonstrated that selective ablation of these ganglion cells eliminates entrainment of the rhythms of clock gene expression in different tissues as well as the rhythm in corticosterone secretion (Kofuji *et al*., 2016).

Melanopsin-containing ganglion cells project directly into the ventral region of the SCN and its axons form what was first recognized as the retino-hypothalamic tract (RHT) in the early 70´s, when the SCN was described as the circadian oscillator (Gooley *et al*., 2001; Hattar *et al*., 2002; 2006; Moore and Lenn, 1972; Moore *at al*., 1995; Pickard and Silverman, 1981). A study using immunohistochemistry combined with retrograde labeling shown that the cells in the SCN that receive appositions of retinal fibers are those that synthetize VIP (Tanaka *et al*., 1993) and true synapses containing glutamate and pituitary adenylate cyclase-activating peptide (Castel *et al*., 1993; Hannibal *et al*., 1997) were confirmed at the soma and dendrites of these neurons by electron microscopy (Ibata *et al*., 1989).

The light information that travels by the RHT stimulates the expression of several genes in the SCN including early activation genes as c-Fos, transcription factors and Per paralogues. The expression of these genes has been related to behavioral changes in response to light and the RHT is then considered as the main input pathway conveying light information into the circadian system (Albretch *et al*., 1997; Kornhauser *et al*., 1996; Morris *et al*., 1998; Yasufumi *et al*., 1997). The ablation of the primary optic tracts does not affect circadian responses to light whereas section of the RHT results in the loss of entrainment ability. Taken together, these results demonstrate that circadian photoreception depends primarily on the RHT and that the classical visual pathways are not necessary for this process (Moore and Eichler, 1972; Pickard *et al*., 1982; Rusak, 1977; Rusak and Boulos, 1981).

A secondary and indirect photic pathway from the eyes to the SCN has been described in most mammalian species. Some of the axons of melanopsin containing neurons bifurcate before entering to the hypothalamus and contact with neurons in the intergeniculate leaflet (IGL), an anatomical subdivision of the lateral geniculate complexes located in the thalamus (Hattar *et al*., 2002; 2006; Moore and Card, 1994; Pickard, 1985). Neurons in the IGL send neuropeptide Y-containing fibers that innervate the ventral aspect of the SCN (Harrington *et al*., 1985) and it has been shown that neuropeptide Y deficient mice and hamsters with IGL lesions exhibit alterations on the entrainment of activity rhythms and in photoperiodic responses (Freeman et al., 2004; 2006; Kim and Harrington, 2008).

The VIP containing neurons in the ventral SCN also receives synapses from a dense serotoninergic input pathway from the medial raphe nucleus (Francois-Bellan and Bosler, 1992; Kiss *et al*., 1984; Leander *et al*., 1998). Serotonin depletion in this structure or its lesion results in a lengthening of the active phase of the locomotor activity rhythm. This does not occur when the dorsal raphe nucleus, which innervates the IGL, is subjected to the same procedures (Meyer-Bernstein and Morin, 1996). In addition to the previously described pathways, afferent fibers coming from several regions of the thalamus, hypothalamus and limbic system have been reported to end in the dorsal SCN (Moga and Moore, 1997). The information reviewed before clearly stablishes that input sources to the circadian system are topographically organized. The involvement of non-photic pathways of entrainment has

received little attention so it is difficult to speculate about the routes that other environmental cues use to reach the circadian system.

Output pathways

An important question about the mammalian circadian system is how the SCN communicates with effector tissues and organs? Transplant studies revealed that some rhythms are regulated by diffusible signals. Another proof for this theory comes from *in vitro* studies that shown that SCN immortalized cells that are co-cultured with fibroblasts can entrain them without any visible physical contact, while other cells fail to induce rhythmicity in the cultures (Allen *et al*., 2001). The transforming growth factor alpha was proposed to be part of the diffusible signals emitted by the SCN since it is expressed rhythmically in that nucleus and injections of it in the third ventricle result in the inhibition of activity and sleep patterns. Mutants for the receptor of this molecule exhibit exacerbated activity during the light phase and does not present negative masking by light (Krammer *et al*., 2001). Similar results have been found for prokineticin II, in addition, its receptor is present in most of the brain areas that are target of the SCN (Cheng *et al*., 2002).

Endocrine rhythms depend on the integrity of specific neural projections so it is important to analyze the neural projections of the SCN in order to understand how it regulates the rhythmic functions of organs. Early anatomical descriptions of the neural efferent pathways of the SCN stablished that the AVP and VIP containing projections from the SCN overlaps in several areas without particular preponderance of either type (Abrahamson and Moore, 2001). Despite these results, it has been also shown that the efferent pathways are indeed topographically organized and that *core* and *shell* targets are not the same. Among the main targets of SCN fibers are the ventral lateral septum, OVLT, RP3V, medial preoptic nucleus, subparaventicular zone, paraventricular nucleus of the thalamus and hypothalamus, DMH, ventromedial nucleus, bed nucleus of the stria terminalis, paratenial nucleus, tuberomammillary nucleus, lateral and anterior hypothalamic areas, substantia innominatta and the anterior amygdala (Leak and Moore, 2001). It seems that the SCN innervates four kinds of targets: areas outside the hypothalamus that are involved in the regulation of behavior, hypothalamic nuclei related to the passage of information to other brain areas, hypothalamic neurons related to the neuroendocrine control of organs and autonomic neurons that are part of multi-synaptic pathways to different organs and tissues throughout the peripheral nervous system (Buijs and Kalsbeek, 2001; Dibner and Albrecht, 2010; Kalsbeek *et al*., 2006).

Injection of retrograde tracers into different organs revealed that the SCN communicates with them by means of multi-synaptic pathways involving the nuclei where the sympathetic and parasympathetic nervous systems originate. Among the organs that revealed this pattern of connections are the liver (la Fleur *et al*., 2000), pancreas (Buijs et al., 2001), thyroid gland (Kalsbeek *et al*., 2000), adrenal gland (Buijs *et al*., 1999) and ovaries (Gerendai *et al*., 2000) and functional evidence about some of these connections exist. In the case of the

thyroid gland, it was shown that a circadian rhythm in the secretion of thyroid-stimulant hormone (TSH), thyroxin and triiodothyronine exist and that lesion of the SCN disrupts the rhythmicity in thyroid hormones but not on TSH, suggesting a neural circadian control of the gland (Kalsbeek *et al*., 2000). Light presented to rats at the beginning of the scotophase reduces corticosterone blood levels without affecting adrenocorticotropic hormone (ACTH) levels and this effect is inhibited in SCN-lesioned animals (Buijs *et al*., 1999). Similar results were found in the *splitting hamster* model, in which exposure to constant light for a long period results in a dissociation of the activity of the left- and right-SCN. This in turn results in two bouts of activity and two daily peaks in cortisol release. Interestingly, ACTH release is suppressed in those animals while hamsters that does not split in constant light present only one bout of activity and one peak in cortisol and ACTH release per day (Lilley *et al*., 2012). These experiments indicate the presence of a SCN-dependent fast mechanism in the regulation of glucocorticoid hormones that does not depend on the pituitary release of ACTH and probably relies on the gland innervation. In the case of the ovaries, a study reported that the synchronization of local clock gene expression depends on the phase of gonadotropin release, suggesting that the ovarian innervation does not have a role in the regulation of its circadian functions (Yoshikawa *et al*., 2009). Gene expression on this experiment was assessed *in vitro*, which does not necessarily reflect the regulation of the gonad *in vivo*. In addition, researchers did not evaluate the effect of denervation on the circadian expression of genes related to ovarian physiology, raising the possibility that neural information arriving throughout the innervation could indeed play a role on circadian regulation of ovaries.

Finally, it is worth to mention that the SCN could use other organs to convey circadian information to the whole body. Infusion of a glucocorticoid analog into cultures of muscle, liver, kidney and hearth induce phase shifts in the expression of clock genes. This effect does not depend on the time of the circadian day when the drug is infused (Balsalobre *et al*., 2000). Considering this, it is possible that the circadian regulation of hormones as cortisol and corticosterone by the SCN serves as an indirect endocrine pathway that transmits the information about the time of the day to the rest of the body. In this sense, an endocrine pathway would reach all tissues at the expense of much less energy than neural coordination would require.

Oscillation of CG´s in the hypothalamic-pituitary-gonadal axis and reproductive tissues

Regulation of reproduction in vertebrates depends on the hypothalamic-pituitary-gonadal axis (HPG). The two functions of the gonads are the production of gametes and the synthesis and secretion of hormones. Both functions are regulated by the pituitary gonadotropins: LH and FSH. Pituitary activity is in turn regulated by hypothalamic stimulant and inhibitory neuro-hormonal systems i.e., the GnRH and the RFRP-3. Sexual steroids synthesized in the ovaries exerts inhibitory and stimulant feedback to the hypothalamus, which results on distinctive patterns of GnRH and RFRP-3 release (Angelopolou *et al*., 2019; Herbison, 2015). This

section summarizes experimental data regarding the presence of the clock genes in the different components of the HPG axis and other reproductive tissues.

The PER2:LUC model allowed researchers to demonstrate the rhythmic expression of Per2 in coronal slices containing the hypothalamus, in particular in the ARC, dorsomedial nucleus, median eminence and in the ependymal cells of the third ventricle. The ARC and dorsomedial nucleus show rhythmicity not only in gene expression but also in electric activity. The expression rhythm persists in cultures containing only one of the nuclei or even in dissociated neurons (Guilding *et al*., 2009). GnRH neurons located in the medial septum, preoptic area, anterior and lateral hypothalamic areas display a circadian expression or Per2 and Bmal1 *in vivo*. This rhythm can be phase shifted by light pulses that also shift the locomotor activity rhythm, indicating a functional clock inside GnRH neurons in the rat brain (Hickok and Tischkau, 2010).

The immortalized GnRH cell line GT1-7 shows a pulsatile release of GnRH that resembles the release of the peptide *in vivo*. It is interesting to note that GnRH pulses are only possible if the neurons in the culture become synchronized to each other, what seems to be attained by diffusible factors (Martínez de la Escalera *et al*., 1992; Weiner and Martínez de la Esclaera, 1993; Wetsel *et al*., 1992). Some neurons in the culture establish close appositions and noradrenaline or dopamine stimulates GnRH release, indicating that neural input to these neurons could also play an important role in pulse generation *in vivo* (Weiner and Martínez de la Esclaera, 1993). Studies in these cells demonstrated that clock genes are expressed rhythmically, directly regulating GnRH secretion and the sensitivity to VIP and kisspeptin, indicating the presence of a functional circadian clock in GT1-7 cells that drives their neurosecretory apparatus and that modulates the sensitivity to input peptides that are well known to stimulate GnRH release *in vivo* (Chapell *et al*., 2003; Zhao and Kriegsfeld, 2009). The expression of the RFRP-3 in the dorsomedial hypothalamus follows a circadian rhythm in proestrus day, suggesting a role for the circadian clock in the regulation of the neuropeptide that is responsible of the inhibition of GnRH secretion (Gibson *et al*., 2008), however, experimental evidence of the participation of the circadian molecular machinery in the physiology of RFRP-3-cells is still lacking.

In the pituitary, the expression of all the clock genes has been assessed in the cells that regulate the functions of the gonads and the mammary gland, gonadotrophs and lactotrophs, respectively. Lactotrophs in culture express prolactin mRNA rhythmically and the peptide is released in pulses. Prolactin promoter contains an E-box and a punctual mutation of this sequence or downregulation of clock genes results in the disruption of the pulsatile release of prolactin (Bose and Boockfor, 2010; Leclerc and Boockfor, 2005). On the other hand, exposure of gonadotrophs to GnRH induces the expression of Per1 throughout a molecular pathway that is indicative of GnRH-receptor activation (Olcese *et al*., 2006; Resuehr *et al*., 2007). GnRH-receptor promoter contains an E-box and a mutation of this sequence or attenuation of the expression of clock genes results in the disruption of GnRH-receptor

transcription (Resuehr et al., 2007). According to these studies, GnRH stimulates the synthesis of its own receptor throughout a pathway that involves direct transcriptional modulation by clock genes. This agrees with the finding that GnRH regulates the sensitivity of the pituitary to pre-ovulatory hypothalamic signals by stimulating the synthesis of the GnRH-receptor in a time-dependent manner (Aiyer *et al*., 1974; Clayton, 1982; Krsmanovic *et al*., 2000).

There are significant differences in the expression of clock genes between males and females at the level of the gonads. In males, the expression of all CG´s in the testis is constant throughout the day. In the case of Per1, expression is restricted to spermatids in different developmental stages while Clock is present in spermatogonia and spermatocytes (Alvarez *et al*., 2003; Morse *et al*., 2003; Yamamoto *et al*., 2004). This report show that a functional circadian clock is apparently absent in male gonads, but that components of the clock machinery are involved in the regulation of particular developmental stages of gametes. Contrary to the case of testis, it has been shown that CG´s are expressed rhythmically in the prostate, epididymis, vas deferens and seminal vesicles. Since these tissues are required for the proper maturation of gametes, authors hypothesize that a clock in accessory tubes plays a role in male reproductive physiology (Bebas *et al*., 2009).

The ovaries of laboratory rodents show a rhythmic expression of CG´s in a wide variety of cells. In samples from whole ovaries, all the CG´s show a circadian pattern of expression similar than that reported for other peripheral tissues, showing peaks of the positive and negative elements in antiphase. LH stimulates the expression of Per1 and Bmal1 (Karman and Tischkau, 2006). Per1 and Per2 mRNA and proteins oscillates in a circadian fashion in the granulosa and theca cells of preantral, antral and pre-ovulatory follicles as well as in corpora lutea and in the interstitial tissue. It is important to mention that these oscillations are present in tissues from rats sacrificed at each stage of the estrous cycle so it seems that circadian regulation is pivotal during the different stages of follicular development and probably during ovulation (Fahrenkrug *et al*., 2006). A posterior study showed that there are slight differences in the amplitude and phase of the expression rhythm that depends on the stage of the estrous cycle, indicating a direct effect of circulating hormones on the clock machinery (Nakamura *et al*., 2010). Cultured luteal cells exhibit circadian expression of Per proteins while granulosa cells treated with LH display a constitutive expression, this result implies that circadian regulation is present only in fully differentiated cells. In addition, LH induce the expression of clock genes in these cells (He *et al*., 2007a; b). In agreement with these results, gonadotropins seem to play an important role in stablishing the phase of the ovarian clock since isolated ovaries can be entrained by timed LH or FSH administration (Yoshikawa *et al*., 2009). The expression of CG´s has been also reported to occur in the oocyte, but its role in this cell has not been explored (Johnson *et al*., 2002).

Finally, the expression of the components of the molecular clock was also reported in the uterus, oviduct and even in the early embryo. In the oviduct, all the CG´s are expressed

but only Per2 and Bmal1 show a sinusoidal waveform of expression while all others seem to be expressed constitutively. The plasminogen activator inhibitor-1, a molecule related with the regulation of oviduct functions, contains an E-box sequence and is expressed rhythmically in this tissue (Kennaway *et al.*, 2003). Similarly, clock genes are expressed in the uterus of both, pregnant and non-pregnant rodents (Johnson et al., 2002; Nakamura *et al.*, 2010). The pattern of expression is heavily modified depending on the stage of the estrous cycle. For instance, the amplitude of Per proteins is higher in proestrus as compared with the other stages. Administration of estradiol and progesterone, which are both involved in the regulation of the functions of the uterus, phase shifts the rhythm and increases its amplitude (Nakamura *et al.*, 2010). Mouse embryos express all the canonical clock genes but the expression pattern depends on the stage of development. Differences between early zygotes, 2-cell, 8 or 16-cell and blastocyst stages have been reported (Johnson *et al.*, 2002). Taken together, these results suggest that the circadian clock may not only be related to the production and maturation of gametes in the gonads but also in the regulation of early developmental processes and on the interaction between the embryo and the oviduct and uterus before implantation. The evidences reviewed in this section suggest that clock genes may exert local regulation on the physiology of all the elements of the HPG axis and related tissues, however, there is little evidence regarding such functional involvement in both males and females. In the case of females, a great amount of research has rather focused on the circadian regulation ovulation, which is the principal topic in the next section.

SCN regulation of the pre-ovulatory gonadotropin surge

A set of classical experiments demonstrated that neural information, in addition to endocrine signals, is pivotal for proper regulation of the pre-ovulatory surge of gonadotropins that occurs on proestrus afternoon. The following results recapitulate the findings about the participation of the SCN on the regulation of ovulation. Everett and Sawyer shown that the systemic injection of pentobarbital, as well as antagonists of the noradrenergic and cholinergic systems, between 14:00 and 16:00 hours delay ovulation by 24 hours. Subsequent injections can delay ovulation in multiples of 24 hours, indicating that a neural signal that follows a circadian rhythm is pivotal for the regulation of ovulation (Everett *et al.*, 1949; Everett y Sawyer, 1950). Posterior studies shown that the blockade of ovulation on the pentobarbital injected animals is the consequence of the suppression of the pre-ovulatory surge of GnRH/LH (Caligaris *et al.*, 1971; Stetson *et al.*, 1981). It was then speculated that estradiol can only stimulate the pre-ovulatory release of GnRH/LH during a "critical window" that occurs during proestrus afternoon and that is determined by the circadian system.

Further evidence about such circadian signals arising at proestrus afternoon came from experiments in which female rats were ovariectomized and, after full recovery of the surgery, primed with high doses of estradiol by means of subcutaneous injections or the implantation of silastic capsules containing the steroid. These animals display daily peaks in LH secretion

that are similar to the proestrus pre-ovulatory-surge in phase and amplitude (Caligaris *et al*., 1971; Henderson *et al*., 1977; Legan and Karsch, 1975; Legan *et al*., 1975; Ramírez and Sawyer, 1974) and can be inhibited by pentobarbital injections (Sarkar *et al*., 1976). Considering these results, it was postulated that the pre-ovulatory surge of GnRH depends on both, high concentrations of estradiol and a circadian neural signal. Given the systemic nature of the barbiturate injections in these experiments, however, it is not possible to discriminate if the blockade of neural transmission affected the GnRH neurons directly or if other structures or neural connections are also pivotal.

Studies in which afferent fibers that reach the mPOA coming from posterior regions of the brain were sectioned using a *Halaz knife* shown alterations of the estrous cycle, inhibition of LH secretion and a blockade of ovulation (Colombo y Phelps, 1981; Hoffman and Gibbs, 1982; Kawakami y Terasawa, 1972; Phelps *et al*., 1976). It is important to mention that this deafferentation does not severs the GnRH fibers that transport the peptide to the median eminence since GnRH immunoreactivity is still present in this region and the electric stimulation of the mPOA results in the secretion of LH by the pituitary (Hoffman and Gibbs, 1982; Phelps *et al*., 1976). These results allow us to speculate that the circadian signals that are required for GnRH phasic release are originating in the brain and must reach preoptic neurons in order to stimulate its neurosecretory activity.

The SCN is a good candidate to be the origin of the circadian signals since it is the central oscillator of the circadian system. Evidence supporting this idea comes from lesion experiments and from the observation that LH secretion is closely related to the locomotor activity rhythm. Bilateral lesion of the SCN results in abnormalities of the estrous cycle, loss of sexual receptivity and lack of ovulation (Brown-Grant and Raisman, 1972; Raisman and Brown-grant, 1972). These adverse effects can be explained by a reduction in the phasic release of LH that follows the bilateral lesion and even the deafferentation of the SCN, implying that neural projections from this nucleus are necessary for the regulation of GnRH neurons (Gray *et al*., 1978; Kawakami and Terasawa, 1972; Kawakami *et al*., 1980; Weigand and Terasawa, 1982). Regarding the relationship of LH secretion and activity rhythms, it was shown that the LH surge has a stable phase relationship with the onset of the locomotor activity rhythm (Robertson *et al*., 2009; Seegal and Goldman, 1975). Similarly, ovariectomized *tau* mutant hamsters with implanted capsules containing estradiol display a shortening in the period of both the activity rhythm and the appearance of the evoked daily peaks of LH (Lucas *et al*., 1999).

Anatomical evidence supports the idea that the SCN regulates GnRH preoptic neurons. VIP-containing fibers from the SCN makes synaptic contacts on ipsilateral GnRH neurons at the mPOA and, on the other hand, GnRH-containig terminals have been found contacting neurons in the SCN (de la Iglesia *et al*., 2003; van der Beek *et al*., 1993; 1994; 1997a; 1997b). This cross communication between the SCN and the mPOA could imply a direct modulation of the reproductive and circadian systems. Administration of SCN neurotransmitters, AVP

and VIP, to GnRH neurons *in vivo* and *in vitro* resulted in contradictory results; some studies attain a stimulant role (Lasaga *et al*., 1989; Palm *et al*., 2001; Piet *et al*., 2015; Samson *et al*., 1981; van der Beek *et al*., 1999; Williams *et al*., 2011) while others an inhibitory one (Akema *et al*., 1988; Alexander *et al*., 1985; Kimura *et al*., 1987). Functional evidence linking the activity of the SCN with that of the mPOA comes from the fact that *Splitting hamsters* show c-Fos expression in the mPOA ipsilateral to the active SCN (de la Iglesia *et al*., 2003). This neural activation is coupled to the presence of two daily peaks of LH secretion, each related to one of the activity bouts that characterizes these animals (Swann y Turek, 1985). At this date, no experiments assessing the effect of GnRH on the SCN and the functions that it regulates have been reported.

A working model to explain the integration of circadian and estrogenic signals that regulates GnRH neurons has been proposed by Ohkura and colleagues (2009). According to this model, RP3V-kisspeptin neurons would detect the increment in the concentration of estradiol, indicative of follicular maturity at the ovaries, and also the circadian signals generated at the SCN. Kisspeptin would be released at its terminals contacting GnRH neurons in the mPOA and hence the phasic secretion of GnRH would be attained. On the other hand, kisspeptin neurons in the ARC would detect low concentrations of estradiol during the rest of the estrous cycle and in turn stimulate tonic release of GnRH, which regulates tonic secretion of gonadotropins and hence follicular development. This model leaves open the possibility for a direct accessory input from the SCN to GnRH neurons that could modulate its sensitivity depending on the stage of the estrous cycle. The fact that RP3V-kisspeptin neuronal system is synchronized with the *shell* (Smarr *et al*., 2012) points toward an indirect participation of the SCN in which it would stimulate kisspeptin release from these neurons only during proestrus afternoon and hence regulate the timed pre-ovulatory release of GnRH. The SCN also innervates RFRP-3-neurons, which are asymmetrically activated in a pattern opposite to that of GnRH-neurons in *splitting hamsters* (Gibson *et al*., 2008). There is a time-dependent effect of VIP on the activity of RFRP-3-neurons (Russo *et al*., 2015) that suggests a downregulation driven by the SCN during the pre-ovulatory surge of gonadotropins. Further experimental evidence regarding the interaction of the SCN with these neurons during the estrous cycle is needed in order to integrate a model that includes the central stimulant and inhibitory elements modulating ovulation that are subject of circadian regulation (anatomical details described above are summarized in Figure 5).

Evidences for circadian regulation of ovulation at other stages of the estrous cycle

"*Ovulation depends on a surge of luteinizing hormone that occurs on the afternoon of proestrus day*" is a statement recurrently found in papers that discuss female fertility and its relationship with circadian clocks and has led to the incorrect generalization that such LH surge is the only requisite for proper ovulation. In this case, proestrus day would be the only stage of the cycle when important regulative processes occur. This is of course false, Everett

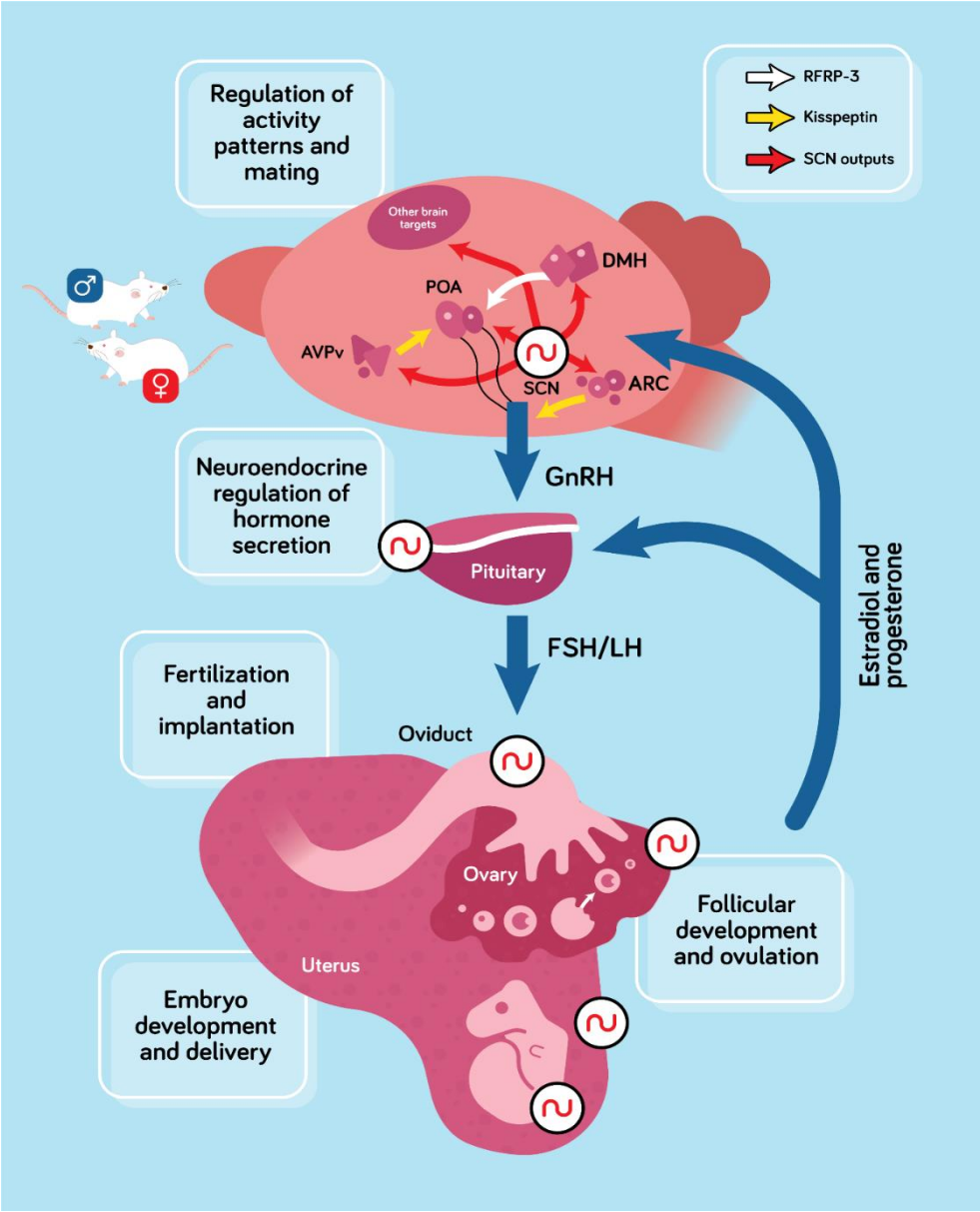

Figure 5. *Circadian regulation of the hypothalamic-pituitary-gonadal axis and related reproductive tissues. At the hypothalamus, clock genes oscillate in the GnRH-neurons of the preotic area (POA). In addition, the suprachiasmatic nucleus (SCN) participates in the regulation of the secretion of GnRH and hence of the follicle stimulating hormone (FSH) and luteinizing hormone (LH). Kisspeptin fibers from the anteroventral periventricular area (AVPv) and the arcuate nucleus (ARC) innervate the soma and terminals of the GnRH-neurons, respectively. These projections convey stimulating information that drives GnRH secretion (yellow arrows). GnRH-neurons also receive RFamide related peptide-3 (RFRP-3) appositions from the dorsomedial hypothalamus (DMH) that inhibits their firing and neurosecretory activity (white arrow). The SCN sends direct projections to all the nucleus mentioned above (red arrows). The connections with GnRH- and kisspeptin-synthesizing cells are thought to represent direct and indirect pathways to stimulate the rhythmic secretion of GnRH. On the other hand, communication with RFRP-3-neurons serves to remove an inhibitory regulative element during the pre-ovulatory surge of GnRH. Circadian control of brain regions related to the organization of daily activity patterns would facilitate the coordination of males and females to mate. Circadian expression of clock genes has been also reported in the gonadotrophs and lactotropes at the pituitary, as well as different cells in the gonads, oviduct, uterus and embryo. Although there is evidence suggesting that these genes play important roles in the physiology of several organs, there has been little interest in explaining their rhythmic expression in reproductive tissues.*

and Sawyer (1950) showed that proestrus afternoon was indeed an important period for the triggering of LH secretion, but a few years after, and using the same paradigm, several groups demonstrated that other stages of the cycle were equally pivotal for the regulation of ovulation. The injection of pentobarbital during the early afternoon (starting at 13:30 and before 16:00) of diestrus stage inhibits the pre-ovulatory surge of LH and also ovulation (Beattie and Schwartz, 1973; Schwartz and Lawton, 1968). Similar results were found in two different studies that, in addition, showed a blockade of ovulation when pentobarbital injections were performed at estrus and metestrus (Domínguez and Smith, 1974; Okamoto *et al*., 1972). These studies reported the results from animals injected at different times of each stage of the estrous cycle and found a circadian pattern in the pentobarbital-dependent blockade of ovulation in which maximal blockade occurs when injections are placed during the "critical window" first described by Everett and Sawyer (1950). Considering the half-life of pentobarbital and its mode of action, it is not possible that injections performed in other stages of the estrous cycle affected the stimulant neural signals that occurs during proestrus afternoon and hence unravel the importance of the "critical window" in other stages of the cycle.

Further evidence about circadian control of gonadotropin release in days other than proestrus comes from a study in which atropine, a muscarinic receptor antagonist, was injected at different times of each stage of the estrous cycle and ovulation was assessed. The authors reported a circadian rhythm in the sensitivity to the drug since a blockade of ovulation could only be elicited at 13:00 hours of each stage. Furthermore, the doses necessary to block ovulation varied during the cycle, lower doses were required during metestrus and diestrus than during estrus and proestrus (Domínguez *et al*., 1982). A similar experiment blocking the dopaminergic and adrenergic systems of female rats by injecting haloperidol or propranolol, respectively revealed differences in both neurotransmitter systems. Adrenergic system blockade resulted in a decrease on the number of ova shed while dopaminergic blockade during estrus, metestrus and proestrus blocked ovulation and decreased FSH serum levels. Estradiol benzoate injections given at diestrus nor GnRH at proestrus restored ovulation on haloperidol treated females, indicating that the dopaminergic system is pivotal to prepare the brain to integrate estrogenic signals and the pituitary to respond to GnRH (Domínguez *et al*., 1987). A relationship between both the dopaminergic and the cholinergic system was revealed by a study in which atropine injection at 13:00 h of metestrus resulted in modifications on the dopaminergic activity at the mPOA that in turn lead to abnormalities in estradiol and LH pre-ovulatory surges and with a 24 h delay in ovulation (Cruz *et al*., 2001).

A general problem with the studies cited before is that systemic injections act at the three components of the HPG axis and hence it is not possible to determine the regulatory step that could be affected. For instance, studies that evaluated the participation of the cholinergic system in the capacity of the ovaries to release estradiol and testosterone in response to unilateral ovariectomy showed that atropine injection modifies the amount of the hormones released by the ovary *in situ* depending on the stage of the estrous cycle (Cruz *et al*., 2006;

Flores *et al*., 2006). This study clearly indicates an involvement of the cholinergic system in the regulation of ovarian steroidogenesis, but it is not clear if atropine exerts a direct effect at the level of the ovary or if the treatment alters the activity of the pituitary, the hypothalamus or both.

Evidence that the cholinergic and dopaminergic systems in the mPOA are related with the regulation of ovulation comes from pharmacological studies assessing the effect of its acute blockade on ovarian functions. Unilateral implants of haloperidol in the left- or right-mPOA at 13:00 h of the different stages of the estrous cycle results on a blockade of ovulation only in rats implanted during estrus and metestrus. On these animals, GnRH injections during proestrus afternoon restored ovulation and the same was true for estradiol benzoate in rats treated at metestrus but not at estrus. This evidence implies that the dopaminergic system is required at the beginning of the estrous cycle to modulate the response of the brain to estrogens and also for the regulation of the pre-ovulatory release of GnRH (Morán and Domínguez, 1995). In a similar study, unilateral implants of atropine crystals at the same time and stages of the cycle results on alterations of the follicular development of the ipsilateral ovary. This result not only demonstrates that the cholinergic system of this area is important in the regulation of ovarian dynamics but also that a neural connection links the mPOA with the ipsilateral ovary (Cruz *et al*., 2014). During diestrus, unilateral microinjection of an atropine solution into the same area results in a decrease of estradiol and LH release during proestrus and in a decrease on the number of ovulating rats the following day. These alterations are rescued by the injection of estradiol benzoate or synthetic GnRH during the afternoon of diestrus and proestrus, respectively. It is evident then that the cholinergic system regulates events occurring prior to proestrus day that are indispensable for the proper triggering of the pre-ovulatory surge of LH (Espinoza-Valdez *et al*., 2016). The presence of M1 and M2 muscarinic receptors has been reported in the mPOA and M2 receptor protein content in this area seems to fluctuate in a daily pattern. Blockade of these receptors by means of selective antagonist results on a decrease or increase on the rate of ovulating females, which depends on the stage of the estrous cycle when blockade was performed (López-Ramírez *et al*., 2017).

At the level of the SCN, we recently observed that bilateral microinjection of tetrodotoxin at 14:00 of each stage of the estrous cycle results in a complete blockade of ovulation at the next expected estrus as compared with vehicle-injected animals. This effect does not occur if the injection takes place outside the critical window. Since tetrodotoxin inhibits the generation of action potentials only for a few hours, these results revealed that the site of origin of the neural signals described by Everett and Sawyer is indeed the SCN (Silva *et al*., 2019; Chapter III). Unilateral injections of atropine into the SCN lowers LH serum levels and blocks ovulation when performed during the morning of proestrus day. The same treatment performed during the afternoon did not modify ovulation, but impairs estradiol and progesterone secretion. Since treatments performed at metestrus did not have any effect, it is concluded that the cholinergic information arriving to the SCN modulates its participation on

the regulation of different processes that takes place at different times of the day and that are stage-specific (Vieyra *et al*., 2016).

This section summarized experimental data that stablished the possible involvement of circadian regulation of ovulation that occurs on days other than proestrus and is represented in Figure 6. Considering this evidence, it is clear that other aspects of circadian regulation of reproduction must be investigated, for instance, the participation of the SCN on the neural regulation of neuroendocrine events that occur on estrus, metestrus and diestrus that are pivotal for proper integration of pre-ovulatory signals. A working model should include the involvement of the local circadian machinery on each component of the HPG axis as well as the involvement of the central oscillator across the 24 hours of the day and of each stage of the reproductive cycle.

Circadian regulation of sexual behavior

In order to reproduce, males and females must coincide to copulate, i.e., it is then necessary that their activity patterns are coordinated. According to early lesion studies, the circadian system allows organisms to organize their daily activity in order to exhibit behavior in optimal phases of the day (DeCoursey *et al*., 1997; 2000; DeCoursey, 2014). This assumption applies to a wide variety of behavioral and physiological functions that are pivotal for reproductive success as the production of viable gametes, courtship, sexual receptivity, mating, fertilization of eggs, parental caring of the youth among others. The involvement of circadian regulation of sexual-related behaviors has not been analyzed in detail. An exploratory study in which young men and women were asked about their sexual habits reported that sexual intercourses seem to follow a daily rhythm peaking at night (Refinetti, 2005). This study, however, cannot assess if there is an endogenous component driving this preference rhythm or if it is only an artifact resulting from the school/work schedules of the subjects.

In male hamsters a true circadian rhythm in copulatory performance has been disclosed. Hamsters were tested with a receptive female at different time points and the mount, intromission and ejaculation frequencies and latencies were assessed. When entrained to a light dark cycle, animals exhibit a rhythm in performance with an acrophase around 09:00 hours. This rhythm is endogenously generated since it persists in constant light. Lesion of the SCN does not prevent copulation as mPOA lesion does, but it abolishes the rhythmic component and hence performance is the same at all time points in lesioned animals (Eskes, 1984). These results are in agreement with previous evidence that showed that blinded males and females exhibit free running locomotor activity rhythms and that pregnancy was only attained when both rhythms overlap (Richter, 1970). Also, when tested in different photoperiods, the peak of performance in mounting behavior of hamsters does not correspond

	Estrus	Metestrus	Diestrus	Proestrus
Kisspeptin from ARC to GnRH-neurones	+	+	+	-
Kisspeptin from AVPv to GnRH-neurones	-	-	-	+
Acetilcholine at SCN	N/A	-	N/A	+
Acetilcholine at POA	N/A	N/A	+	N/A
Acetilcholine at POA through m1 receptor	+	+	-	N/A
Acetilcholine at POA through m2 receptor	-	+	+	N/A
Dopamine at POA	+	+	±	-
Dopamine	+	+	±	+
Noradrenaline	-	-	±	±
SCN "critical window" signal	+	+	+	+

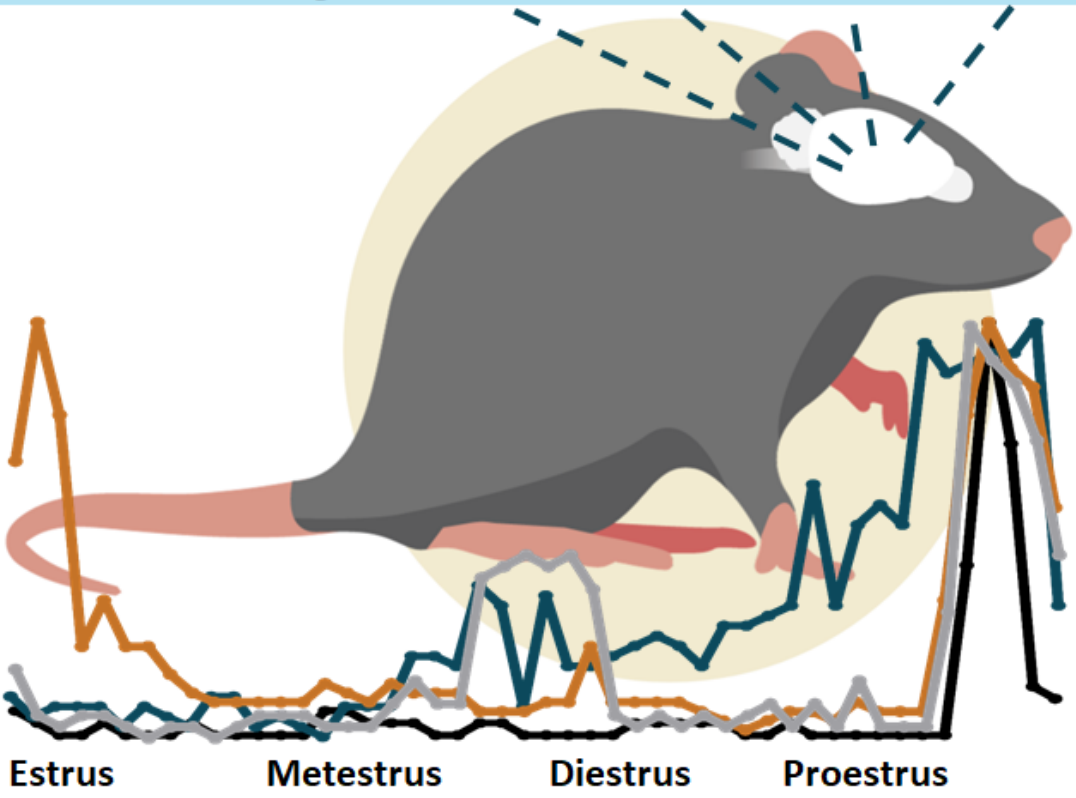

Figure 6. *Summary of the central circadian regulation of ovulation during the estrous cycle. The blue box depicts the activity of several neurotransmitter systems that show a circadian pattern on their regulation of ovarian functions throughout the rodent estrous cycle. Such activity can be pivotal (+), not indispensable (-) or partially indispensable (±). N/A indicates the lack of experimental evidence showing a role for that neurotransmitter in that specific stage. Anetroventral periventricular area (AVPv), arcuate nucleus (ARC), preotic area (POA), suprachiasmatic nucleus (SCN). The secretion profiles of estradiol (blue), progesterone (gray), luteinizing hormone (black) and follicle stimulating hormone (yellow) through the rodent estrous cycle is depicted below for reference (based on data from our laboratory).*

with the dark phase, but with the subjective night as calculated by the phase angle of entrainment with the locomotor activity rhythm (Morin and Zucker, 1978). In addition to this data, it has been shown that male mice and rats can anticipate mating time when a female is presented at a restricted time each day. Social interaction does not explain these results since

exposure to another male does not result in anticipation (Glenn *et al*., 2012; Hsu *et al*., 2010). It is then possible that the circadian system modulates rewarding systems in order to prepare the animal to take advantage of available resources related to reproduction. This is interesting since it has been demonstrated that, in rodents, copulation must occur close to ovulation or the outcome of the embryos is compromised (Sakai *et al*., 1988).

Effects of the disruption of the circadian system on reproduction

As mentioned in previous sections, lesions of the SCN and exposure to constant conditions that impairs the activity of the SCN result in alterations of reproductive function, indicating a direct role for the master circadian clock in reproduction. It was also discussed that local interference of the function of clock genes disrupts the physiology of the tissue. In addition, it has been recognized that dysregulation of circadian system by changes in the entraining stimuli impairs reproduction. Shift work is defined as scheduled work that occurs before 19:00 h and before 09:00. Humans in these regimens are then exposed to light during the dark period and forced to be active at the opposite phase of their natural daily cycle. Jet lag, on the other hand, results from shifts in the entraining signals due to transmeridional travels or to rotating schedules of activity or work. These shifts must be compensated by the circadian system and a re-synchronization usually occurs after a few days of transient desynchronization (Mahoney, 2010). In recent years, several studies have shown that humans recurrently exposed to these adverse situations are prone to develop reproductive malfunction. Shift work has been associated with negative outcomes in pregnant women as pre-term delivery of the product, small size and weight for the gestational age, increment in pregnancy-related hypertension and pre-eclampsia (Palmer *et al*., 2013). Another study found a positive correlation between the risk of miscarriage and the exposure to shift work, permanent work during the night and recurrent performance of demanding physical activities during the night (Bonde *et al*., 2013). Abnormal menstrual cycles and infertility have been also reported for the general population in reproductive age that is exposed to shift work (Stocker *et al*., 2014).

Nurses represent a population that is usually exposed to long working schedules, shift work and physically demanding activities due to the specific demands of their job. A study on a group of nurses wanting to become pregnant revealed that a loss of fecundity results from the exposure to shift work and long schedules of more than forty hours per week (Gasking *et al*., 2015). It has been also reported that nurses experiment abnormal menstrual cycles that are very short (21 days) when they are exposed to shifts on their work schedules or very long (32-50 days) when exposed to more than forty hours of work per week (Lawson *et al*., 2015). Experimental evidence from murine models has shown that continuous phase shift of the light dark cycle decreases fertility depending on the direction of the shift i.e., advance or delay (Summa *et al*., 2012). A negative effect of light pollution on the reproduction of a marsupial mammal has been reported. According to this study, the establishment of artificial lightning in regions were populations of wallabies mate and breed results in alterations in the circadian

and circalunar patterns of melatonin secretion. This leads to an increase of the seasonal breeding duration, which in turn result on the birth of young wallabies at moments of the year when predation and competence for resources is high and hence the survival rate decreases. Additionally, an increment in the percentage of product reabsorption was found (Robert *et al*., 2015).

Concluding remarks

This review summarized experimental data regarding the presence of several components of the circadian timing system across the HPG axis, as well as functional evidence linking this system with reproductive success. As the reader can infer, several experiments have been performed in order to understand the circadian regulation of the phasic release of GnRH in female rodents, which in turn drives the pre-ovulatory secretion of gonadotropins. On the other hand, little attention has been put on the possible role of the circadian system on the regulation of the tonic release of gonadotropins and in other neuroendocrine events that are pivotal for both phasic and tonic release of GnRH. In addition, since core clock genes have been found to be rhythmically expressed in different reproductive tissues, it would be interesting to explore the particular role that they play on the regulation of the functions of those tissues. As new information becomes available, it is more evident that reproduction is regulated in a circadian fashion in different tissues and organs, and this varies depending on the stage of the reproductive cycle.

Research question

The early studies by Everett and Sawyer about the blockade of ovulation following the injection of sodium pentobarbital and its circadian nature (Everett and Sawyer, 1950) paved the way for a great number of studies to explain the nervous regulation of ovulation. Since then, 70 years of research effort has mainly focused in the pre-ovulatory events that occurs during the "critical window" during proestrus afternoon, neglecting the fact that pentobarbital has the same effect during estrus, metestrus and diestrus (Beattie and Schwartz, 1973; Domínguez and Smith, 1974; Okamoto *et al*., 1972; Schwartz and Lawton, 1968). Considering this, it is evident that neural signals generated during each stage of the estrous cycle participate on the modulation of neuroendocrine events that occurs before proestrus and that are indispensable for a proper release of oocytes.

In a previous study we found that unilateral lesions of the SCN performed in the four stages of the estrous cycle blocks ovulation. This is accompanied by alterations of the cycle when the lesion occurs at estrus or metestrus, but not diestrus nor proestrus. Injection of synthetic GnRH at 14:00 hours of the expected day of proestrus stimulates ovulation in most of the lesioned animals. These results coincide with the reports from other laboratories that related this nucleus with the circadian regulation of the pre-ovulatory surge of gonadotropins, but such restricted role for the SCN does not explain the alterations of the cycle. Injections of estradiol benzoate in a dose has been reported to stimulate gonadotropin release does not restore the cycle nor the ovulatory outcome and hence we asked if the SCN participates in the preparation of the hypothalamus to respond to the estradiol feedback arising at the ovaries during the early stages of the cycle. The aims of this study were to investigate if the SCN is a pivotal structure for the regulation of ovulation during the "critical window" of each stage of the estrous cycle and if the ARC, as an important integrative area of the estrogenic feedback, participates in this process.

Hypotheses

Our first hypothesis is that the SCN generates a circadian neural signal during the "critical window" of each stage of the cycle that is transmitted to areas of the brain, as the ARC, that prepare the system to respond to a wide variety of signals that in turn regulates ovulation. If this is true, the transitory inhibition of the SCN neural activity at this time will lead to the blockade of ovulation.

Since the ARC participates in the regulation of the tonic secretion of gonadotropins that occurs during most of the estrous cycle, informs the metabolic state to the reproductive system and is anatomically and functionally linked to the SCN, we hypothesize that it participates in the circadian regulation GnRH secretion during the "critical window" of all the

stages of the estrous cycle and hence the inhibition of its electric activity at this time will also lead to the blockade of ovulation.

Objectives

1. Analyze the effect of the transitory blockade of sodium-dependent action potentials in the SCN at 14:00 hours of estrus, metestrus, diestrus or proestrus on the progression of the estrous cycle and ovulation.

2. Analyze the effect of the transitory blockade of sodium-dependent action potentials in the ARC at 14:00 hours of estrus, metestrus, diestrus or proestrus on the progression of the estrous cycle and ovulation.

Chapter III

Chapter III. Based on:

"A neural circadian signal essential for ovulation is generated in the suprachiasmatic nucleus during each stage of the estrous cycle"

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Abstract

Reproduction is a highly timed process that depends on both the reproductive and circadian systems. The core oscillator of the latter resides at the SCN and it is pivotal for the regulation of the proestrus pre-ovulatory surge of gonadotropins in females. There is evidence to suggest that this system may be involved in the regulation of neuroendocrine events that are essential for ovulation and that occur prior to proestrus. We explored this possibility by transiently inactivating the SCN. Female rats were implanted with guide cannulas aimed at the SCN. After recovery of the estrous cycle, animals were injected with TTX, artificial cerebrospinal fluid (ACSF) or saline solution while freely moving. Injections were performed at 14:00 h of each stage of the estrous cycle. Animals were killed on the next predicted estrus day, the number of ova shed was counted and intact rats at estrus stage were used as absolute control. ACSF did not modify ovulation. Saline solution blocked ovulation in estrus- and diestrus-injected rats. Irrespectively of the stage of the estrous cycle, TTX blocked ovulation. These results lead us to suggest that a neural circadian signal, pivotal for triggering the gonadotropin pre-ovulatory surge, arises from the SCN during the critical window of proestrus. We also suggest that a similar signal, needed for the regulation of other events that are indispensable for proper regulation of ovulation, is also generated in this nucleus during the other stages of the cycle at a similar time.

Introduction

Perpetuity of all mammalian species relies on sexual reproduction, which implies the production and interaction of gametes. In the case of females, oocytes develop in association with somatic cells forming a functional unit known as the ovarian follicle. Follicular development is regulated by neural information that arrives at the ovary through its innervation and also by the secretion patterns of the two pituitary gonadotropins: FSH and LH (Domínguez and Cruz-Morales, 2011). In the process of ovulation, mature oocytes capable of being fertilized are released from the follicles and deposited into the oviduct once every estrous/menstrual cycle. In laboratory rodents, this process is gated by a surge-like secretion

of LH and FSH that results from a timed increase in the firing and secretory activity of the hypothalamic neurons that synthesize the GnRH (Conti *et al.*, 2012; Richards, *et al.*, 1998; Russell and Robker, 2007).

It has been shown that the pre-ovulatory surge of gonadotropins occurs at a specific time of the proestrus stage with strict phase angle with the circadian rhythm of locomotor activity (Robertson *et al.*, 2009; Seegal and Goldman, 1975). Furthermore, the study by Everett and Sawyer (1950) showed that an injection of sodium pentobarbital during pro-estrus blocks ovulation only if it is performed between 14:00 and 16:00 h, the so called 'critical window'. Females that did not ovulate with this injection retained high estradiol levels, as inferred from uterine distention on the day of killing, and ovulated 24 h later. The authors reported that by repeating the treatment during the critical time window of the following days, ovulation could be delayed by multiples of 24 h. It was then proved that the blockade of ovulation in pentobarbital-treated rats is the result of the downregulation of GnRH/LH secretion (Stetson *et al.*, 1981).

Ovariectomized rodents primed with high doses of estradiol display daily surges of GnRH/LH similar in amplitude and phase to the naturally occurring proestrus surge (Caligaris *et al.*, 1971; Henderson *et al.*, 1977; Legan and Karsh, 1975; Legan *et al.*, 1975; Ramírez and Sawyer, 1974). These surges can be entrained to a light–dark cycle, implying that they are regulated by an endogenous circadian pacemaker. Furthermore, a specific mutation that decreases the period of the locomotor activity rhythm also decreases the period of these daily-occurring gonadotropin surges (Lucas *et al.*, 1999). Taken together, these results suggest that ovulation depends on the occurrence of a neural circadian signal in a background of high estradiol levels and that such a signal originates in the SCN. In concordance, bilateral ablation of the SCN not only results in the inhibition of the gonadotropin surge and the consequent anovulatory state, but also in alterations of the estrous cycle and aberrant sexual behavior (Brown-Grant and Raisman, 1977; Gray *et al.*, 1978; Raisman and Brown-Grant, 1977).

The SCN seems to coordinate ovulation by means of synaptic outputs to effector areas since its surgical deafferentation results in similar alterations previously found in lesion studies (Kawakami and Terasawa, 1972; Kawakami *et al.*, 1980; Weigand and Terasawa, 1982). In addition, SCN transplants into lesioned female hosts restore the rhythm in locomotor activity but not the estrous cycle nor the phasic secretion of gonadotropins. This has been attributed to the destruction of well-structured neural pathways from the SCN to specific targets involved in the regulation of reproductive functions, which cannot be restored by the non-specific neural outgrowths from the graft (Meyer-Bernstein *et al.*, 1999). It is commonly accepted that the participation of the SCN in the regulation of ovulation is restricted to the generation of a neural signal that stimulates the pre-ovulatory surge of gonadotropins. This can be achieved either by a direct pathway linking the SCN with the preoptic GnRH neurons, or by a multisynaptic pathway involving interneurons that in turn

stimulates GnRH release, or by both (de la Iglesia and Schwartz, 2006; Miller and Takahashi, 2013; Ohkura *et al.*, 2009).

An interesting finding is that pentobarbital injections between 14:00 and 16:00 h of estrus, metestrus and diestrus also results in the blockade of ovulation (Beattie and Schwartz, 1973; Domínguez and Smith, 1974; Okamoto *et al*., 1972; Schwartz and Lawton, 1968). This implies that the critical window first described in the study of Everett and Sawyer (1950) is not exclusive to the proestrus stage. These experiments were performed on animals with both ovaries in situ, and thus estradiol levels fluctuated naturally. This allows us to suggest that the neural circadian signal required for ovulation is generated every day, approximately at the same time, irrespective of the concentration of estradiol. Additionally, we recently found that unilateral lesions of the SCN performed at estrus or metestrus results in acute alterations of the estrous cycle followed by anovulation, while lesions performed at diestrus or proestrus blocks ovulation without modifying the cycle (in preparation). We hypothesize that the circadian signals generated during each stage of the estrous cycle originate at the SCN and are pivotal for the regulation of ovulation because they regulate processes other than the pre-ovulatory surge of gonadotropins. We tested this hypothesis in the present study by analyzing ovulation in female cycling rats after a transitory blockade of the SCN's voltage-gated Na^+ channels, and thus of the generation of action potentials, on the critical window of each stage of the estrous cycle.

Methods

Ethical approval.

All experiments were approved by the Ethics Committee of Facultad de Estudios Superiores Zaragoza, UNAM under the license UNAM; FESZ/DEPI/CI/374/18. This institution operates in strict accordance with the Mexican rules for animal handling, Official Norm NOM-062-ZOO-1999. The authors declare that they understand the ethical principles under which Experimental Physiology operates and confirm that our procedures comply with the principles and regulations for animal reporting as described by Grundy (2015).

Animals and housing

A total of 150 3-month-old female hooded rats (CIIZV strain) weighing between 230 and 260 g were used in this study. Animals were provided by the bioterium at Facultad de Estudios Superiores Zaragoza, UNAM. Rats were housed in groups of four in standard polypropylene rat cages and kept in a room with a 14:10 light–dark photoperiod with lights on from 05:00 to 19:00 h. Temperature was set at 22 ± 2 °C, humidity at 40% with food and tap water available at all times. Vaginal smears were taken every day between 11:00 and 12:00 hours. Five intact rats were used as absolute controls and the rest were implanted with bilateral guide cannulas aimed at the SCN.

Cannula construction and preoperative care

Bilateral guide cannulas were made of two 23G stainless steel hypodermic needles cut into 14 mm segments and aligned 400 μm apart from each other. Both needles were glued together at the superior end with non-saturated polyester resin of low reactivity. To ensure patency of the cannulas throughout the study, 30G stainless steel hypodermic needles occluded from both sides were used as obturators. All the cannulation surgeries were performed under aseptic conditions in rats at diestrus (n = 145). Anesthesia was induced with 4% isoflurane (PiSA Agropecuaria, Jalisco, México) in 100% oxygen inside an anesthetic chamber for rats connected to a vaporizer (Kent Scientific Corp., Connecticut, USA). To avoid distress to the animals, the chamber was not prefilled. After loss of the righting reflex was observed, a surgical plane of anesthesia was confirmed by pupil dilatation and the loss of pain measured by the tail and ear pinch tests. The rats were immediately removed from the chamber and anesthesia effects maintained with 2.5% isoflurane through a nose cone. The hair of the scalp was removed with clippers and a surgical alternating scrub of iodopovidone and 70% ethanol was performed in the shaved area three times. A dose of 2 mg kg^{-1} of meloxicam (Aranda, Mexico City, México) and 5 mg kg^{-1} of enrofloxacin (Senosiain, Mexico City, México) was injected subcutaneously as a non-steroidal anti-inflammatory/analgesic and as an antibiotic, respectively. Hypromellose artificial tears (Sophia Labs, Mexico City, México) were applied to each eye to avoid desiccation during the surgery.

Guide cannula implantation

Once prepared, the animals were placed on a model 900 stereotaxic apparatus (David Kopf Instruments, California, USA) and anesthesia was maintained through a face mask (David Kopf Instruments). A 1.5 cm incision was made in the skin and muscle, the skull was cleaned with sterile saline solution and a craniotomy of about 2 mm diameter was made. To place the cannula in the superior border of the SCN, stereotaxic coordinates from Paxinos and Watson (2014) were modified to match this rat strain: 0.3 mm posterior to bregma, ± 0.3 mm lateral to the midline and 8.9 mm ventral to the skull; the tooth bar was set at −5 mm below the ear bars. To avoid damaging the superior sagittal sinus while lowering the cannula, we followed the protocol of Wirtshafter and colleagues (1979). The cannula was anchored to the skull with three surgical screws and dental cement. Animals received thermal support through the surgical procedure and until they fully recovered from anesthesia and started moving around the recovery cage. Lost fluids were replaced by applying an intraperitoneal injection of 0.5 ml of physiological sterile and warm saline. After cannula implantation procedures, the rats were single housed and post-operative doses of enrofloxacin and meloxicam were provided 24 and 48 hours after the surgery. No other manipulation was performed on the rats during this recovery period. Vaginal smears were resumed on the third day after surgery and continued until the day of killing.

Hormonal replacement of non-cycling rats

As determined by vaginal smears conducted up to 30 days after the implantation of the cannula, 25 of the implanted rats did not recover their estrous cyclicity (n = 25). These non-cycling animals were assigned to the gonadotropin replacement protocol described by Cruz and colleagues (1992) in order to identify the endocrine pathway altered by the cannulation procedure. Briefly, injection of human chorionic gonadotropin (hCG), which has an LH-like effect on rodent ovarian follicles, allowed us to test the hypothesis that the acyclic state of these rats was the result of alterations in the phasic release of LH, while tonic secretion of gonadotropins was unaltered. Animals were injected at 14:00 h with 20 IU of hCG (Sigma-Aldrich, Missouri, USA) and killed 24 h after (n = 13). As expected, most of the animals injected with hCG did not ovulate (see Results) and so we then tested the hypothesis that the cannulation altered both the tonic and the phasic release of FSH and LH, but not the responsiveness of the ovaries to gonadotropins. Animals were injected at 14:00 h with 8 IU of pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich), which has an FSH- and LH-like effect on rodent ovarian follicles, and 56 h later were injected with 20 IU of hCG, before killing 24 h later (n = 12).

Experimental groups and microinjection procedure

Rats displaying at least three consecutive 4-day estrous cycles after the cannulation surgery were assigned to the TTX experiment. TTX citrate was purchased from Alomone labs (Jerusalem, Israel) as lyophilized powder and reconstituted in sterile 0.9% saline solution following vendor instructions and then diluted to 10 ng μl^{-1} and later to 1 ng μl^{-1}. Since animals treated with the saline solution as vehicle into the SCN showed deleterious effects on ovulation, a different vial of TTX was reconstituted in artificial cerebrospinal fluid (ACSF; BASi, Indianapolis, USA) and diluted to 10 ng μl^{-1}. Animals were divided into four groups depending on the stage of the estrous cycle (number of animals in each group after histological confirmation was: estrus n = 21, metestrus n = 20, diestrus n = 21, proestrus n = 38) and then they were further subdivided into the following groups (see rationale and number of rats per group in Results). Proestrus: saline at 14:00 h; saline at 17:00 h; ACSF at 14:00 h; TTX diluted in saline (1 ng μl^{-1}) at 14:00 h; TTX diluted in saline (10 ng μl^{-1}) at 14:00 h; TTX diluted in ACSF (10 ng μl^{-1}) at 14:00 h; TTX diluted in ACSF (10 ng μl^{-1}) at 17:00 h. Estrus, metestrus and diestrus: saline at 14:00 h; ACSF at 14:00 h; TTX diluted in saline (10 ng μl^{-1}) at 14:00 h; TTX diluted in ACSF (10 ng μl^{-1}) at 14:00 h.

To avoid distress to littermates, animals were transported to a separate room in their own housing cages and the microinjection was performed while freely moving. Microinjection was achieved by gently removing the obturators and 30G microinjectors, connected to 10 µl Hamilton syringes through Teflon tubing (MF-5164; BASi), were inserted into the guide cannulas. These microinjectors were cut to a final length that allowed a protrusion of 1/2 mm out of the cannulas. Syringes were prefilled with the vehicles/drugs described above and controlled with a two-channel microinjection pump model 101 (KD

Scientific, Massachusetts, USA) set at an infusion rate of 50 nl min^{-1} for 4 min to achieve a total volume of 200 nl. This volume was selected considering other studies where TTX was infused into the SCN (Mintz *et al.*, 1999; Paul *et al.*, 2004) and was tested in previous experiments from this laboratory that confirmed that it fully covered the SCN with minimal or no leaking into adjacent areas. To avoid reflux of the infusate, the microinjectors were left in place for an additional 2 min after microinjection. The procedure took less than 10 min per individual. After infusion treatment the animals were returned to their home room. Animals were killed at 09:00 h on the predicted day of estrus, which matched with a cornified vaginal smear in all but three rats (see Results).

Killing and tissue processing

Animals received an intraperitoneal injection of sodium pentobarbital (80 mg kg^{-1}; PiSA Agropecuaria) and were decapitated after showing loss of consciousness. Brains were extracted and frozen at −20 °C. With the aid of a cryostat (Leica, Germany), 50 µm-thick brain sections were cut throughout the coronal plane of the hypothalamus. Sections from the anteroventral periventricular area to the arcuate nucleus (Figs 32–69 from Paxinos and Watson, 2014) were obtained, stained with the Nissl technique and subsequently analyzed to assess the final position of the cannulas and the dispersion of the infusate under an optic microscope. A successful microinjection into the SCN was considered only when we confirmed that the cannula and the dispersion of the fluid was confined to the suprachiasmatic area (Figs 39–42 from Paxinos and Watson, 2014). Results from animals where the cannula/dispersion was outside this area are also discussed in relation to the areas affected by the microinjection (see Results). During the autopsy, ovaries were dissected and the oviducts excised. Oocytes from each oviduct were extracted under a stereomicroscope, stained with hematoxylin and eosin and then counted under an optical microscope.

Data analysis

The fraction of cyclic animals at each time point was compared using X^2 test and then expressed as the cumulative percentage of cyclic rats with respect to recovery time. We calculated the rate of ovulation for each experimental group as the number of ovulating rats/n and compared them using Fisher's exact test and then represented it as the percentage of ovulating animals. As the observations regarding the number of ova shed are non-negative integers (count data), we analyzed these data using non-parametric tests; the Mann-Withney U test was used to compare between two groups and the Kruskal–Wallis test, followed by Dunn's test, was used when comparing multiple groups. We did not find any difference in the number of oocytes shed by the left and right ovaries and thus they are expressed as the median and interquartile range of the total shed by both ovaries. Statistical comparisons were made using GraphPad Prism 7.0 software for Windows (GraphPad Software Inc., California, USA). The threshold for statistical significance was set at $\alpha = 0.05$. The power to detect a 90% reduction in the ovulation rate (effect size) in a one-tailed test for independent proportions with $\alpha = 0.05$ was performed using G*power (version 3.1.9.4 for Windows, University of

Dusseldorf, Germany) to ensure that the number of individuals in each experimental group was adequate.

Results

All of the rats provided by the animal house were included in the study since they displayed at least three consecutive 4-day estrous cycles before surgery or killing. In the first cycle after the implantation of the cannula, 80% of the animals showed an elongation of their estrous cycle due to the presence of a third day of diestrus. The animals gradually recovered estrous cyclicity. By the fifth cycle after surgery, approximately 15% of the rats remained acyclic (Figure 7A). Animals that did not recover their estrous cycle after surgery had smears with a predominance of leukocytes for several days. Such days were usually interrupted by 3–4 days of cornified smears. We did not find smears containing nucleated cells that could be interpreted as prooestrus in these animals. Therefore, these rats were injected with hCG or PMSG+hCG. None of the rats injected with hCG alone ovulated (Intact: 5/5 vs. hCG: 0/13, P = 0.0001, Fisher's exact test) while 10/12 rats injected with PMSG+hCG ovulated (P > 0.9999, Fisher's exact test; Figure 7B). Histological analysis of their brains showed that the cannulas were placed in similar regions to those of the rest of the animals in this study and hence we could not associate the acyclic status to a particular localization of the cannula.

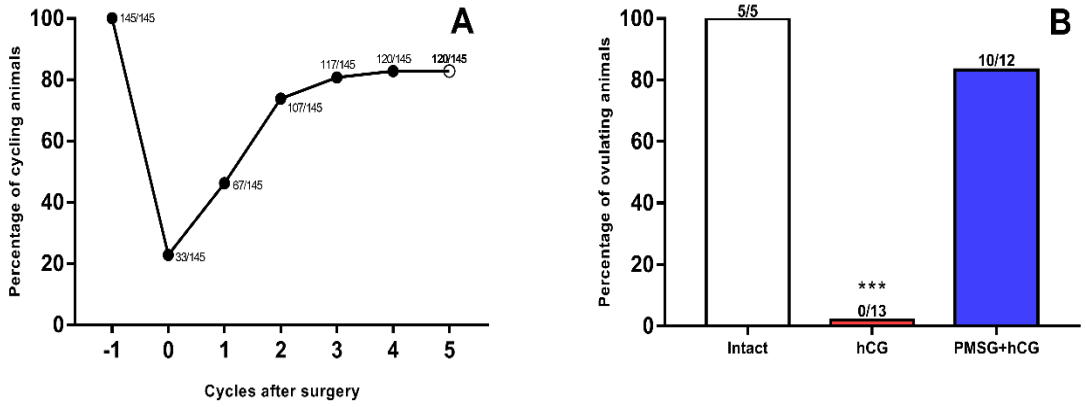

Figure 7. *(A) Cumulative percentage of cycling animals before and after the implantation of the guide cannula. It is important to note that a significant decrease in the percentage of cycling animals was observed even in the fifth cycle ($p \leq 0.0001$, one-tailed X^2 test). (B) Percentage of ovulating females submitted to the hormonal replacement with human chorionic gonadotropin (hCG) or pregnant mare serum gonadotropin (PMSG)+hCG after determination of acyclicity (***$p \leq 0.001$ vs. intact, Fisher´s exact probability test).*

Animals that recovered their estrous cycle after the cannulation surgery were randomly distributed to the microinjection groups established above. All but three of the animals did not show alterations of the estrous cycle after microinjection treatment, and hence the predicted day of estrus coincided with a cornified vaginal smear that is characteristic of the estrus stage.

To determine the amount of TTX that effectively inhibited the Na^+-dependent action potentials in the SCN, we tested a concentration of 10 ng μl^{-1}, which has been successfully used in in vivo experiments of pharmacological disconnection of several areas of the brain, but not the SCN (Bohenke and Rasmusson, 2001; Freund *et al.*, 2010; Pereira de Vasconcelos et al., 2006; Zhuravin and Bures, 1991). For purposes of comparison, we tested a lower dose of 1 ng μl^{-1}, which is similar to the concentration used for both *in vitro* and *in vivo* experiments working with the SCN (Harlan *et al.*, 1983; Mintz *et al.*, 1999; Paul *et al.*, 2004; Rothfeld *et al.*, 1986; Sandford *et al.*, 2005; Schwartz *et al.*, 1987; Shibata and Moore, 1993).

Animals in proestrus were microinjected with saline solution or TTX diluted in saline (at concentration of either 1 or 10 ng μl^{-1}) into the SCN at 14:00 h (critical window) and were killed the next morning. As shown in Figure 8A, all the animals injected with saline ovulated, 4/6 animals ovulated when microinjected with the low dose of TTX and none of the animals that received the high dose of TTX ovulated. Compared to the saline-treated group, the lower ovulation rate observed in low TTX dose-treated animals was not significant (Saline: 7/7 vs. TTX 1 ng μl^{-1}: 4/6, P = 0.1923, Fisher's exact test). The high TTX dose blocked ovulation completely (Saline: 7/7 vs. TTX 10 ng μl^{-1}: 0/5, P = 0.0013, Fisher's exact test). Based on these results, the TTX dose of 10 ng μl^{-1} was used for the rest of the experiment. No difference in the number of oocytes shed by ovulating animals was observed in this experiment (Table 1).

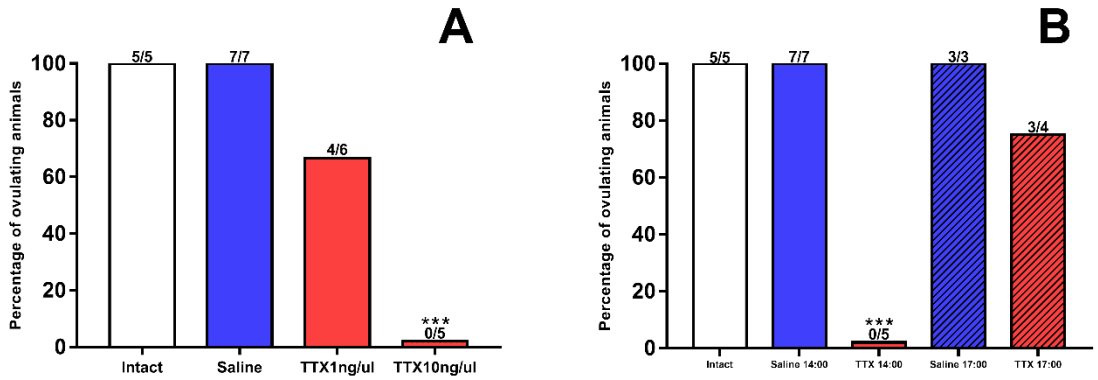

Figure 8. *(A) Percentage of ovulating animals in the group microinjected with saline solution or with tetrodotoxin (TTX) diluted in saline solution at a concentration of 1.0 ng/µl or 10.0 ng/µl. Microinjections were performed at 14:00 hours of proestrus (***p≤0.001 vs. saline, Fisher´s exact probability test). (B) Percentage of ovulating animals microinjected with saline or with TTX in a dose of 10.0 ng/µl at 14:00 or 17:00 hours of proestrus (***p≤0.001 vs. saline at 14:00 h, Fisher´s exact probability test).*

To validate our TTX model, we replicated part of the experiment by Everett and Sawyer (1950). Animals were microinjected with saline solution or TTX (10 ng μl^{-1}) during or outside the proestrus critical window (14:00 or 17:00 h, respectively) and killed the next morning. Irrespective of the hour of treatment, all saline-treated animals ovulated.

Microinjection of TTX at 17:00 h did not modify ovulation (Saline: 3/3 vs. TTX: 3/4, P > 0.9999, Fisher's exact test). None of the rats treated with TTX at 14:00 h ovulated (Saline: 7/7 vs. TTX: 0/5, P = 0.0013, Fisher's exact test; Figure 8B). These results show that the TTX treatment blocks the neural signals originating from the SCN during the critical window of proestrus. Consequently, TTX microinjection treatment was performed at 14:00 h in rats on the other stages of the estrous cycle.

Table 1. Median and interquartile range (IR) of the number of oocytes shed by experimental animals

Group	Estrus		Metestrus		Diestrus		Proestrus	
	Median	IR	Median	IR	Median	IR	Median	IR
Intact	11.0	9.0-13.0	-	-	-	-	-	-
Saline at 14:00 hours	7, 7*	N/A	12.0	10.0-13.0	N/A	N/A	13.0	11.0-13.0
TTX in saline (1 ng/µl) at 14:00 hours	-	-	-	-	-	-	13.0	9.75-15.5
TTX in saline (10 ng/µl) at 14:00 hours	12*	N/A	8*	N/A	N/A	N/A	N/A	N/A
Saline at 17:00 hours	-	-	-	-	-	-	14.0	13.0-17.0
TTX in saline (10 ng/µl) at 17:00 hours	-	-	-	-	-	-	13.0	12.0-14.0
ACSF at 14:00 hours	14.0	10.0-14.0	12.0	7.5-14.0	11.5	8.0-12.75	13.0	10.0-14.0
TTX in ACSF (10 ng/µl) at 14:00 hours	N/A	N/A	12*	N/A	N/A	N/A	N/A	N/A

**Number of oocytes shed by animals when the number of observations was too low to perform the proper statistics, N/A means that there were no ovulating animals in the group. Blank spaces mean that an experimental group was not performed on a particular stage of the estrous cycle (see results for rationale). Artificial cerebrospinal fluid (ACSF), tetrodotoxin (TTX).*

Without considering the stage when treatments were performed, we found a significant decrease in the rate of ovulating females injected with saline as compared with the intact animals (intact: 5/5 vs. saline: 15/30, P = 0.0478, Fisher's exact test). Despite this decrease in the ovulation rate, a difference between saline- and TTX diluted in saline (TTX(S))-treated animals was found (saline: 15/30 vs. TTX(S): 2/20, P = 0.0033, Fisher's exact test). Considering that the saline solution administered at certain stages of the estrous cycle has a deleterious effect on ovulation, we decided to include the use of ACSF as a vehicle for this experiment. Compared to intact rats, ACSF microinjection did not lower the rate of ovulating animals. A significant decrease in the ovulation rate was observed in animals treated with TTX diluted in ACSF (TTX(A)) (ACSF: 17/19 vs. TTX(A): 1/13, P = 0.0001, Fisher's exact test).

Figure 9 shows the effect of each treatment when performed at a specific stage of the estrous cycle. Saline solution injected during estrus blocked ovulation in 7 out of 9 animals, a significantly lower rate compared with the intact group (intact: 5/5 vs. saline: 2/9, P = 0.0105, Fisher's exact test). Compared to the control group, ACSF microinjection decreased ovulatory rate, but not significantly (P = 0.2222). Even in this case, it is ambiguous to compare the TTX treated animals against either of the vehicle solutions. We found a significant decrease when comparing TTX-treated animals vs. the intact group (intact: 5/5 vs. TTX(S): 1/4 or TTX(A): 0/3, P = 0.0476 and P = 0.0179, respectively, Fisher's exact test).

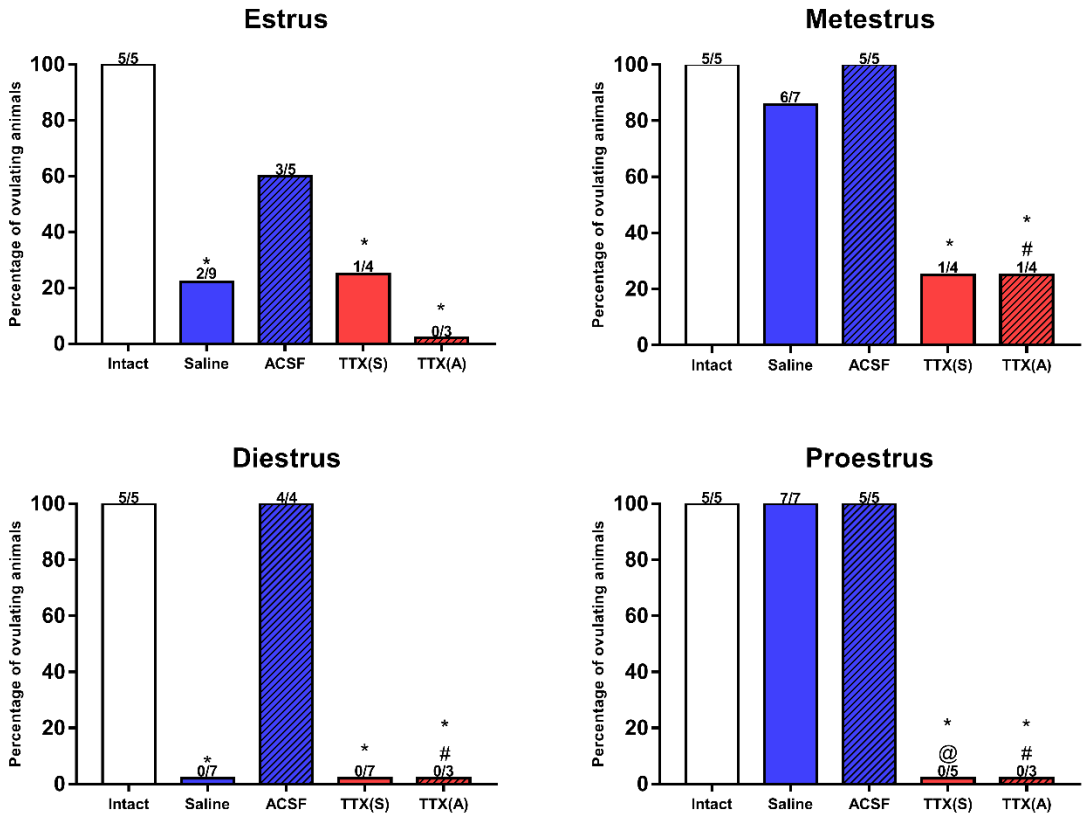

Figure 9. *Percentage of ovulating animals microinjected with saline, artificial cerebrospinal fluid (ACSF), tetrodotoxin diluted in saline [TTX(S)] or tetrodotoxin diluted in artificial cerebrospinal fluid [TTX(A)] at 14:00 of each stage of the estrous cycle. Notice that the intact group was added to each stage for comparison purposes (* p≤0.05 vs. intact; @ p≤0.05 vs. saline; # p≤0.05 vs. ACSF, respectively, Fisher´s exact probability test).*

In rats at metestrus, saline solution only blocked ovulation in one animal and compared to rats treated with TTX dissolved in saline solution, the ovulation rate difference was not significant (saline: 6/7 vs. TTX(S): 1/4, P = 0.0879, Fisher's exact test). All the animals injected with ACSF successfully ovulated while only one of the TTX dissolved in ACSF rats ovulated (ACSF: 5/5 vs. TTX(A): 1/4, P = 0.0476, Fisher's exact test). At diestrus, none of the animals injected with saline or with TTX dissolved in saline ovulated (intact: 5/5

vs. saline: 0/7 or TTX(S): 0/7, P = 0.0013, Fisher's exact test). All of the animals injected with ACSF ovulated and a significant decrease was found in TTX dissolved in ACSF animals (ACSF: 4/4 vs. TTX(A): 0/3, P = 0.0286, Fisher's exact test).

All the animals at proestrus treated with either saline solution or ACSF ovulated, while none of the animals treated with TTX dissolved in either vehicle ovulated (saline: 7/7 vs. TTX(S): 0/5, P = 0.0013; ACSF: 5/5 vs. TTX(A): 0/3, P = 0.0179, Fisher's exact test). Five experimental animals removed one of the obturators; consequently, unilateral infusions on the side that retained patency were performed. On these animals, treatment with TTX dissolved in saline was performed at 14.00 h of the proestrous day. Only one of these animals ovulated and, compared to animals treated bilaterally with saline solution, showed a significantly lower rate of ovulation (saline: 7/7 vs. unilateral-TTX(S): 1/5, P = 0.0012).

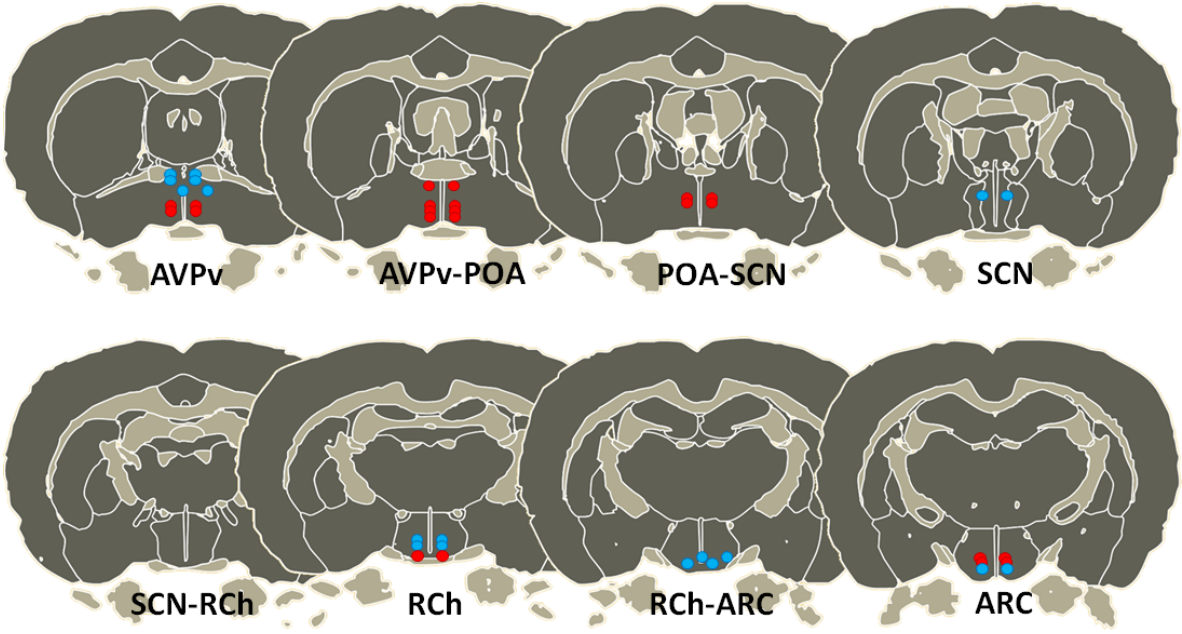

Figure 10. *Final location of the guide cannulas´ tip that were determined to be outside the suprachiasmatic nucleus. Each pair of dots represent the location of the bilateral cannula in one animal. Red dots represent cannulas that resulted in anovulation while blue dots represent cannulas from animals that ovulated. Anteroventral periventricular area (AVPv), preoptic area (POA), suprachiasmatic nucleus (SCN), retrochiasmatic area (RCh), arcuate nucleus (ARC).*

After histological confirmation, the cannulas of 20 animals (not considered in the statistical analysis above) were determined to be outside of the SCN. The location of the cannulas ranged from the anterior hypothalamus, at the anteroventral periventricular area (AVPv), to the posterior hypothalamus near the ARC. Since these animals were intended to belong to several of the 14:00 h groups, at the different stages of the cycle, it is not possible to do a stage-by-stage analysis; however, we found an association between the final position of the cannula and the ovulatory outcome of the animal. Irrespective of treatment (vehicle or

TTX), most rats in which the cannulas were placed on the AVPv, the POA, or the ARC showed anovulation. Two of the animals injected with saline into the AVPv and one into the ARC showed longer estrous cycles characterized by three leucocytic smears following microinjections. Irrespective of the treatment type, cannulas inserted in the anterior commissure or the retrochiasmatic area resulted in ovulation (summarized in Figure 10).

Discussion

The main findings of this study were as follows. (a) During the critical window of proestrus, the inhibition of Na^+-dependent action potentials in the SCN results in the blockade of ovulation. This result implies that the SCN is the locus of the neural circadian signal that stimulates the pre-ovulatory surge of gonadotropins. (b) Inhibition of Na^+-dependent action potentials at 14:00 h during estrus, metestrus or diestrus also blocked ovulation. We propose that a neural circadian signal originating in the SCN during the afternoon of every stage of the estrous cycle is required to regulate ovulation properly. (c) Saline solution administration into the SCN during estrus or diestrus impairs ovulation. This was not observed in animals treated with ACSF. This may be an indication of the SCN's different capacities to compensate for changes in ion concentrations. Such differences probably depend on the concentration of hormones that fluctuate during the estrous cycle, such as estradiol and progesterone.

Alterations in the estrous cycle of the animals that were implanted with a cannula can be explained by the activation of the stress axis. It has been previously shown that female rats are particularly susceptible to stress due to a higher activation of the Corticotropin Releasing Factor neurons at the paraventricular nucleus and other stress-related areas as compared with males (Babb *et al.*, 2013). Both Sharp *et al.* (2002) and Babb *et al.* (2013) report that the deleterious effects of stress on reproductive function do not depend on the stage of the estrous cycle. However, we have observed that recovery time increases if stereotaxic surgeries are performed at estrus or metestrus (unpublished observation). In general, hormones released during stressful situations inhibit the activity of the hypothalamic–pituitary–ovarian axis (Kalantaridou *et al.*, 2004). Animals exposed to acute surgical stress exhibit an increase in the concentration of progesterone, probably of adrenal origin, and estradiol, which alters the phasic release of gonadotropins (Nequin *et al.*, 1975). In addition, different stressors presented during the estrous cycle stimulate the angiotensin system through the AT1 and AT2 receptors, resulting in a decrease in the number of ova shed and the lordosis quotient during the next estrus (Cecconello *et al.*, 2010; Donadio *et al.*, 2007).

Finally, it has been reported that exposure to stress desynchronizes the estrous cycle due to alterations in the release of steroid hormones, which in turn feed back into the central mechanisms governing the cycle (Pollard *et al.*, 2004). These results possibly explain the fact that most of our rats exhibited a transient disturbance of their cycles after the surgery, which were re-established a few days after the stressor was eliminated. The hormonal replacement protocol performed on non-cycling rats revealed that both the tonic and the phasic release of

gonadotropins were altered. It has been reported that structures dorsal to the SCN, such as the medial POA, are pivotal for gonadotropin secretion in males and females (Colombo and Phelps, 1981; Kawakami *et al.*, 1970). The histological analysis of the brain sections did not reveal any peculiarity on these rats that could serve to differentiate them from the cyclic rats and thus we concluded that alterations of the estrous cycle observed in the present study are the result of individual susceptibility to stress. Another possibility is that damage of fibers and/or the destruction of neuronal bodies that are important in conveying the GnRH signals to the median eminence occurred to a greater extent. The results obtained from the cycling rats are thus free of the acute effects of the surgical stress. The continuity of the cycles allows us to suggest that their tonic and phasic secretion of gonadotropins was normal at the time of treatment and hence that any observed effect is due to the microinjection of the vehicle/drug.

The molecular machinery that drives the circadian rhythmicity of the SCN, i.e., clock genes, is not altered by TTX treatment and therefore its period and phase continue unaltered after treatment (Mintz *et al.*, 1999; Paul *et al.*, 2004; Schwartz *et al.*, 1987; Shibata and Moore, 1993). Consequently, the anovulation observed in this study is the result of the blockade of a pivotal neural efferent signal that depends on voltage-gated Na^+ channels and not on the delay or advance of it. The occurrence of a neural signal originating at the SCN has been proposed to explain the circadian triggering of the pre-ovulatory surge of GnRH during proestrus. This assumption was based on data from experiments in which the SCN was lesioned or deafferented several days before the sampling procedure (Brown-Grant and Raisman, 1977; Gray *et al.*, 1978; Kawakami and Terasawa, 1972; Kawakami *et al.*, 1980; Raisman and Brown-Grant, 1977; Weigand and Terasawa, 1982). The alterations of sexual behavior, gonadotropin secretion and ovulation following a prolonged exposure to constant light (Campbell and Schwartz, 1980; Hardy, 1970; Hoffman, 1967), as well as the aberrant tonic/phasic release of gonadotropins and poor reproductive performance exhibited by clock gene mutant mice, also support this idea (Dolatshad *et al.*, 2006; Kennaway *et al.*, 2004). The permanent disruption of the circadian system in all of these studies, however, does not permit the determination of whether the anovulatory outcome is the result of the inhibition of a SCN efferent signal or the effect of the alteration of physiological variables that are governed by the circadian system that in turn impact reproduction, for example corticoid and melatonin secretion.

The study by Everett and Sawyer (1950) disclosed the presence of the neural circadian signal itself, but since barbiturates act at different structures, no evidence of its origin nor its neurotransmitter nature was shown. To our knowledge, our study is the first to analyze the effects of the transitory inhibition of the SCN's Na^+-dependent action potentials on ovulation. In the case of the proestrus stage, the fact that only rats injected inside the critical window did not ovulate allows us to suggest that the SCN is the generator of the neural circadian signal that triggers the pre-ovulatory surge of GnRH/gonadotropins. Following the discovery of the SCN as a circadian clock, there has been evidence about the presence of monosynaptic-ipsilateral and reciprocal pathways linking it with the preoptic areas related to the regulation

of reproduction. Such fibers contain the two predominant neurotransmitters of the SCN, AVP and VIP (van der Beek *et al.*, 1993, 1994; 1997a; 1997b). Furthermore, timed administration of AVP into the POA results in the generation of a surge-like release of LH (Palm *et al.*, 1999; 2001) while administration of VIP inhibits the estradiol-induced surge of LH in ovariectomized rodents (Akema *et al.*, 1988; Kimura *et al.*, 1987).

The results listed above are in favor of a direct signal from the SCN that regulates the pre-ovulatory surge of gonadotropins acting at the POA. We hypothesize that this pathway may be responsible for conveying the neural signal to the GnRH neurons. Experiments by van der Beek *et al.* (1994) and de la Iglesia *et al.* (2003) revealed that the SCN neurons and the GnRH neurons innervated by them are co-activated during the pre-ovulatory surge of gonadotropins. Studies evaluating the electrical activity of the POA, as well as the phasic secretion of gonadotropins in the SCN TTX-silenced model with or without preoptic injections of VIP/AVP could serve to fully disclose the nature of the communication between these two areas.

The interaction of the SCN with other neuronal populations involved in the regulation of ovulation also needs to be considered to fully understand the circadian regulation of the process. It has been shown that the SCN and the POA share reciprocal connections with the neurons located in the AVPv and the ARC (de la Iglesia *et al.*, 1995; 1999; Hahn and Coen, 2006; Kalló *et al.*, 2013; Saeb-Parsy *et al.*, 2000; Simodian *et al.*, 1999; Yi *et al.*, 2006). There is evidence to suggest that the SCN projects to kisspeptin neurons in the AVPv, modulating their circadian activation during the pre-ovulatory surge of gonadotropins (Smarr *et al.*, 2013; Vida *et al.*, 2010; Williams *et al.*, 2011). On the other hand, a direct projection linking ARC kisspeptin neurons with the SCN has not been demonstrated. AVPv and ARC kisspeptin neurons stimulate the secretion of GnRH in a background of high and low estradiol levels, respectively (Ohkura *et al.*, 2009). It is believed that AVPv kisspeptin neurons are responsible for driving the phasic release of gonadotropins while ARC kisspeptin neurons drive the tonic release. The inhibition of the crosstalk between the SCN and the AVPv could serve as an alternative explanation for the results obtained from rats treated in proestrus. In this scenario, TTX injected into the SCN would result in the lack of a stimulant signal to the kisspeptin neurons in the AVPv and hence GnRH neurons would not show a preovulatory activation.

The communication of the SCN with the ARC can explain the anovulation that was observed when TTX was injected at stages of the estrous cycle other than proestrus. It has been reported that TTX maximum blockade is attained about 30 min after microinjection into the brain and its blocking effects lasts between 2 and 24 h, which seems to depend on the system studied (Harlan *et al.*, 1983; Zhuravin and Bures, 1991). This effective period is consistent across several studies even though the molecule can be detected by immunohistochemistry up to 32 h after administration (Freund *et al.*, 2010). Considering this, the anovulation observed after TTX administration at estrus, metestrus or diestrus can hardly

be the effect of the blockade of the neural signal that occurs during the proestrus afternoon. The pivotal role of the communication between the SCN and the ARC has been proved in a study that shows that micro-cuts severing the fibers that links both nuclei result in the loss of circadian rhythmicity in locomotor activity, body temperature and hormone secretion (Buijs *et al.*, 2017).

The participation of the ARC kisspeptin neurons in the regulation of reproduction has been also demonstrated by means of selective ablation of this population of cells in mice. These animals showed not only alterations in the circadian organization of food consumption and locomotor activity but also in LH secretion, which is accompanied by abnormal estrous cycles (Padilla *et al.*, 2019). Mittelman-Smith and colleagues (2016) reported that selective ablation of the rat ARC-KNDy neurons, which co-synthesize kisspeptin, neurokinin B and dynorphin, results in low levels of LH in serum, a permanent state of diestrus, ovarian follicular development with augmented incidence of atresia in large follicles and a reduction in the number of endometrial glands in the uterus. Interestingly, ovariecomized females submitted to the ARC-KNDy ablation showed an increase in LH secretion in response to an estradiol+progesterone priming protocol known to induce a surge-like release of gonadotropins. This increase was not accompanied by an increase in the number of AVPv or GnRH neurons that are activated, suggesting that the ARC-KNDy neurons modulate the amount of GnRH that is released during the surge by providing an inhibitory signal acting either at the median eminence, where the GnRH terminals are located, or at the kisspeptin neurons in the AVPv (Mittelman-Smith *et al.*, 2016).

In favor of the last hypothesis mentioned above, Helena and colleagues (2015) found evidence indicating that dynorphin from the KNDy neurons acts directly at the AVPv, decreasing the amount of GnRH/LH that is secreted during the surge. As in the experiment cited above, the authors reported an increase in LH in the serum of female rats submitted to the ablation of the KNDy neurons and implanted with estradiol capsules. In these animals, an increase in the content of kisspeptin in the AVPv was evident and injection of dynorphin into the same area resulted in the secretion of LH in amounts similar to those of the control animals. On the other hand, there is also evidence of the neurokinin B/dynorphin system acting at the level of the ARC-median eminence. In the goat, it has been shown that the electric activity of the ARC-KNDy neurons is related to the secretion of GnRH/LH. In this model, the injection of neurokinin B into the lateral ventricle results in the induction of ARC pulsatile activity while the injection of dynorphin inhibits such activity. The authors hypothesize that neurokinin B and dynorphin represent a stimulant and inhibitory output signal, respectively, which acts at the GnRH terminal and also at the KNDy neuron (Wakabayashi *et al.*, 2010).

We speculate that the SCN participates in the preparation of the AVPv and the ARC to respond to the rise in estradiol levels that starts at diestrus and peaks during proestrus and hence TTX microinjection would result in the indirect blockade of the preovulatory surge of

gonadotropins. In support of this idea, previous data from our laboratory shows that rats bearing unilateral lesions of the SCN have normal follicular development but cannot properly respond to an ovulation-inducing injection of estradiol (in preparation). The AVPv and the ARC are believed to integrate estrogenic signals arising at the ovaries since both express the α-isoform of the estrogen receptor (Ohkura *et al.*, 2009). Accordingly, destruction of either of these areas results in the loss of estrous cycles, alterations in the sexual behavior and anovulation (reviewed in Barbacka-Surowiak *et al.*, 2003). These results also explain the blockade of ovulation that we observed in the animals that had cannulas located in the AVPv–POA or ARC. It seems then that the transitory inactivation of these neurons during the critical window of each stage of the cycle is sufficient to impair the reception of estrogenic signals and/or the direct stimulation of the preoptic GnRH neurons by them. The idea that the SCN may act directly on the kisspeptin neurons is supported by a study that showed concomitant activation of the AVPv kisspeptin neurons with the dorsomedial SCN neurons (Smarr *et al.*, 2013). Further experiments assessing the expression of estradiol receptors in the ARC and AVPv kisspeptin neurons in animals with TTX injected into the SCN are in progress to assess the validity of this hypothesis.

Finally, an unexpected result from this study was the deleterious effect of saline solution on the ovulation rate of estrus- and diestrus-treated groups. Saline solution does not match the physiological ion concentrations found in the intercellular fluids of the brain (Kazim *et al.*, 2010) and thus we can expect it to induce alterations in the function of neurons. This is supported by studies that have shown an increase in fever, headaches, inflammation and apoptosis in humans and in animal models in which saline solution or other perfusates that differ from the cerebrospinal fluid were used (Miyajima *et al.*, 2012; Oka *et al.*, 1996). Changes in the shape and volume of neurons as a result of alteration in the extracellular ion concentration are also associated with cell death by necrosis, which in turn can impact adjacent neurons (Pasantes-Morales and Tuz, 2006). The fact that ACSF injection did not modify the ovulation rate in animals injected at diestrus indicates that anovulation in saline-treated rats was due to the change in the concentration of ions and not to the dilution of other nutrients such as glucose. However, the partial restoration observed at estrus could be the effect of an increase in sensitivity to changes in ion concentration during this stage. It has been shown that even ACSF as perfusate can result in deleterious effects if the concentration of ions such as Mg^{2+} are not the same as those found in the physiological cerebrospinal fluid (Mori *et al.*, 2013). In this scenario, it is clear that the sensitivity of neurons located at the SCN to changes in the concentration of ions in the extracellular fluids is modified as a function of the estrous cycle. To our knowledge, it is not known if these changes are related to the function of the SCN as a circadian pacemaker in females or if similar changes occur in other hypothalamic nuclei involved in the regulation of ovulation. If this is the case, an evaluation of the biological and clinical significance of such changes on the sensitivity to the extracellular concentration of ions is needed.

In conclusion, based on present and previous results from our lab, we suggest that the SCN generates a circadian neural signal every day around the same time that is pivotal for proper regulation of ovulation. This signal is related to the pre-ovulatory release of gonadotropins but also to other neuroendocrine events occurring prior to proestrus. The participation of the SCN in the regulation of ovulation seems to be more than just the triggering of GnRH/gonadotropins release during the afternoon of proestrus and a role of this nucleus in the preparation of structures that regulate such release is suggested. Studies aimed to fully disclose the events that could be coordinated by the SCN, needed for proper regulation of ovulation, are already in progress.

Chapter IV

Chapter IV. Based on:

"The neural activity of the arcuate nucleus is required during the critical window for a proper progression of the rat estrous cycle and ovulation"

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Abstract

Sexual reproduction demands the expenditure of a high amount of energy so it is dependent on the metabolic state. In addition, it is under the control of the circadian system to ensure that events as hormone secretion, ovulation and mating occurs at the most advantageous time. We have shown that a circadian neural signal that is pivotal for ovulation is generated at 14:00 hours of each stage of the estrous cycle in the SCN, the main oscillator of the circadian system. We hypothesize that such signals are sent to central structures related with the preparation of the system to respond to the rise in estradiol levels that in turn triggers ovulation. In the present study we explored the participation of the ARC as one of such structures by transiently blocking its neural activity. Guide cannulas aiming to the ARC were implanted in female rats and, upon recovery of their estrous cycles, were microinjected with ACSF or TTX at 14:00 hours of one of the four stages of the cycle. The progression of the cycle, ovulation and ovarian, uterine and adrenal mass were analyzed on the next predicted estrus. Both treatments decreased the mass of the ovaries when performed in estrus, leaving the rest of the parameters unaltered. The estrous cycles were profoundly affected when TTX was microinjected in metestrus or diestrus, resulting in an anovulatory state and in an increase of the uterine and adrenal mass. Finally, TTX in proestrus blocked ovulation without altering the cycle or the mass of the organs. These results suggest that the neural information generated in the ARC during the afternoon of metestrus, diestrus and proestrus is pivotal for the proper regulation of gonadotropin release and hence for the development of ovarian follicles and ovulation.

Introduction

In female mammals, the development of oocytes, their eventual ovulation and the secretion of hormones take place at the ovaries under the regulation of the pituitary gonadotropins: FSH and LH. Their secretion is modulated by the GnRH, synthesized by neurons located in the POA that send axonal projections to the median eminence. At this level, GnRH is secreted into the portal circulation through which it reaches the pituitary and hence it is considered to be the last stimulant signal from the brain to regulate fertility (Schwartz, 2000; Stamatiades and Kaiser, 2017). In this sense, the brain must interpret the information arising at several

sensory systems that rely information about the internal and external milieu and then organize input signals to the GnRH system. As a result, its secretion is precisely controlled to increase the reproductive fitness of the species and it occurs in harmony with the environmental changes that result from geophysical cycles as the alternation of the days and seasons (Giesel, 1976; Kennaway, 2005; Tena-Sempere, 2013; Wood and Loudon, 2014).

In concordance, several neurotransmitter systems provide direct input to the GnRH-neurons to regulate different aspects of reproduction (Dudas and Merchenthaler, 2006; Hrabovszky and Liposits, 2013). For example, estrogens regulate the pulsatile secretion of gonadotropins, but GnRH-neurons do not express the ERα, which is essential for the process (Herbison and Theodosius, 1992; Herbison and Pape, 2001; Shughrue *et al.* 1997; Watson *et al*., 1992). ERα-expressing neurons in the RP3V and the ARC detect the fluctuations in estradiol levels and then regulate the activity of the GnRH-neurons (Franceschini *et al*., 2006; Gottsch *et al*., 2004). The ERα-neurons in both regions co-express kisspeptin, the most potent stimulator of GnRH release, and their synthetic and electric activity respond in opposite ways to the steroid, i.e., inhibited in the former and enhanced in the later. Considering this, both regions are essential to mediate the hormonal communication between the ovaries and the hypothalamus. The ARC mediates the negative feedback that results in the tonic secretion of gonadotropins, driving the development of ovarian follicles while the RP3V mediates the positive feedback that triggers the pre-ovulatory surge, responsible for the rupture of the follicular wall and the release of oocytes (Adachi *et al*., 2007; Smith *et al*., 2006b).

In addition to estrogens, the metabolic state plays a critical role in the regulation of reproduction. From an evolutionary point of view, natural selection has favored strategies that optimizes the intake and storage of nutrients, as well as the efficient expenditure of the energy obtained. The production of gametes, mating, pregnancy, lactation and parental caring are energy demanding and thus are stimulated in high energy states and inhibited in low ones (Evans and Anderson, 2012; Schneider, 2004; Schneider *et al*., 2013). This is clear in the reports of the inhibition of the tonic and phasic secretion of LH in laboratory rodents by forced exercise or restricted feeding, which leads to a delay of puberty, gonadal involution, absence of follicular growth and anovulation (Bronson, 1986; Campbell *et al*., 1977; Howland, 1980; Manning and Bronson, 1989; 1991). Such deleterious effects are the result of the direct inhibition of GnRH release since there is an increase in the concentration of the peptide in the brain and proper feeding, or pulsatile infusion of GnRH, restores reproductive functions. Additionally, the activity of GnRH-neurons and the expression of the gene encoding the precursor of the peptide decreases in fasted animals (Berriman *et al*., 1992; Gruenewald and Matsumoto, 1993).

As in the case of estrogens, GnRH-neurons do not respond directly to many metabolic signals as leptin (Hakansson *et al*., 1998; Quennell *et al*., 2009) and insulin (Divall *et al*., 2010; Evans *et al*., 2014), although ghrelin could be an exception (Evans and Anderson, 2012; Farkas *et al*., 2013). Due to its anatomical features, which allow it to sense the concentration

of metabolites in the blood (Chronwall, 1985), the ARC regulates reproduction by communicating the state of the internal milieu to the reproductive system. Kisspeptin neurons in the region co-express neurokinin B and dynorphin A, which stimulates and inhibits the release of GnRH, respectively (Navarro *et al*., 2009). These cells were named KNDy-neurons and their selective destruction or inactivation by optogenetic or pharmacological agents suppresses the pulsatile release of GnRH (Clarkson *et al*., 2017; Mittelman-Smith *et al*., 2016; Wakabayashi *et al*., 2010). The expression of the three peptides is modulated by the metabolic state (Li *et al*., 2012; Matsuzaki *et al*., 2011; Nakao *et al*., 2018) and these neurons also contain leptin and insulin receptors. However, the metabolic information seems to be received from other neurons in the ARC since the selective deletion of both receptors from KNDy-neurons does not impact on fertility (Donato *et al*., 2011; Evans *et al*., 2014).

KNDy-neurons receive most of their monosynaptic input from other neurons in the ARC that express peptides related with the regulation of metabolism as proopiomelanocortin (POMC)/α-melanocyte stimulating hormone (α-MSH), cocaine- and amphetamine-regulated transcript (CART), agouti-related peptide (AGRP), neuropeptide Y (NPY) and pituitary adenylate cyclase-activating peptide (PACAP) and also the receptors to sense the concentration of metabolites in the blood (Moore *et al*., 2019; Yeo *et al*., 2019). A particularly interesting input to KNDy-neurons arises at the vasopressin-neurons of the SCN (Yeo *et al*., 2019). This is added to the body of anatomical evidences about neural projections that link the SCN with different populations of neurons in the ARC (Berk and Finkelstein, 1981b; de la Iglesia *et al*., 1999; Saeb-Parsy *et al*., 2000; Stephan *et al*., 1981; Swanson and Cowan, 1975; Yeo *et al*., 2016; Yi *et al*., 2006; Zhang *et al*., 2009). It is hypothesized that the SCN sets the time at which feeding and ovulation occurs in order to coordinate them with the active phase of the animal. In this sense, lesion or deafferentation of the SCN results in the loss of the circadian pattern of feeding and in the permanent abolition of reproduction (Angeles-Castellanos *et al*., 2010; Brown-Grant and Raisman, 1972; Challet, 2019; Gray *et al*., 1978; Kawakami and Terasawa, 1972; Kawakami et al., 1980; Nagai *et al*., 1978; Raisman and Brown-grant, 1972; Robertson et al., 2009; Seegal y Goldman, 1975; Stoyney and Ikonomov, 1987; Weigand y Terasawa, 1982).

In the previous chapter we showed that a circadian neural signal is generated in the SCN every day to regulate ovulation. This occurs between 14:00 and 17:00 hours, a time previously described in proestrus as the "critical window" (Caligaris *et al*., 1971; Everett and Sawyer, 1950; Stetson *et al*., 1981). First interpretations of this phenomena indicated that such daily signals have the role of triggering the pre-ovulatory surge of GnRH only in the presence of high estradiol levels (Caligaris *et al*., 1971; Henderson *et al*., 1977; Legan and Karsch, 1975; Legan *et al*., 1975; Ramírez and Sawyer, 1974; Sarkar *et al*., 1976). This agrees with the reports of neural activation of the SCN, the RP3V and the GnRH neurons during the pre-ovulatory surge (de la Iglesia *et al*., 2003; Smarr *et al*., 2013). Our data, however, points towards other roles for the signals occurring at other stages of the cycle. We hypothesize that the ARC receives circadian signals from the SCN during the "critical window" to regulate

processes involved in the preparation of the system to respond to stimulant signals that eventually leads to ovulation. To test this hypothesis, we transiently blocked the electric activity in the ARC of female rats at 14:00 hours and then evaluated the progression of their estrous cycle and ovulation.

Research design and methods

Animals

We used 79 3-month-old female hooded rats (CIIZV strain) from our own stock, weighing approximately 260 g. The Ethics Committee of Facultad de Estudios Superiores Zaragoza, UNAM, approved the following experiments (UNAM; FESZ/DEPI/CI/374/18). This institution operates in strict accordance with the Mexican rules for animal handling, Official Norm NOM-062-ZOO-1999. Rats were grouped-housed in standard polypropylene cages and kept in a room with a 14:10 light–dark photoperiod with lights on from 05:00 to 19:00 hours. Temperature was set at 22 ± 2 °C, humidity at 40% with food and tap water available at all times. Vaginal smears were taken every day between 11:00 and 12:00 hours.

Implantation of bilateral cannulas in the ARC

Bilateral guide cannulas were made of two 23G stainless steel hypodermic tubes cut into 14 mm segments and aligned 400 μm apart from each other. Both tubes were soldered together at the superior end with lead-free solder and 30G stainless steel wires were used as obturators to avoid clogging. On the day of diestrus, animals were anesthetized with 4% isoflurane (PiSA Agropecuaria, Jalisco, México) in 100% oxygen inside an anesthetic chamber for rats connected to a vaporizer (Kent Scientific Corp., Connecticut, USA). After reaching a surgical plane of anesthesia, the effects were maintained with 2.5% isoflurane through a face mask. The head was shaved, a surgical scrub performed and 2 mg kg^{-1} of meloxicam (Aranda, Mexico City, México) and 5 mg kg^{-1} of enrofloxacin (Senosiain, Mexico City, México) were provided subcutaneously as a non-steroidal anti-inflammatory/analgesic and as an antibiotic, respectively. Hypromellose artificial tears (Sophia Labs, Mexico City, México) were applied to each eye to avoid desiccation.

Rats were mounted on a model 900 stereotaxic apparatus (David Kopf Instruments, California, USA) above a warming pad set at physiological temperature and an anterior-posterior incision of about 1.5 cm was made in the skin and muscle. A craniotomy of about 2 mm diameter was made to insert the cannula in the superior border of the medial aspect of the ARC (reference from Bregma: anterior-posterior -2.1 mm, medial-lateral ± 0.3mm, dorsal-ventral -9.8 mm; the tooth bar was set at −5 mm below the ear bars). The superior sagittal sinus was set aside to avoid hemorrhages during the procedure (Wirtshafter *et al*., 1979). We used dental cement and three surgical screws to secure the cannula to the skull. After the surgery, the animal was injected with 1.0 ml of sterile saline solution at physiological temperature to replace lost fluids and then allowed to recover with thermal support until the

effects of anesthesia disappeared. The rats were immediately single-housed and post-operative doses of enrofloxacin, meloxicam and saline solution were provided 24 and 48 hours after. Vaginal smears were resumed on the third day after surgery and continued until the euthanasia was performed. A total of 72 rats were implanted with guide cannulas.

Experimental groups and microinjection procedure

Only rats that showed at least three consecutive 4-day estrous cycles after the implantation of the cannula were included in the study. They were divided in four groups: estrus, metestrus, diestrus and proestrus and then further subdivided into vehicle and TTX groups. The vehicle consisted in a microinjection of ACSF (BASi, Indianapolis, USA) performed at 14:00 hours while the TTX group received a solution of TTX citrate (Alomone labs, Jerusalem, Israel) diluted in ACSF to a concentration of 10 ng μl^{-1}. On the selected stage of the estrous cycle, animals were transported to a separate room in their own cages and the microinjection was performed while freely moving. Obturators were removed and 30G microinjectors connected to 10 µl Hamilton syringes through Teflon tubing (MF-5164; BASi) were inserted into the cannulas. The syringes were previously loaded with the infusates and a two-channel microinjection pump model 101 (KD Scientific, Massachusetts, USA) was used to control them at an infusion rate of 50 nl min^{-1} for 4 min (total volume = 200 nl). After the injection, we left the microinjectors in place for two minutes to avoid reflux. Obturators were replaced and the rats returned to the colony and euthanized at 09:00 hours on the next predicted day of estrus. Seven rats that were not cannulated were euthanized as an intact control group.

Euthanasia and tissue processing

The rats received an intraperitoneal overdose of sodium pentobarbital (80 mg kg^{-1}; PiSA Agropecuaria, México) and were decapitated after showing loss of consciousness. The heads were immersed in a 10% formalin solution for at least one week before removing the brains, which were preserved at -20 °C. 50 µm-thick brain sections were cut throughout the coronal plane of the hypothalamus with a cryostat (Leica, Germany). The sections were stained with the Nissl technique and subsequently analyzed to assess the final position of the cannulas and the dispersion of the infusate under an optic microscope. During the autopsy, we obtained the adrenal glands, the uterus and the ovaries, the oviducts were excised under a dissecting microscope and then all fat tissue was removed from the organs before weighing them in a precision balance. The oocytes were extracted from each oviduct, stained with hematoxylin and eosin and then counted under an optical microscope.

Statistical analysis

We determined that the minimum number of observations per group needed for this study was four (n = 4). This was done by running a hypothetical experiment trying to detect an effect size of a 90% reduction in the ovulation rate (blockade of ovulation) by a one-tailed test for independent proportions with α set at 0.05 (G*power version 3.1.9.4 for Windows,

University of Dusseldorf, Germany). We calculated the rate of ovulation for each experimental group as the number of ovulating rats/n and compared them using the Fisher's exact test and then represented it as the percentage of ovulating animals. The same test was used to compare the fraction of cyclic animals, which was expressed as the percentage of cycling animals. The number of ova shed by both ovaries was summed, expressed as the median and interquartile range (IR) of the total shed by both ovaries, and compared using the Mann-Withney U test or the Kruskal–Wallis test, followed by Dunn's test, when comparing two or multiple groups, respectively. The mass of the organs, relative to 100 g of the corporal weight of the animal, was calculated and submitted to the D´Agostino and Pearson and Saphiro-Wilk normality tests. As the data passed the tests and showed homoscedasticity, we compared them using the t-test or ANOVA followed by Tukey and express the data as the mean ± standard error of the mean (SEM). In the case of ovaries and adrenal glands, the weight of the left and right organ was summed before tests. Comparisons were made using GraphPad Prism 7.0 software for Windows (GraphPad Software Inc., California, USA).

Results

As reported in our previous study, most of the animals experienced alterations in their estrous cycles after the implantation of the cannula, but overcome this state and gradually showed consecutive 4-day cycles within a month of the treatment. From the 72 rats that were operated, only four did not recovered their characteristic 4-day cycle during this time, two of them showing consistent 5-day cycles while the other two were acyclic; one showed persistent estrus and the other persistent diestrus. The histological examination of their brains revealed cannulas located into the ARC, near to the median eminence, what can modify the secretion of GnRH and hence explain the aberrant cycles. These animals were excluded from the rest of the analysis and hence 68 rats where microinjected.

We first analyzed the effect of the microinjection of ACSF or TTX in the animals with cannulas confirmed to be in the superior border of the ARC (n = 44), without considering the stage of the cycle when the treatment was performed. As shown in Figure 11A, the females injected with TTX tended to become acyclic after the microinjection more often than those injected with the vehicle (ACSF: 20/21 vs. TTX: 14/23, $P = 0.0074$, Fisher´s exact test). The intact rats where cyclic during all the experiment and hence there was a significant difference against the TTX group (Intact: 7/7 vs. TTX: 14/23, $P \leq 0.05$, Fisher´s exact test) but not against the ACSF one (Intact: 7/7 vs ACSF: 20/21, $P = 0.7500$, Fisher´s exact test). Figure 11B shows how the fraction of ovulating females in the TTX group decreased in comparison to both the intact (Intact: 7/7 vs. TTX: 7/23, $P = 0.0017$, Fisher´s exact test) and the vehicle groups (ACSF: 18/21 vs. TTX: 7/23, $P = 0.0002$, Fisher´s exact test). No differences were found between the control groups (Intact: 7/7 vs. ACSF: 18/21, $P = 0.4060$, Fisher´s exact test). The number of oocytes shed by the rats that ovulated, however, was similar in all three groups (Intact: median 10, IR 9-15 vs. ACSF: median 14, IR 11-15 vs. TTX: median 13, IR

13-15, P < 0.05, Kruskal-Wallis, followed by Dunn´s test; Figure 11C). Regarding the mass of the organs, no differences were found in the case of the adrenal glands nor the uterus. On the other side, a slight but statistically significant decrement was observed in the ovaries of TTX-treated females in comparison to those from the intact group (Table 2).

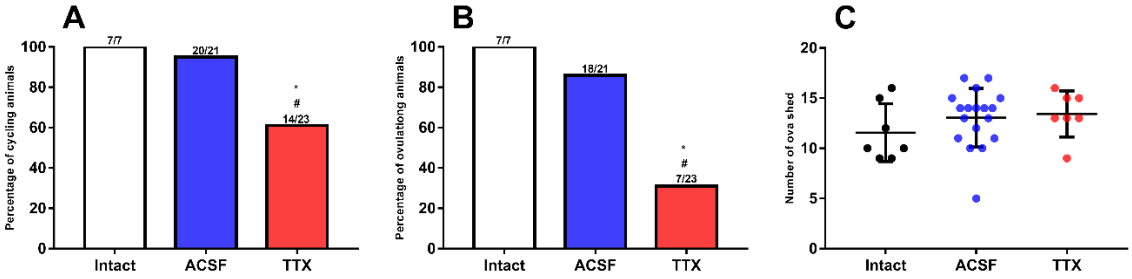

Figure 11. *(A) Percentage of cycling animals in the intact, microinjected with artificial cerebrospinal fluid (ACSF) or tetrodotoxin (TTX) groups (*P ≤ 0.05 vs. Intact, #P = 0.0074 vs. ACSF, respectively, Fisher´s exact probability test). (B) Percentage of ovulating animals (* P = 0.0017 vs. Intact, # P = 0.0002 vs. ACSF, respectively, Fisher´s exact probability test). (C) Median and interquartile range of the number of ova shed by the animals that ovulated from each group. The fractions above each bar represent the rate of cycling/ovulating animals.*

Table 2. Mean and standard error of the mean (SEM) of the relative mass of the organs without considering the stage of the estrous cycle when the treatment was performed

Organ	Intact		ACSF		TTX	
	Mean	SEM	Mean	SEM	Mean	SEM
Adrenal glands	22.97	0.94	24.03	0.99	23.53	0.81
Uterus	151.50	8.58	173.23	4.83	167.81	5.36
Ovaries	30.53	1.78	27.04	1.10	25.35*	0.87

**P = 0.0297 vs. Intact group, ANOVA followed by Tukey. Artificial cerebrospinal fluid (ACSF), tetrodotoxin (TTX).*

As the main objective of this experiment was to describe the participation of the ARC in the different stages of the estrous cycle, we analyzed the data from each stage separately. Figure 12 describes the effect of the treatments on the progression of the estrous cycle. When the ACSF or the TTX where microinjected in estrus a slight, but not statistically significant, decrease was detected in the percentage of cycling animals. In metestrus, the TTX treatment completely inhibited the cyclicity in comparison with the intact (Intact: 7/7 vs. TTX: 0/4, P = 0.0030, Fisher´s exact test) and ACSF groups (ACSF: 4/4 vs. TTX: 0/4, P = 0.0143, Fisher´s exact test). In diestrus, TTX resulted in a tendency to decrease in the percentage of cycling animals that was not statistically significant. No effects were found for proestrus stage. When the animals were described as acyclic, it was due to an increase in the number of days with a

predominance of leukocytes in the vaginal smear, indicative of metestrus-diestrus. This resulted in either a leukocytic or a proestrus-like smear at the expected day of estrus. Only one of the animals showed a persistent estrus after microinjection of TTX in estrus.

In Figure 13 the effect of the treatments on the ovulation rate is depicted. As described for the percentage of cycling animals, a small reduction in this parameter was found in estrus, but was not significant. TTX blocked ovulation when injected in metestrus (Intact: 7/7, ACSF: 4/4 vs. TTX: 0/4, P = 0.0030 vs. intact and P = 0.0143 vs. ACSF, Fisher´s exact test). A decrement was also found in diestrus, but it escaped the statistical significance, demonstrating that more animals should be added to this group to fully disclose the true effect of the treatment. As in the case of metestrus, TTX injected in proestrus completely blocked ovulation (Intact: 7/7, ACSF: 4/4 vs. TTX: 0/7, P = 0.0003 vs. intact and P = 0.0030 vs. ACSF, Fisher´s exact test).

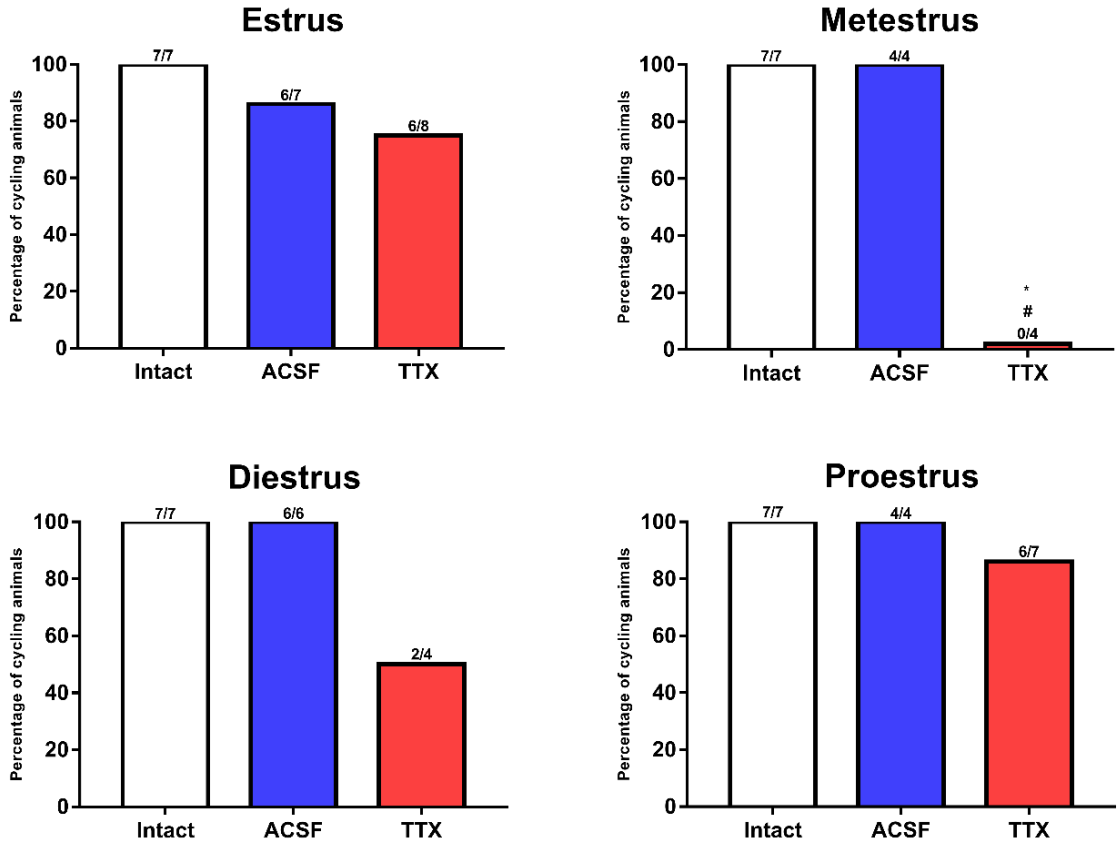

Figure 12. *Percentage of cycling animals in the intact, microinjected with artificial cerebrospinal fluid (ACSF) or tetrodotoxin (TTX) groups in each stage of the estrous cycle. (*P = 0.0030 vs. Intact, #P = 0.0143 vs. ACSF, respectively, Fisher´s exact probability test). Fractions above each bar represent the rate of cycling animals.*

Table 3 contains the mean and standard error of the mean of the data regarding the mass of the organs evaluated. In estrus, both treatments resulted in a decrease of the ovarian

mass when compared against the intact group (P = 0.0177 vs. ACSF and p = 0.0436 vs. TTX, ANOVA followed by Tukey). The adrenal (P = 0.0165 vs. ACSF, ANOVA followed by Tukey) and uterine mass (P = 0.0385 vs. ACSF, ANOVA followed by Tukey) decreased in the animals injected with TTX in metestrus. In diestrus no changes were detected and in proestrus the uterine mass increased significantly when compared with the intact group and just escaped significance against the ACSF group (P = 0.0069 vs. intact and P = 0.06 vs. ACSF, ANOVA followed by Tukey). These results were directly related with the observation of a ballooned, fluid-filled appearance of the uterus, indicative of a disturbance in the secretion of estradiol, when TTX was injected in proestrus day (ACSF: 0/4 vs. TTX: 5/7, P = 0.0455, Fisher´s exact test). The fraction of ballooned uteri in the animals treated in estrus (ACSF: 0/7 vs. TTX: 1/8), metestrus (ACSF: 1/4 vs. TTX: 3/4) and diestrus (ACSF: 0/6 vs. TTX: 2/4) was not significantly different with a probability greater than 0.05 according to the Fisher´s exact test.

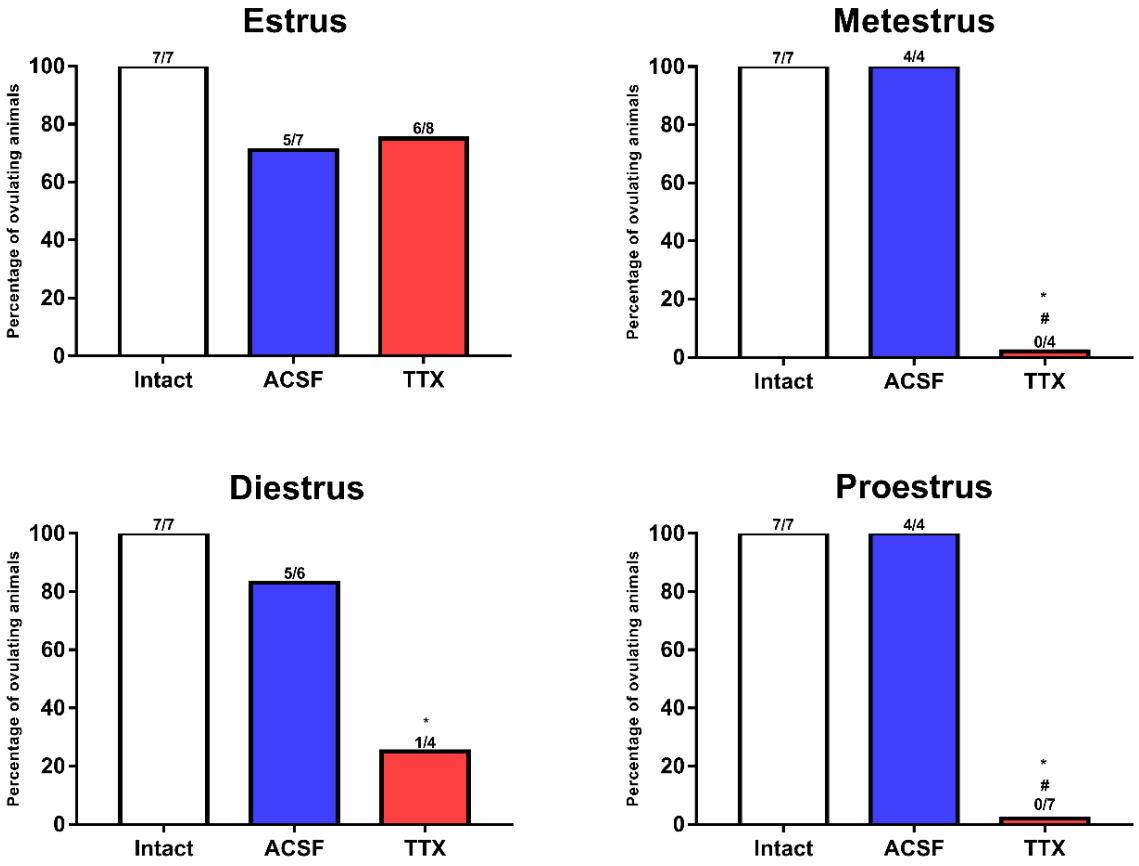

Figure 13. *Percentage of ovulating animals in the intact, microinjected with artificial cerebrospinal fluid (ACSF) or tetrodotoxin (TTX) groups in each stage of the estrous cycle. Metestrus: p = 0.0030 vs. Intact, #p = 0.0143 vs. ACSF, respectively. Diestrus: *p = 0.0242 vs. Intact. Proestrus: *p = 0.0003 vs. Intact, #p = 0.0030 vs. ACSF, respectively. Fisher´s exact probability test was used for all comparisons. Fractions above each bar represent the rate of ovulating animals.*

The cannulas of 24 of the rats that recovered their estrous cycle were determined to be outside of the ARC. Since these animals were distributed in all the experimental groups, it was not possible to do a separate analysis for each stage of the cycle and the data was analyzed depending on the region and treatment. Figure 14 shows that the final position of these cannulas was either dorsally, just below the dorsomedial hypothalamic nucleus (DMH), or too lateral, resulting in an injection into the third ventricle. All the animals with cannulas near the DMH ovulated independently if they were injected with ACSF (3/3) or TTX (4/4). 5/8 animals ovulated when injected in the ventricle with ACSF, which was not different against the intact group (P = 0.1231, Fisher´s exact test). Contrary to this, ovulation was blocked in the rats injected with TTX into the ventricle (Intact: 7/7 vs. TTX: 3/9, P = 0.0105, Fisher´s exact test). Animals that ovulated were previously cyclic and the opposite was true for the non-ovulating females and hence a separate analysis of cyclicity was not performed.

Table 3. Mean ± standard error of the mean of the relative mass of the organs obtained from animals treated in the different stages of the estrous cycle

Group	Estrus			Metestrus			Diestrus			Proestrus		
	Adrenals	Uterus	Ovaries	Adrenals	Uterus	Ovaries	Adrenals	Uterus	Ovaries	Adrenals	Uterus	Ovaries
Intact	22.97 ±0.94	151.5 ±8.58	30.53 ±1.78	-	-	-	-	-	-	-	-	-
ACSF	23.44 ±1.13	180.6 ±8.04	24.66 ±0.98*	27.60 ±2.87	191.4 ±4.32*	29.78 ±1.14	22.47 ±2.26	161.7 ±9.47	26.12 ±1.87	23.83 ±1.90	159.4 ±8.64	29.85 ±4.19
TTX	23.69 ±0.59	153.6 ±8.30	25.65 ±1.16*	19.63 ±0.64#	156.1 ±6.13#	22.85 ±3.40	25.03 ±2.78	164.1 ±8.10	24.48 ±1.79	24.74 ±1.78	192.8 ±8.27	26.94 ±1.41*#

** vs. Intact group, # vs. Artificial cerebrospinal fluid (ACSF), $p \leq 0.05$, ANOVA followed by Tukey. Tetrodotoxin (TTX).*

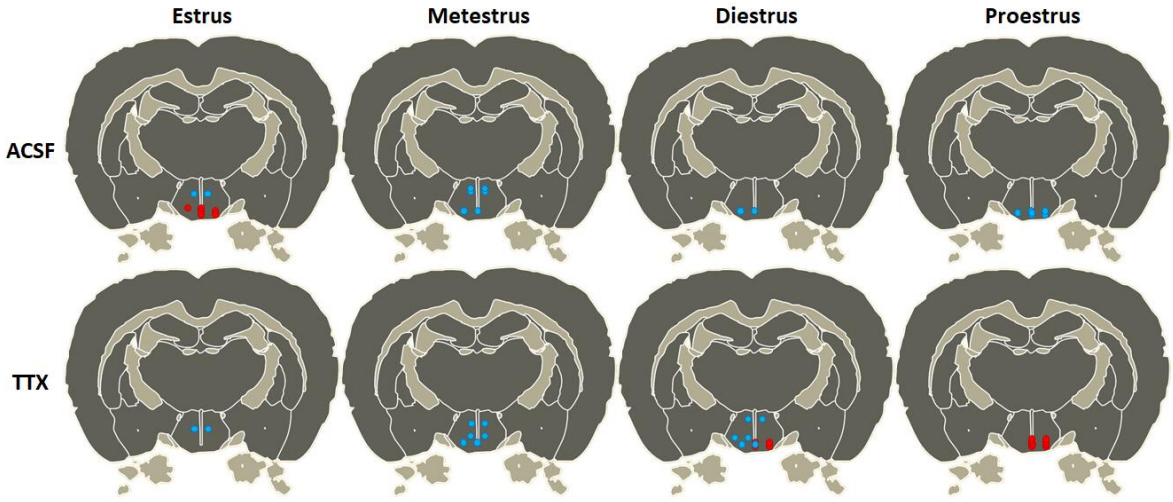

Figure 14. *Final location of the guide cannulas´ tip that were determined to be outside the desired region in the upper border of the arcuate nucleus. Columns represent the stage of the cycle when microinjection took place and rows represent the treatment: Artificial cerebrospinal fluid (ACSF) or tetrodotoxin (TTX). Each pair of dots represent the location of the bilateral cannula in one animal. Red dots represent cannulas that resulted in anovulation while blue dots represent cannulas from animals that ovulated.*

Discussion

The main finding of this study is that the transient blockade of the electric activity in the ARC, for a few hours starting at 14:00 hours, has a detrimental acute effect on the estrous cycle and ovulation. We also described that this is dependent on the stage of the estrous cycle when the treatment takes place i.e., treatments do not have any effect in estrus, TTX disrupts the cycle and ovulation when performed in metestrus or diestrus and blocks ovulation without a previous effect in the cycle when performed in proestrus. Our results are in general agreement with the reports from other laboratories that describes the ARC as an important hypothalamic region involved in the regulation of GnRH release and hence of the estrous cycle and ovulation. For example, early lesion studies shown that the electrolytic destruction of the ARC in non-human primates and rodents results in the permanent inhibition of FSH and LH release. This is the consequence of a reduced secretion of GnRH since the sensitivity and biosynthetic activity of the pituitary were not affected by the surgery (Lampberti and Baldwin, 1979; Plant *et al*., 1978; Soper and Weick, 1980). Similarly, chemical degeneration of neuronal processes in the ARC impairs the secretion of gonadotropins, leading to small ovaries with follicular cysts, suppression of the estrous cycle and anovulation (Brawer *et al*., 1978). The release of LH is rescued by GnRH administration but not by POA electric stimulation, demonstrating a critical role for the inputs and outputs of the ARC in the regulation of GnRH release (Brawer *et al*., 1980).

Due to its permanent nature, however, lesion experiments do not permit to study the punctual participation of a brain structure in a process along a specific time frame because the effects of the treatment can be evaluated only after a prolonged recovery period. This can lead to the summation of the deleterious effects in other physiological events that are under the control of the same structure or to the homeostatic compensation that results from the regeneration or rearrangement of the implicated neuronal circuits (Vaidya *et al*., 2019). Considering this, and that several neuronal populations with different neurochemical identity into the ARC have been reported to regulate reproduction, we decided to inhibit all the sodium-dependent action potentials in the region by the microinjection of TTX in order to evaluate its participation on the progression of the estrous cycle and ovulation.

Without considering the stage of the cycle when treatments were performed, we observed aberrant estrous cycles and anovulation accompanied by a significant reduction in the mass of the ovaries in the animals injected with TTX. Such reduction in the ovarian mass could reflect a deficit in the development of the follicles. This makes sense considering that the ARC modulates the negative feedback that estradiol exerts at the hypothalamus (Smith *et al*., 2005; 2006b). It is hypothesized that this region senses the concentration of circulating estrogens, which results from the steroidogenic activity of the developing follicles, and stimulates the tonic release of the GnRH in a low estradiol environment through a kisspeptin-dependent mechanism (Wang and Moenter, 2020). The tonic secretion of GnRH preferentially stimulates the release of FSH, allowing the follicles to continue growing (Dalkin *et al*., 1989;

Haisenleder *et al*., 1991; Kanasaki *et al*., 2005). An impairment of their development would in turn explain the alterations of the estrous cycle and the anovulatory state observed in 40% and 70% of the rats, respectively.

Since the histological analysis of the follicular dynamics at the ovaries of experimental females is not finished, we cannot confirm such hypothesis at this moment, but there is experimental data that support this idea. The KNDy-neurons are considered to be pivotal for the maintenance of fertility along the lifetime of female mammals (Luo *et al*., 2016) and can be selectively ablated by an intra-ARC injection of a saporin toxin conjugated with a neurokinin 3-receptor agonist. Thirty days after the injection, the animals show a decrease in the concentration of LH, but not of FSH. Their estrous cycles were characterized by long periods of leukocytic smears and the ovaries were smaller than those of control animals as a result of an increase in the incidence of atresia. Ovulation was not assessed, but it probably did not occur as revealed by the absence of fresh corpora lutea in the ovaries (Mittelman-Smith *et al*., 2016). These results are similar than ours, indicating that a transient inactivation of the ARC can evoke the effects of its permanent ablation, at least for a few days after treatment. If this is true, it implies that the activity of the ARC is necessary during a short period each day to regulate the development of the ovarian follicles that would otherwise ovulate during the estrous cycle under study and probably of those ovulating in the following cycles. Surprisingly, Mittelman-Smith and colleagues (2016) did not find differences in the concentration of FSH as we would expect, however, they assessed only one time point, which does not throw information about the pulsatile pattern of secretion, which is even more important than the amount secreted *per se*.

KNDy-neurons regulate the release of GnRH through kisspeptin so our results are probably the consequence of kisspeptin release. In this sense, the viral transfection of an antisense kisspeptin probe directly into the ARC results in the acute decrease of the amplitude of LH pulses. This leads to a lengthening of the estrous cycle, which supports the idea of an alteration in the pulsatile pattern of gonadotropin release (Beale *et al*., 2014). The authors reported that no differences in the number of antral follicles nor corpora lutea were observed, but this discrepancy with our study is probably due to a compensatory regulation considering that the ovaries were collected 100 days after the transfection. In addition, they did not assess the population of the smaller follicles, the incidence of atresia nor the weight of the ovaries and hence alterations in the follicular dynamics may have passed unnoticed. In a different study, microinjection of peptide 234, a kisspeptin antagonist, into the ARC also resulted in an immediate alteration of the tonic release of LH (Li *et al*., 2009). This was conducted in ovariectomized females and hence no data of their estrous cycles nor ovarian morphology was obtained, however, it brings evidence about the acute deleterious effects of the specific blockade of the ARC-kisspeptin system in a narrower window.

The model presented above deserves attention because the authors reported an alteration that lasts for about 1-3 hours after the injection, which coincides with the times

reported in other species by the research team that developed the drug (Roseweir *et al*., 2009). With an affective time of action similar to that reported for TTX (Zhuravin and Bures, 1991), and selectivity for the kisspeptin system, this drug represents a valuable tool to further characterize the role of the ARC during the "critical window" in the absence of the blockade of other neuronal populations. This is important because most of the experimental models that explore the role of the ARC in the regulation of reproduction are based in the permanent disruption of the neurons in the region, leading to the loss of information about the importance of the timing of the signals generated by them. In addition, it is worth to mention that most of these models use ovariectomized rats, which lack the neural and most of the hormonal information that is generated in the gonads and then transmitted to the brain.

As revealed in the stage-by-stage analysis of our data, the ARC is necessary during the afternoon of metestrus and diestrus for a proper progression of the estrous cycle and during proestrus for ovulation to occur, while it seems to be silent in estrus. Such differences between the stages probably reflect the modulation that circulating hormones exert on the neuronal circuits in the ARC. For instance, our data correlates with the anatomical evidence about the changes in the number of axo-somatic synapses into the ARC along the estrous cycle. It has been shown that the amount is relatively constant and high during metestrus and diestrus, then starts to decrease during the afternoon of proestrus, reaching a minimum that lasts until the morning of estrus and then starts to increase gradually. This pattern of neuronal remodeling could be the anatomical basis for an estrous cycle-dependent change in the input tone that modulates the activity of the ARC (Naftolin *et al*., 1996; Olmos *et al*., 1989). This seems be regulated by estradiol since the pattern of decrease in the number of synapses is inverse to the profile of secretion of this hormone (Smith *et al*., 1975). In addition, administration of estradiol to castrated rats modifies the number of synapses in the ARC depending on the stage of development and the presence of neural inputs (Matsumoto and Arai, 1981).

Early electroencephalographic recordings showed that the electric activity of the ARC-neurons is correlated with the changes in the synaptic density described above and follows a circadian pattern with an acrophase in the late morning, preceding the "critical window". In metestrus, the multi-unit activity is low, increasing in diestrus and proestrus, which both concentrate the highest level of activity of all the stages, and then falls to the minimum in estrus (Kawakami *et al*., 1980). These results have been confirmed using modern photometry techniques and are apparently the result of the activity of the KNDy-neurons. A mouse model expressing GCaMP6 under the kisspeptin promoter allows the description of the activity of these neurons along the estrous cycle. This is characterized by the presence of spontaneous episodes of synchronization on their firing activity that precedes the pulses of GnRH/gonadotropin secretion. In metestrus, diestrus and proestrus, such episodes occur with a much higher frequency than in estrus, when most of these neurons are quiescent (McQuillan *et al*., 2019). Considering this, it is likely that the disruption of the estrous cycle experienced by animals treated in metestrus and diestrus is due to the inhibition of the stimulant signals

that drives the secretion of GnRH. This would result in the inhibition of gonadotropin release for as long as the TTX remains occupying the sodium channels and hence in an alteration of the development of ovarian follicles.

In favor of this, the inhibition of gonadotropin release by a systemic injection of a GnRH-antagonist at 12:00 hours impair the estrous cycle. This is due to the increment in the number of atretic follicles and to an early luteinization of some of the larger ones, which in turn results in the inhibition of ovulation as inferred by the absence of implantations after successful copulatory events. It is worth to mention that FSH levels in these animals are low for several days, explaining the aberrant follicular dynamics (Sharpe *et al.*, 1990). Administration of a GnRH antiserum at 11:00 hours of metestrus or diestrus results in a decrease in the serum levels of LH and an increase in those of FSH within 6 hours of the injection. This reveals a profound alteration in the secretion of gonadotropins, probably responsible for alterations in the development of the follicles since the authors reported the inhibition of the pre-ovulatory rise in estradiol levels, which is the result of their maturation. Additionally, they found that metestrus-treated females did not ovulate in response to hCG priming, while ovulation was only partially impaired in the diestrus-treated rats (Arai *et al.*, 1998). This study is interesting because perfectly matches the effects that we observed in our TTX-treated rats. For example, microinjections performed in metestrus resulted in the alteration of the cycle and in anovulation in all the rats, while the same treatment in diestrus only impaired the cycle in 50% of the animals and blocked ovulation in 75%.

Inconsistencies between the animals treated along the estrous cycle would be expected since the population of follicles in different states of maturity varies during each stage and hence the impact on the ovary is not equal. The lowest number of healthy follicles with a diameter between 300 µm and 400 µm is found in proestrus, it increases in estrus, reaches the highest number in metestrus and then starts to decline again in diestrus. These follicles are considered to be the ones that can reach the pre-ovulatory maturity within the estrous cycle under study (Hirshfield, 1983) and hence the inhibition of their development in metestrus would result in the decrease of the ovarian mass, deficit in estradiol synthesis (as evaluated by the low uterine weight), lack of estrous cycles and anovulation observed in this study. Alterations in LH secretion during this specific stage does not modify the number of small antral follicles in the ovaries, but increases the percentage of atresia (Devorshak-Harvey *et al.*, 1985), which would explain that the treatment does not result in a 24-hour lengthening of the estrous cycle, but in a longer disruption as revealed by the leukocytic smears found in estrus.

An increase in the number of large follicles with a diameter higher than 450 µm, along with a decrease in the smaller antral follicles, is observed in diestrus (Hirshfield, 1983). It has been shown that the secretion of LH during this stage is primordial for the developmental changes leading to the pre-ovulatory state and this include the increase in estradiol synthesis (Richards *et al.*, 1980). This is relevant because it can explain the blockade of ovulation preceded by only a partial alteration of the estrous cycles and, in addition, the lack of effect on

the uterine weight and morphology as observed in metestrus (low) and proestrus (high). In support of this, it was shown that the presence of the ovaries between 10:00 and 16:00 hours is sufficient to stimulate the uterine growth characteristic of proestrus (Schwartz, 1964) and we would expect that the ovarian follicles continue to secrete estradiol for at least a few hours after the treatment. Finally, the small antral follicles reach their minimum amount in proestrus (Hirshfield, 1983), explaining the failure of TTX to disrupt the estrous cycle, however, this stage concentrates the pre-ovulatory healthy follicles that will be ovulated the next morning. The inhibition of the activity in the ARC during the "critical window" is expected to block the pre-ovulatory surge of gonadotropins and, in consequence, ovulation. The ARC has been mainly related with the tonic secretion of GnRH, however, there is also evidence about a role in the phasic release. In sheep and rats, kisspeptin-neurons show an increased transcription of c-Fos and Kiss1 genes during proestrus afternoon, just preceding the pre-ovulatory surge (Estrada *et al*., 2006; Kinoshita *et al*., 2005; Smith *et al*., 2009). As shown by Everett and Sawyer (1950), the blockade of neurotransmission during this time does not result in immediate atresia and ovulation usually occurs 24 hours later. This explains the ballooned and fluid-filled uteri that we found, showing that the large follicles continued secreting estradiol and they could probably ovulate during the next day.

The results presented in this study seems to reflect the direct inhibition of the KNDy-neurons by TTX, however, important signals related to the metabolic state of the animals are generated in the region and hence also impaired by the toxin. Neurons in the ARC detect the levels of circulating molecules as leptin, insulin and ghrelin and then inhibit or enhance the release of kisspeptin and, consequently, of GnRH. For example, ARC-CART neurons innervate the KNDy-neurons and CART has a depolarizing effect on them (True *et al*., 2013). On the other hand, intra-ventricular injection of an agonist of POMC downregulates the expression of kiss1 gene in the ARC (Backholer *et al*., 2009). Considering this, the effects on reproduction of the inhibition of such information by the microinjection of selective antagonists should be analyzed in further experiments. In addition, the ARC has been also associated with the regulation of reproduction because it conveys information about the metabolic state directly to the POA, as discussed below.

ARC-CART neurons innervate the pre-optic GnRH neurons that are activated during proestrus afternoon and the peptide seems to depolarize them (Leslie *et al*., 2001; Rondini *et al*., 2004; True *et al*., 2013). Similarly, POMC/α-MSH stimulates the firing activity of GnRH-neurons (Roa and Herbison, 2012; Xu *et al*., 2011). In contrast, NPY and AGRP have an inhibitory effect in the same population (Roa and Herbison, 2012). The content of NPY in the ARC increases during lactation, when GnRH is down regulated, and GnRH neurons in the POA and their terminals in the median eminence receive direct contacts from ARC NPY-neurons (Li *et al*., 1999). Chronic exposure to NPY results in a decrease in the firing activity of GnRH-neurons (Xu *et al*., 2009) and in a decrease in the weight of the pituitary and the ovaries. These abnormalities were associated with alterations in the estrous cycle and anovulation (Catzeflis *et al*., 1993), very similar to the results obtained in this study so we

cannot rule out the possibility of the blockade of a direct ARC-POA communication by the TTX microinjection.

An interesting aspect of this study is the timing of TTX microinjection, this was chosen because of the occurrence of neural circadian signals between 14:00 and 17:00 hours that regulate ovulation (Caligaris *et al*., 1971; Everett and Sawyer, 1950; Stetson *et al*., 1981). An early study shown that the electric stimulation of the ARC between 13:00 and 16:00 hours of diestrus results in alterations in the intrinsic pulsatile mechanism that drives GnRH secretion. This results in an early luteinization of the ovarian follicles, leading to the presence of leukocytic vaginal smears for several days and in the inhibition of ovulation (Sherwood, 1977). This experiment is interesting because, to our knowledge, is the only previous evidence about the deleterious effects of the transient disruption of the ARC electric activity at the "critical window" on the processes driven by hypothalamic-pituitary-ovarian axis. In the previous experiment we demonstrated that the circadian signals generated during the "critical window" arise at the SCN and we hypothesized that they might activate the ARC and RP3V to regulate the activity of the ovaries in a circadian fashion.

As mentioned before, the ARC and the SCN are linked by reciprocal projections. With this in mind, it is reasonable to think that the pivotal role of the ARC during the "critical window" represents one of the output signals transmitted by the SCN. In fact, it has been shown that the communication between these nuclei is indispensable for the expression of some circadian functions. For example, removal of their neural connections by surgical deafferentation abolishes the phasic release of LH (Blake and Sawyer, 1974), the circadian rhythm of activity, temperature and hormone secretion and inhibits the SCN response to metabolic cues (Buijs *et al*., 2017). Additionally, the SCN seems to modulate the activity of certain populations of ARC neurons to adjust to physiological events linked to the metabolic state (Guzmán-Ruiz *et al*., 2014; Herrera-Moro *et al*., 2015). Considering that the ARC also conveys information to the SCN, modulating its activity, it is possible that an intrinsic mechanism also participates in the generation of the neural information that is pivotal during the "critical window".

In line with this, it has been shown that the pulsatile release of gonadotropins persists in rodents with a complete isolation of the ARC-median eminence complex, while inhibited if the ARC is separated from the median eminence (Blake and Sawyer, 1974; Ohkura *et al*., 1991; 1992). Summated to the evidence presented above regarding the activity of KNDy-neurons, it is believed that the ARC is an autonomous GnRH pulse generator that drives the tonic secretion. On the other hand, a circadian pattern in the electric activity of the ARC, with acrophase near the pre-ovulatory surge, has been described, suggesting a role on the phasic secretion (Kawakami *et al*., 1970). This may be just the effect of the control exerted by the SCN, but this hypothesis does not explain the maintenance of such rhythm by ARC explants from female, but not male, rats cultivated *in vitro*, in isolation from the SCN (Yeoman and Jenkins, 1989). When co-cultured with hemi-pituitaries containing the anterior lobe, these

explants result in a circadian rhythm of FSH and LH secretion at the time that the intact animal exhibits the pre-ovulatory surge (Lamperti, 1985). Considering these studies, it is possible that the inhibition of an intrinsic neural signal also contributed to the effects overserved in the present study.

Considering that the misplaced injection of TTX did not modify the estrous cycle nor ovulatory outcome, we suggest that the neural activity that is generated in the ARC, or transmitted to it by afferent neurons, at 14:00 hours participate in the regulation of the ovarian cycle. This is important because it highlights the pivotal role of the ARC during the "critical window" and suggests its implication in the circadian regulation of ovulation. The follicular dynamics in the ovaries of the treated animals is under study to describe the effects of TTX on follicular development and in the incidence of atresia. Further experiments assessing the pattern of secretion of estradiol and gonadotropins after TTX injection are also in progress to clarify the effects observed in the organs. In the future, it will be important to use selective antagonists to block the activity of specific neuronal systems in the ARC in order to fully disclose the nature of the neural signals inhibited in this study that leads to the blockade of ovulation.

Chapter V

General discussion

In the present thesis we evaluated the participation of the SCN in the generation of the daily neural signals that are indispensable for ovulation and that occur in every stage of the estrous cycle between 14:00 and 17:00 hours, a period known as the "critical window". The existence of such signals was demonstrated in proestrus by the studies of Everett and Sawyer (1950) and further work showed that they also occur in estrus, metestrus and diestrus (Domínguez and Smith, 1974; Okamoto *et al*., 1972). In all of the mentioned studies, an intraperitoneal injection of sodium pentobarbital was used as a model to evaluate the effects of the general blockade of neurotransmission on ovulation, demonstrating that it depends on both, endocrine and neural information. Unfortunately, this method does not allow to elucidate the anatomical substrate implicated in the generation of the signals due to its systemic nature.

Although no evidence was provided at the time, the SCN was considered to be the source of those signals since they seem to follow a circadian rhythm. Besides, lesion and deafferentation studies revealed a crucial role of the SCN in the regulation of several aspects of reproduction, from gene expression to behavior. Nevertheless, these experiments lack of temporal resolution since their effects are permanent and, in addition, true findings are difficult to separate from the unspecific side effects of the surgery on other parameters that indirectly impact on reproduction. To our knowledge, this thesis is the first study that analyzes the effect of the blockade of the electric activity of the SCN in the different stages of the estrous cycle on the regulation of ovulation. With this, we sought to elucidate if the SCN is indeed the structure that generates the mentioned signals. The following sections provide a description of the possible signals that may have been blocked by the TTX treatment, explaining the anovulation observed in our experiments along the estrous cycle (Figure 15).

The stimulant VIP-signal that is transmitted directly to the POA by the SCN to regulate the secretion of GnRH

Our experiment in which TTX was microinjected into the SCN in proestrus showed that the local inhibition of the Na^+-dependent action potentials results in the blockade of ovulation when it occurs inside the "critical window" (injection at 14:00 hours), but not outside (injection at 17:00 hours). This is consistent with the results of the pentobarbital studies and hence supports the general idea that the SCN is the locus of the pre-ovulatory signal that triggers the phasic release of GnRH and gonadotropins. The anatomical substrate that explains the circadian regulation of the pre-ovulatory surge of gonadotropins has been subject of investigation for more than two decades. Early studies pointed towards a direct regulation of GnRH secretion by the SCN that depends on the high estradiol background characteristic of proestrus day. First, the existence of a monosynaptic projection from the SCN that stablishes true synapses on the GnRH-neurons located in the POA was demonstrated in female rats (van der Beek *et al*., 1997). The increase in the expression of c-Fos in the SCN and the POA, just

before the LH surge, represent a functional evidence of the participation of this projection (Tsukuhara, 2006). In addition, a study reported the asymmetric activation of the GnRH-neurons only on the hemisphere in which the SCN is active in ovariectomized *splitting* hamsters primed with estradiol, also discarding the participation of humoral signals in the regulation of the pre-ovulatory surge (de la Iglesia *et al*., 2003).

On the basis of this anatomical substrate, we hypothesized that the TTX-treatment at 14:00 hours of proestrus blocked an efferent neural signal from the SCN that directly contributes to the generalized depolarization of GnRH-neurons in the POA. Since the most common efferent neurotransmitters of the SCN are VIP and AVP, it is possible that such signal relies on them and there is evidence supporting a direct regulation through the former. It has been long known that VIP is a stimulant of GnRH secretion, for example, systemic immunoneutralization by the intravenous administration of an anti-VIP antibody decreases LH serum levels (Lasaga *et al*., 1989). The site of action of VIP is the hypothalamus since the ventricular injection of the peptide increases the secretion of LH, but not FSH, in ovariectomized rats primed with estrogens. Contrary, the exposure of pituitary explants to the peptide has no effect unless hypothalamic synaptosomes are present in the culture (Vijayan *et al*., 1979; Samson *et al*., 1981). Furthermore, the injection of an anti-VIP antibody into the lateral ventricle before the expected onset of the LH surge delays it and decreases its amplitude (van Der Beek *et al*., 1999).

It has been shown that VIP increases the firing rate of the GnRH-neurons and this is inhibited in the presence of a VIP-antagonist (Piet *et al*., 2006). Around 40% of the GnRH-neurons in the POA express receptors for this peptide and most of them are synaptic targets of VIP-containing fibers (Smith *et al*., 2000). In favor of a direct regulation of the circadian release of GnRH, the axonal projection that links the SCN with the POA contains VIP (van der Beek *et al*., 1993) and GnRH-neurons that receives VIP-input are activated at the beginning of the pre-ovulatory surge along with the SCN-neurons (Tsukuhara, 2006; van der Beek *et al*., 1994). An attenuation of VIP synthesis into the SCN by the microinjection of antisense oligonucleotides results in a delay of the LH surge and in a decrease of its amplitude (Harney *et al*., 1996). Additionally, females show a sexually dimorphic circadian rhythm of VIP expression in the SCN that peaks in the early afternoon of proestrus (Krajnak *et al*., 1998b). This rhythm, as well as the number of VIP-cells and the content of the peptide in the SCN are attenuated in old animals experimenting the natural reproductive senescence as the follicular reserve is exhausted, which does not occur in the case of AVP (Krajnak *et al*., 1998a). Such a decline in the VIP rhythmic output results in a decrease in the activation of GnRH-neurons (Krajnak *et al*., 2001) and this parameter, as well as the concomitant decrease in LH phasic secretion, can be rescued by the ventricular injection of VIP during the "critical window" (Sun *et al*., 2012). This agrees with the report by Christian and Moenter (2008), which found that VIP stimulates the firing activity of GnRH neurons in a time-dependent manner with a peak at the onset of the pre-ovulatory surge and almost no sensitivity at other times.

The evidence presented above suggests that, when the TTX was microinjected at 14:00 hours of proestrus, we blocked a VIP-signal that originates in the SCN and directly stimulates the phasic release of GnRH by acting at the POA. It is worth to mention, however, that not all the experimental evidence supports a stimulant role for VIP in the regulation of gonadotropin release. An example of this is the report by Akema and colleagues (1988) in which the microinjection of VIP directly into the POA of ovariectomized rats inhibited the tonic release of LH. This agrees with other experiments that found similar results by intraventricular infusion of the peptide (Stobie and Weick, 1989; Weick *et al*., 1992), which are explained by a reduction in the synthesis of the α- and β-LH chains (Gajewska *et al*., 2005). Such an inhibitory effect can be explained by the fact that, in contrast to most of the experiments reporting a stimulant role, these studies analyzed the secretion of LH under conditions that mimicked the negative feedback by using ovariectomized rats without estradiol priming.

Evidence of a dependence on estradiol for the response of the GnRH-neurons to the afferent information can be found in the experiment by Alexander and colleagues (1985), who showed that ventricular injection of VIP lowers the frequency and amount of LH secreted by ovariectomized rats, but not by rats that were previously primed with estradiol. This result implies that the role of VIP in the regulation of gonadotropin secretion is not only dependent on the time of the day but also on the stage of the estrous cycle. In this sense, VIP would stimulate the release of GnRH in a high estradiol background, but inhibit its secretion when estradiol levels are low and hence the results found in this study on other stages of the estrous cycle would be explained by the blockade of a different signal. In contrast to this hypothesis, Piet and colleagues (2006) found that the GnRH-neurons are depolarized by VIP in diestrus and proestrus and hence concluded that there is not a dependence on the stage of the cycle, however, they did not consider that the concentration of estradiol is already high during diestrus afternoon, sharply contrasting with those found in estrus and metestrus (Smith *et al*., 1976).

The circadian AVP-signal that regulates the secretion of GnRH through kisspeptin interneurons

There is compelling evidence about a role for AVP in the regulation of GnRH release. Systemic administration of this peptide in ovariectomized rats primed with estradiol advances the LH surge and increases its amplitude (Salisbury *et al*., 1980). Also, in intact rats, intracerebral injection of an AVP-antagonist during the critical window of proestrus decreases the amplitude of the LH surge (Funabashi *et al*., 1999). The source of this stimulating signal seems to be the SCN since co-cultures of POA and SCN display an estradiol-dependent circadian rhythm in GnRH and AVP secretion that peaks at the same time, out of phase with a VIP rhythm that is also present. Only-POA cultures do not display rhythmic secretion of GnRH but the addition of AVP, but not VIP, elicit the acute release of the decapeptide (Funabashi *et al*., 2000). In clock$^{-/-}$ mutants, a circadian rhythm in the expression of AVP in

the SCN that can be observed in wild type animals is absent and this results in a decrease in the expression of the AVP receptor at the hypothalamus and also in the blockade of the LH surge. Injection of AVP during the afternoon of proestrus induces the phasic secretion of LH and this is inhibited in the presence of an AVP-antagonist (Miller *et al*., 2006). In addition, lesion of the SCN inhibits the cyclic release of LH in ovariectomized rats treated with estradiol and this is rescued by POA administration of AVP (Palm *et al*., 1999). In SCN-intact females, the same treatment stimulates the secretion of LH when performed in the afternoon, but not in the morning (Palm *et al*., 2001). This result suggests that the SCN sets a sensitivity window for its own efferent signals and it is logical to think that it may be mediated through the regulation of the AVP receptor, as shown in the study by Miller and colleagues (2006).

Despite all these evidences, reports indicating a direct AVP-pathway from the SCN to the POA are scarce. For instance, Mahoney and Smale (2005) found that only a small population of GnRH-neurons in the POA of the diurnal rodent *Arvicanthis niloticus* is contacted by AVP-containing fibers. In this study, however, there is no evidence suggesting that such fibers originate at the SCN and, to our knowledge, there are no other published reports regarding this kind of innervation of the GnRH neurons in conventional rodent models. In concordance, AVP receptors are rarely expressed in GnRH-neurons in the POA, suggesting that the peptide acts through interneurons (Kalamatianos *et al*., 2004). The fact that AVP injected into the POA triggers the phasic release of LH only in a small fraction of the animals treated, and that an AVP-antagonist injected in the same region does not inhibit its stimulant effect (Palm *et al*., 2001), may suggest that this neurotransmitter is not acting at the POA, but in other region that in turn modulates the activity of the GnRH-neurons. Considering this, the anovulation observed in our experiment performed in proestrus can also be explained by the blockade of an indirect signal that originates in the SCN and is transmitted to interneurons that rely the information to the POA.

In this line of thinking, it is known that the SCN communicates with ERα-expressing neurons in the RP3V through a reciprocal neural pathway (de la Iglesia *et al*., 1995; 1999; Hahn and Coen, 2006). Of interest to our study are the appositions found in neurons that also contain kisspeptin since it is well-stablished that they stimulate the pre-ovulatory surge of GnRH. Although there is evidence suggesting that the stimulant effects of VIP mentioned above partially depend on the activation of the RP3V kisspeptin-neurons (Kauffman *et al*., 2014), the SCN fibers that contact these neurons only contain AVP and the number of appositions increases in a high estradiol background, suggesting a direct role in the control of the pre-ovulatory surge (Vida *et al*., 2010; Williams *et al*., 2011). A functional role for this communication is the fact that the SCN and the RP3V are co-activated just before the LH surge. In addition, animals bearing unilateral lesions of the SCN show a decrease of the kisspeptin content in the ipsilateral RP3V. Further evidence was found using a protocol that desynchronizes the activity of the ventrolateral and dorsomedial portions of the SCN. In this model, kisspeptin expression and the LH surge are synchronized with the dorsomedial-SCN, in which AVP is synthesized, although the amplitude of the surge seems to be also dependent

on the ventrolateral region (Smarr *et al*., 2012). Finally, kisspeptin-neurons in the RP3V express AVP receptors and are activated in response to ventricular administration of AVP irrespectively of the time of the day (Vida *et al*., 2010; Williams *et al*., 2011). A similar effect was found *in vitro*, where AVP depolarizes these neurons irrespectively of the time of the day, but in an estradiol dependent manner (Piet *et al*., 2015). This can be explained by the fact that there is a circadian rhythm in the expression of the AVP receptor and kisspeptin in these neurons that is driven by clock genes only in the presence of estradiol (Smarr *et al*., 2013). This contrasts with the time-dependent stimulant effect at the POA reported by Palm and colleagues (2001) and suggests that the SCN may be involved in the imposition of a circadian rhythm of sensitivity to kisspeptin at the GnRH neurons, probably mediated by the modulation of the expression of the GPR54 receptor by the VIP fibers that contact those neurons. Additionally, the sensitivity of the RP3V kisspeptin-neurons to circadian input from the SCN may depend on a functional endogenous clock inside them that controls the expression of the AVP receptor.

The importance of the circadian removal of an RFRP-3 inhibitory signal that blocks the secretion of GnRH

In addition to kisspeptin, it has been proposed that RFRP-3 is also the target of a circadian control to regulate the secretion of gonadotropins (Angelopoulou *et al*., 2019; Beymer *et al*., 2016; Khan and Kauffman, 2011). This peptide inhibits the stimulant signaling mediated by kisspeptin and VIP in the GnRH neurons (Son *et al*., 2016) and hence its activity must be suppressed during the afternoon of proestrus so the pre-ovulatory surge of gonadotropins can occur. A circadian removal of its influence has been in fact reported, for example, the number of RFRP-3 neurons in the DMH and their activity follow a circadian rhythm with minimum levels at the time of the LH surge. This pattern is estrogen-dependent and therefore is not observed in other stages of the estrous cycle (Gibson *et al*., 2008; Henningsen *et al*., 2017; Poling *et al*., 2017).

Such rhythm of activity depends on the SCN since the RFRP-3 neurons receive afferent fibers containing VIP and AVP and the central injection of the former, but not the later, inhibits their activity in a time-dependent manner (Russo *et al*., 2015). Moreover, in *splitting* hamsters, the activity of RFRP-3 neurons occurs in antiphase with that of the SCN and the POA (Gibson *et al*., 2008). Despite this evidence, the regulation by the SCN seems to be indirect since RFRP-3 neurons do not express VIP receptors (Russo *et al*., 2015). These results suggest that the SCN sends a VIP-signal to the DMH that act through interneurons to inhibit the activity of the RFRP-3 and hence the blockade of such signal by TTX would result in the presence of an inhibitory tone impeding the phasic secretion of GnRH and hence in the blockade of ovulation.

The neural circadian signals that occur in the different stages of the estrous cycle to regulate ovulation

Some of the signals that the SCN sends to regulate the secretion of gonadotropins do not depend on a high concentration of estradiol. This suggests that the circadian regulation of ovulation is not restricted to the occurrence and timing of the pre-ovulatory surge of gonadotropins, but also includes other processes that prepare the hypothalamus, and probably the pituitary, to respond to ovarian information. These signals occur in stages when the concentration of estradiol is low and hence its blockade can explain the anovulatory state of our rats. In Chapter II we reviewed several studies that shown the deleterious effects on ovulation after blocking the adrenergic, dopaminergic, cholinergic and muscarinic systems as well as its dependence on the time of the day and the stage of the cycle. In general, those studies revealed abnormalities in the secretion of ovarian and pituitary hormones, as well as a decrease in the sensitivity of the hypothalamus to the stimulant feedback of estradiol, explaining the results obtained here.

Similarly, we found in a previous experiment that the unilateral lesion of the SCN acutely blocks ovulation irrespectively of the stage of the cycle when the surgery is performed. What is interesting is that the lesion impairs the estrous cycle only in estrus- and metestrus-treated females and they do not respond to a stimulant estradiol-paradigm that induces ovulation, but ovulate in response to GnRH (Silva, 2016). These results allow us to propose that the SCN modulates the sensitivity of the hypothalamus to estradiol, probably by regulating the expression or ERα in the RP3V or the ARC. This make sense considering that the mRNA and protein of the estrogen receptors in the POA are higher in estrus and metestrus and low in diestrus and proestrus, which is opposite in the ARC (Shughrue *et al*., 1992; Zhou *et al*., 1995). This suggests that the brain responds dynamically to the fluctuation of estradiol levels by diminishing the amount of its receptors when the concentration is high. Despite this evidence, the pattern of expression of this receptor has not been described through a 24-hour cycle and daily variations that depend on the stage of the cycle have been not described either. Considering that there is ample evidence about the regulation of the circadian system by the estrogen receptors (Hatcher *et al*., 2018), but not the opposite, it will be interesting to explore if the expression of these receptors follows a daily rhythm and if the blockade of the activity of the SCN impairs it. An alternative hypothesis to the lack of effect of the estradiol regimen is that the SCN modulates the expression of the GPR54 in GnRH neurons, altering they capability to respond to stimulant input. In this case, an evaluation of its expression as the proposed for the estrogen receptors should be performed.

Another example is the study by Murakami and Takahashi (1983), who described a circadian rhythm in the concentration of adenosine-3',5'-monophosphate (cAMP) in the SCN of adult male rats. This rhythm peaks in the afternoon and is also present in the RP3V of females in every stage of the estrous cycle, as well as in ovariectomized rats primed or not with estradiol (Chappell *et al*., 2000). This explains the observation of an activity rhythm in

the same region that is independent on the concentration of estradiol, implying that circadian regulation of this structure is pivotal to regulate functions aside the proestrus LH surge (Williams et al., 2011). There is a circadian rhythm of VIP synthesis into the SCN of ovariectomized rats with or without estradiol replacement (Krajnak *et al*, 1988b) and injection of VIP-antisense oligonucleotides into the SCN blunts the local and RP3V rhythms in cAMP, leading to an inhibition of the GnRH-neurons (Gerhold *et al*., 2005). This suggests that the VIP rhythmic output regulates the activity of the RP3V in a daily basis, probably indirectly by modulating the activity of the dorsomedial SCN, which in turn communicates with kisspeptin neurons through AVP. This makes sense considering that the GnRH-neurons are sensitive to that stimulant input in diestrus and proestrus (Piet *et al*., 2016). Finally, VIP-knockout mice exhibit abnormally long estrous cycles characterized by several leukocytic smears, probably due to an inhibition of follicular development, leading to a decrease in spontaneous LH surges (Loh *et al*., 2014). These results indicate that the VIP signals from the SCN that originate during each stage of the cycle are needed to regulate the phasic and tonic secretion of GnRH.

The cAMP produced during the rhythms discussed above can evoke LH surges in the appropriate estradiol context. This seem to be dependent on the activation of the progesterone receptor since they are inhibited by the microinjection of a progesterone antagonist, but it is not clear if progesterone binding is necessary or if it is a transactivation phenomenon (Chappell *et al*., 2000). This result allows to link the circadian regulation of the RP3V to progesterone, which is well-known for its participation in the regulation of the estrous cycle and the modulation of the frequency and amplitude of the tonic and phasic secretion of GnRH by acting directly at the ARC and RP3V, respectively (He *et al*., 2017). The implication of this relationship should be analyzed at the light of the secretion profile of progesterone as there are two major peaks during the estrous cycle, one in metestrus and the other in proestrus and its specific role in the regulation of ovulation is not clear (Kosaka *et al*., 1988; Smith *et al*., 1975).

The interplay between the ovarian and metabolic signals that impact on female fertility

The evidence presented above sought to explain the blockade of ovulation in the TTX-animals treated in estrus, metestrus or diestrus as an effect of the inhibition of a circadian signal that acts at the RP3V. This do not completely explain our results, as discussed in Chapter IV, reproductive success depends on the integration of information regarding the time of the day and the metabolic state of the individual at the level of the GnRH-neurons. This ensures that ovulation occur only in the presence of a proper availability of nutrients and at the active phase of the animal, increasing the probability of mating and of a non-risk pregnancy for both the mother and the offspring. The ARC is an important hypothalamic area that mediates the negative feedback of estradiol and the metabolic regulation of GnRH secretion (Méndez-Hernández *et al*., 2020; Roa, 2013). The first indications of the participation of the ARC in the regulation of the secretion of gonadotropins came from lesion studies. Large, but

not partial, electrolytic lesions of the ARC impair the pulsatile release of LH (Soper and Weick, 1980), however, this was difficult to analyze due to the proximity of the nucleus to the median eminence, which usually results in the destruction of the GnRH-terminals that release the peptide into the portal circulation (Sisk *et al*., 1988). In ovariectomized rats, chemical lesions that destroy neuronal bodies in the ARC, leaving the GnRH-terminals intact, result in a decrease in the amplitude and frequency of the release of the decapeptide and hence in lower serum levels of LH. In these animals, GnRH injections result in the release of LH, which is not inhibited by pentobarbital sedation, indicating a disruption of the negative feedback of estradiol at the brain (Sridaran *et al*., 1981).

Modern experimental tools have shown that the ARC regulates the tonic secretion of GnRH by releasing kisspeptin at the POA and median eminence through neurokinin B and dynorphin A (Wakabayashi *et al*., 2010). Ablation of the KNDy-neurons in ovariectomized rats lowers the secretion of gonadotropins, leading to a failure of follicular development and an increase in the incidence of atresia. This disrupts the estrous cycle causing a permanent leukocytic smear (Mittelman-Smith *et al*., 2012; 2016; Padilla *et al*., 2019). In addition, the body mass increases and the feeding rhythm becomes disorganized (Padilla *et al*., 2019). The results described above highlight the pivotal role of the KNDy-neurons in the regulation of the tonic secretion of GnRH. Considering this, it was expected that the blockade of its activity in any stage of the estrous cycle would lead to an acute alteration of the estrous cycle and hence in a delay in ovulation. In fact, TTX injections in the ARC differ from those in the SCN by the alterations in the estrous cycle that follows them. The absence of such an alteration when the TTX was microinjected in estrus probably reflects the capability of the system to compensate for a temporal inhibition of stimulant input if enough time is given.

The destruction of the KNDy-neurons increases the amount of LH secreted in response to estradiol priming, revealing a direct role in the modulation of the pre-ovulatory surge in addition to its indisputable role in the regulation of tonic secretion. This is not accompanied by an increment in the number of GnRH- nor kisspeptin-neurons activated in the POA and the RP3V (Mittelman-Smith *et al*., 2016). Irrespectively of neuronal activation, the amount of kisspeptin in the RP3V increases in these animals and this seems to be mediated by the abolition of a dynorphin A inhibitory tone (Helena *et al*., 2015). Even considering the modulation of the RP3V during the pre-ovulatory surge by the ARC, it was not clear if the "critical window" was an important period for a structure usually related with the tonic secretion. Our results demonstrate that the transient blockade recapitulates the effects of a permanent lesion on ovulation, probably explained by alterations in the secretion of gonadotropins, highlighting the importance of the ARC during this time. Considering this, we expected that TTX treatment in proestrus would result in ovulation but we observed the contrary.

The anovulation in proestrus-treated rats can be explained by the inhibition of neural signals that originates in the ARC, but not in the KNDy-neurons, conveying information about the metabolic state to stimulate the secretion of gonadotropins. For instance, the GnRH-neurons in the POA receive input from a wide variety of classic neurotransmitters that are

present in the ARC, among them are the γ-aminobutyric acid (GABA; Leranth *et al*., 1985), glutamate (Kiss *et al*., 2003), catecholamines (Hoffman, 1985). Additionally, several peptides as galanin (Merchenthaler *et al*., 1990), melanin concentrating hormone (Wu *et al*., 2009; Ward *et al*., 2009), orexin (Campbell *et al*., 2003), endorphins (Chen *et al*., 1989), substance P (Hrabovszky *et al*., 2013), neuropeptide Y (NPY; Li *et al*., 1999; Tsuruo *et al*., 1990; Ward *et al*., 2009), neurokinins (Ciofi *et al*., 2006; Ward *et al*., 2009), dynorphins (Goodman *et al*., 2004) and kisspeptin (Yeo and Herbison, 2011; Yeo *et al*., 2016). Functional evidence is sometimes available, for example, it has been shown that the GABA-neurons in the ARC directly stimulate the secretion of GnRH by acting at the somas located in the POA by a $GABA_A$-dependent mechanism. Furthermore, abnormalities in the activation of this pathway leads to the disruption of the estrous cycle and polycystic ovarian syndrome (Silva *et al*., 2019). In the case of the peptides, it has been shown that NPY stimulates the release of GnRH (Kalra, 1993). The actions of this peptide seem to occur directly at the POA since its concentration in the POA and ARC follows a circadian rhythm peaking just before the gonadotropin surge and this rhythm is absent in old rats showing reproductive senescence (Sahu and Kalra, 1998). In addition, NPY stimulates the firing activity of cultured GnRH-neurons in a kisspeptin-dependent manner (Verma *et al*., 2014).

The regulation of ovarian clocks by the gonadotropins and multi-synaptic pathways through the autonomic nervous system

As mentioned in Chapter II, the SCN is connected to the regions in the central nervous system in which the autonomic nerves originate and there is evidence to suggest that some endocrine organs receive circadian information through this pathway (Bartness *et al*., 2001; Ueyama *et al*., 1999). For example, light stimulation increases the activity of sympathetic nerves while suppresses that of parasympathetic ones, which is inhibited in animals with bilateral lesions of the SCN (Nijimi *et al*., 1992; 1993). Also, the daily rhythm in the secretion of thyroid and adrenal hormones is altered after manipulations of the SCN without matching modifications in the secretion of TSH and ACTH, respectively (Buijs *et al*., 1999; Kalsbeek *et al*., 2000; Lilley *et al*., 2012). In the case of the ovaries, a similar pathway has not been conclusively demonstrated in mammals because the secretion of GnRH is immediately disrupted after alterations of the circadian system. Considering this, it is difficult to differentiate if the alterations in ovarian functions are the result of the inhibition of the hormonal or the neural signals that reach the gonad. In addition, and contrary to the thyroid and adrenal glands, most of the physiological outputs from the ovary that can be measured, as the secretion of steroid hormones, the development of follicles and ovulation, does not follow circadian patterns.

Despite the infradian-like physiology of the mammalian ovaries, the core CG´s are expressed in a circadian fashion in the different cells inside them (Fahrenkrug *et al*., 2005; He *et al*., 2007b; Karman *et al*., 2006) and this is a good circadian output from the gonads that can be used as a tool to explore their regulation. It has been reported that the ovaries of Bmal1 knockout mice display an abnormal expression of several genes that encode important enzymes for the synthesis of steroid hormones. These deleterious effects, plus alterations in

the expression of the prostaglandins associated with ovulation, can be replicated in the ovaries of wild type mice by a siRNA treatment that interfere with the expression of Bmal1, demonstrating that such effects are not due to a global disruption that affects the central circadian machinery in the SCN (Chen *et al*., 2013; Wang *et al*., 2020). The conditional knockout of Bmal1 in granulosa or theca cells does not alter the pre-ovulatory peak of LH, the morphology of the ovary nor the sexual behaviors. However, the sensitivity to LH is completely disrupted and slight modifications can be detected in the estrous cycle of the animals bearing the mutation in theca cells. This is explained by alterations in the expression of the LH, but not FSH, receptors (Mereness *et al*., 2016). Finally, a decrease in fertility due to an implantation failure was also shown in these animals. This seems to be caused by an alteration in the secretion of progesterone and can be rescued by the transplantation of wild type ovaries (Liu *et al*., 2014). Together, these results demonstrate that the local clocks in the mammalian ovary play a critical role in the regulation of hormone secretion, ovulation and fertility.

How is the ovarian clock maintained in tune with the environment? is maybe the most interesting question derived from the previous paragraph. The gonadotropins are obvious candidates and there is a generalized acceptance of their role as synchronizing agents for the ovary based on the following evidence. The expression of clock genes has a low amplitude and is arrhythmic in the ovaries of prepubertal and hypophysectomized adult rats and rhythmicity can be induced by the injection of gonadotropins (Gräs *et al*., 2012). This appears to be organ-specific since liver tissue does not entrain to this stimulus in rats nor birds (Karman and Tischkau, 2006; Tischkau *et al*., 2011). A resetting of the rhythm of clock gene expression can be observed in the ovaries of animals that face a 6-hour phase shift and this still occur after sympathetic denervation and even transplantation of the organ into the subcutaneous tissue. In addition, both gonadotropins induce large phase shifts in cultured ovaries. These results suggest that the diffusible signals that reach the ovary are enough to synchronize their local clocks (Yoshikawa *et al*., 2009).

Most of the studies mentioned above did not take into account that the secretion of gonadotropins does not follow a circadian rhythm, but an infradian one instead. This is well conserved in mammals although the length of the rhythm depends on the species, for example, 4-5 days in rodents and around 28 days in humans. To this date, no studies have been made in laboratory animals with the explicit objective of assessing if the secretion of gonadotropins show daily variations in a parameter that the ovaries could use to synchronize to a circadian pacing as the amplitude, frequency or hormone composition. The classic descriptions of the concentration of these hormones along the estrous cycle did not have the resolution to draw a conclusion, but at the time it was stated that the only significant differences were noted during proestrus afternoon (Smith *et al*., 1975). Similar results have been obtained for the content of GnRH in the anterior pituitary, where its target cells are located (Park and Ramírez, 1989). In concordance, a study shown the absence of circadian rhythmicity in the secretion of FSH and LH during the early follicular phase of women under

constant conditions in which other endocrine circadian rhythms were perfectly preserved (Klingman *et al*., 2011). Considering this, it is not clear how an infradian signal could synchronize the circadian clocks in the ovary, although possible, it is especially unlikely in those species with very long cycles. This topic, however, must be revisited since there are reports of daily changes in the electric activity of hypothalamic centers as the POA and ARC occurring on other stages of the estrous cycle in association with the "critical window" (Kawakami *et al*., 1970). Daily changes in the secretion of GnRH, FSH and LH have been also reported to occur in estrus, metestrus and diestrus, but the pattern is not the same on each stage (Chiappa and Fink, 1977). Even more conclusive is the fact that, after removal of the ovarian inhibitory feedback, FSH, LH, ACTH and TSH are secreted in a circadian fashion closely related with the secretion of their respective hypothalamic releasing hormones, which are in turn modulated by a neural circadian mechanism (Szafarczyk *et al*., 1980).

Even if the gonadotropins somehow transmit circadian information to the ovaries, there is evidence of a circadian rhythm in the sensitivity to them. This can be observed by the injection of cetrorelix, a long-lasting antagonist of GnRH (half-life of about 65 hours) during the morning of proestrus. In this model, the endogenous release of gonadotropins is inhibited and timed injections of LH can be applied to measure the responsivity of the gonad at different time points. A circadian rhythm of sensitivity to LH that regulates ovulation in mice and rats was demonstrated with an acrophase during proestrus afternoon/night and nadir during the early morning of estrus (Mereness *et al*., 2016; Sellix et al., 2010; Sen and Sellix, 2016). A similar result was previously reported to occur in the pituitary, in which the sensitivity to GnRH also follows a daily rhythm (Wilkinson and Moger, 1981). These results strongly indicates that local clocks, or other temporal cues, prepares the organ to interpret gonadotropic information. In this case (and also in the case that gonadotropins do not offer a reliable circadian input as stated above), analyzing the role of the autonomic innervation in the entrainment of the ovary is a logical step.

The first element in the multi-synaptic pathway that could transmit circadian information to the ovaries are the neurons in the SCN that project to the paraventricular nucleus of the hypothalamus (Kalsbeek et al., 2011; Sofroniew and Weindl, 1978; Ueyama *et al*., 1999). This region is considered to be an output center that communicates information generated in the central nervous system to the rest of the body through humoral and neural signals. For this, the magnocellular cells that it contains project to the posterior lobe of the pituitary and secrete oxytocin and AVP into the bloodstream. Its parvocellular cells, further divided into neuroendocrine and pre-autonomic neurons, project to the median eminence, where they secrete releasing factors, and innervate the pre-autonomic cells, respectively (Swanson and Sawchenko, 1980). Such pre-autonomic neurons are located in the dorsal vagal complex, parabrachial nucleus, nucleus of the solitary tract and the intermediolateral column, where the autonomic nerves that reach peripheral organs as the pancreas, liver, adrenal, thyroid and ovaries originate (Buijs *et al*., 1999; 2001; Gerendai *et al*., 2009; Kalsbeek *et al*., 2000; la Fleur *et al*., 2000; Ueyama *et al*., 1999). It is important to mention, however, that

fewer neurons are labeled in the SCN when retrograde tracers are injected in the ovary, as compared with the adrenal and thyroid gland (Gerendai *et al*., 1998; 2000; 2002).

The SCN contains different subsets of neurons that modulate the pre-parasympathetic and pre-sympathetic neurons in the paraventricular nucleus. This suggests that it can modulate the activity of peripheral organs by a dynamic control of the opposing roles of these branches of the autonomic nervous system rather than by the transmission of a rigid daily signal (Buijs *et al*., 2003). The ovaries receive sympathetic information through the superior ovarian nerve and the nerve of the ovarian plexus and parasympathetic information via the *vagus* nerve (Burden and Lawrence, 1980: Gerendai *et al*., 2005; Lawrence and Burden, 1980). This innervation develops before birth and contacts vascular and primordial cells inside the organ. During the perinatal period, before evident sensitivity to gonadotropins appears, ovarian functions seem to be regulated by this pathway (Aguado, 2002; Malamed *et al*., 1992).

There is a great amount of experimental evidence indicating that the brain regulates ovarian functions through its innervation also during adulthood. For example, electric stimulation of the RP3V-POA region during the "critical window" of intact proestrus rats increases the secretion of gonadotropins and ovarian steroids (Kawakami *et al*., 1979). This treatment retains its effectivity to elicit the increase in steroid secretion in hypophysectomized and adrenalectomized rats and this is inhibited if ovarian denervation is performed prior to the stimulation. This result strongly suggests that the information that is generated in the anterior hypothalamus regulates the secretion of ovarian hormones through the autonomic innervation (Kawakami *et al*., 1981). This pathway seems to also regulate the development of ovarian follicles since unilateral implants of atropine crystals into the POA disrupts this process only in the ipsilateral ovary, which cannot be explained by an endocrine mechanism that would otherwise impact both gonads (Cruz *et al*., 2014). In addition, surgical or chemical denervation of the ovary, as well as local pharmacological manipulation of neurotransmitter systems result in abnormalities in the ovarian functions, the sensitivity to gonadotropins and the compensatory growth (Domínguez and Cruz-Morales, 2011).

The role of the ovarian innervation in the transmission of circadian signals has received little attention. Yoshikawa and colleagues (2009) shown that the section of the superior ovarian nerve does not prevent the synchronization of the ovarian clock after a 6-hour shift. On the other hand, a slight but disrupted synchronization was found in excised ovaries wrapped in a semipermeable membrane and implanted below the skin. They concluded that the ovary is entrained by humoral signals, however, the abnormalities observed in the implanted ovaries and the fact that not all the ovarian nerves were considered raise questions about the validity of such conclusions. Some observations from our lab suggest that the ovulatory and hormone secretion outcome after the section of either, the superior ovarian nerve or *vagus* nerve, depends on the time of the day when the procedure is performed (Domínguez and Cruz-Morales, 2011). This suggest that the information that the ovaries receive from these fibers is not equal through the day and that it only regulates their

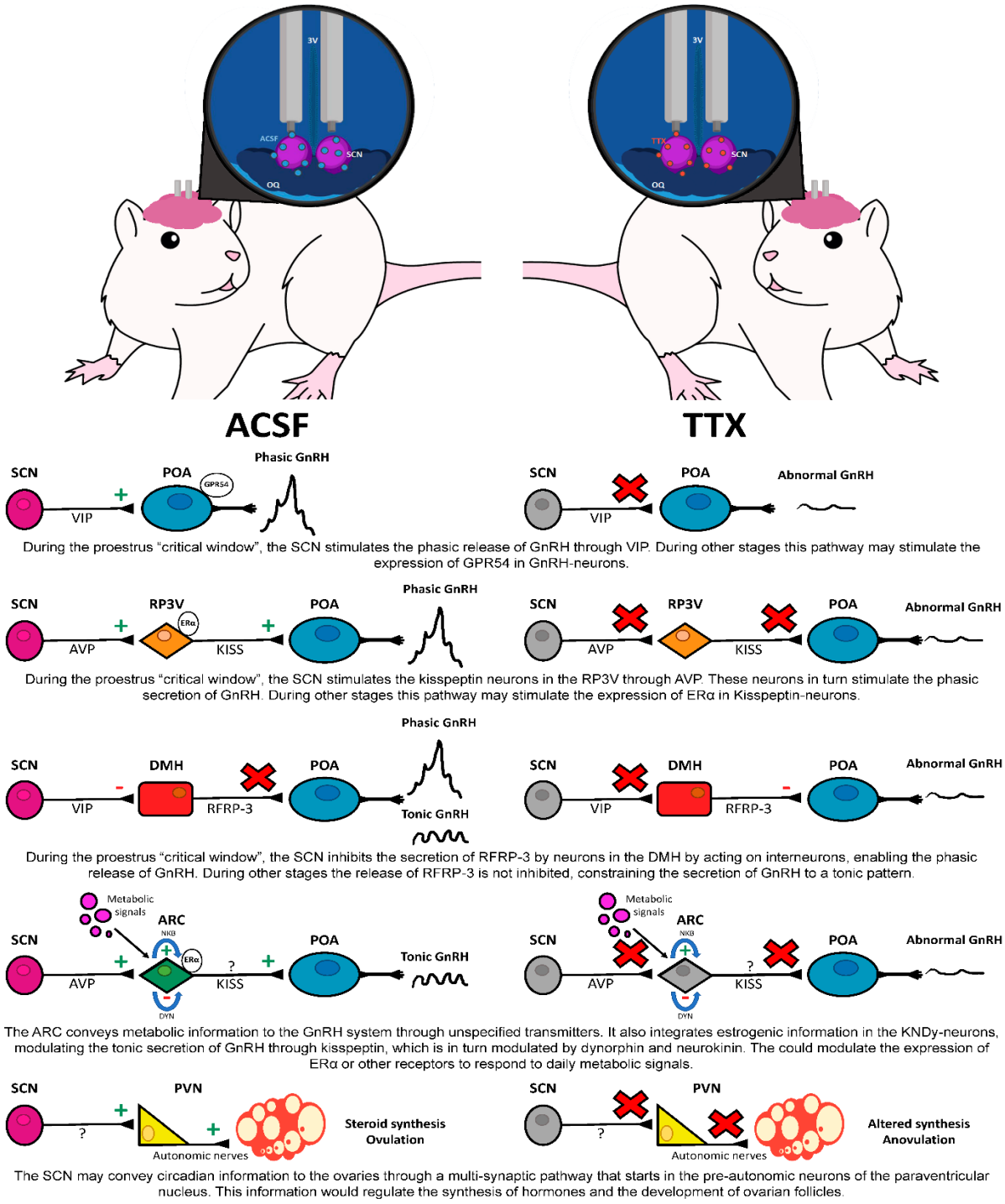

Figure 15. *The participation of the suprachiasmatic nucleus (SCN) in the regulation of gonadotropin secretion and ovarian functions. The possible signals that are altered by the microinjection of artificial cerebrospinal fluid (ACSF) or tetrodotoxin (TTX) into the SCN are described in the left and right column, respectively. The nuclei inhibited in these experiments are depicted with gray. Arcuate nucleus (ARC), dorsomedial nuelcues (DMH), paraventricular nucleus (PVN), preoptic area (POA), rostral periventricular region of the third ventricle (RP3V) Stimulant (+), inhibitory (-) and blocked (X) signals of defined or undefined (?) neurochemical identity are highlighted. Arginine-vasopressin (AVP), dynorphin (DYN), kisspeptin (KISS), neurokinin B (NKB), RFamide related peptide-3 (RFRP-3), vasoactive intestinal polypeptide (VIP) See text in this chapter for details.*

functions at specific moments. In support of this hypothesis, the lesion of the SCN followed by ipsilateral section of the superior ovarian nerve during proestrus reduces the number of oocytes ovulated by the ipsilateral ovary only. Furthermore, this effect only occurs when the surgeries are performed in the morning, but not in the afternoon (Ramírez *et al.*, 2016). As it can be concluded from the previous paragraphs, there is no conclusive information available regarding this important topic and dedicated experiments must be performed to disclose the participation of the ovarian innervation and the daily humoral communication between endocrine glands on its circadian regulation, as it has occurred for other organs.

The involvement of the SCN in the regulation of seasonal reproduction

Until this point, we have focused our analysis of the circadian regulation of ovulation in non-seasonal polyestrous breeders that ovulate spontaneously, as rats, mice and humans. In these species, reproductive cycles are repeated all year long and ovulation occurs at the end of each one regardless if mating occurred or not. It is important to mention that not all species follow this pattern, for example, seasonal mammals alternate between periods of gonadal quiescence followed by the resumption of their functions during the breeding season (Bronson, 1985; 2009; Weir and Rowlands, 1973). Considering the inherent differences in the reproductive patterns of seasonal and non-seasonal breeders, an interesting question is whether the conclusions drawn from the latter type can be extrapolated to the former one.

Outside the regions located along the equatorial line, in which the proportion of day and night hours is symmetrical (12:12), the length of the day varies as the Earth completes its translational movement. Reproduction of seasonal breeders display photoperiodic responses, i.e., changes as a function of day length to adapt to the environmental pressures resulting from this geophysical cycle. The start of the breeding season is determined by the secretion of melatonin, a hormone synthesized by the pineal gland during the night, and also depends on the duration of the gestational period. The breeding season of animals with a short gestation, as the hamster, starts at the beginning of the long-day months while animals with long gestation as the sheep starts breeding during the short-day months. This ensures that births in both species occur during the spring and summer, when the environmental conditions and the availability of food are optimal for the development of the offspring (Guh *et al.*, 2019; Hut *et al.*, 2014).

In seasonal breeders, melatonin plays a pivotal role in the determination of reproductive status because it allows the discrimination of the short and long days by serving as an interplay between the visual and neuroendocrine systems (Guh *et al.*, 2019; Ikegami and Yoshimura, 2013; Nishiwaki-Ohkawa and Yoshimura, 2016). Light stimulation of the retina induces the release of glutamate at the RHT terminals, which depolarizes SCN-neurons enabling the entrance of Ca^{2+}, this initiates a transduction pathway that involves the phosphorylation of CREB and hence the modulation of gene expression (Meijer and Schwartz, 2003). The SCN acts as a luminance detector that interpret the photoperiod and

then transmits the information to the pineal gland through the paraventricular nucleus-intermediolateral column-superior cervical ganglion pathway (Illnerová, 1991; Klein and Moore, 1979). During the night, in the absence of light, the SCN sends stimulant signals that result in the release of noradrenaline by neurons in the superior cervical ganglion. This catecholamine binds to β1 receptors in the pinealocytes, driving the expression of the enzymes that participate in the synthesis of melatonin. As the hormone is soluble in water and lipids, it is immediately released to the general circulation. Considering that the synthesis of melatonin ceases during the light period by inhibitory signals following the pathway mentioned before, it is used to convey information about the length of the light and dark portions of the photoperiod to the rest of the body (Claustrat *et al*., 2005).

Binding of melatonin to its receptors in the thyrotropes located in the *pars tuberalis* of the pituitary stimulates the release of thyroid stimulating hormone, which in turn binds to its receptors in the ependymal cells of the medio-basal hypothalamus. This promotes the expression of the DIO2 gene, which encodes for the thyroid hormone-activating enzyme that converts thyroxine into its active form, triiodothyronine. This hormone mediates changes in the anatomy of the GnRH-terminals located in the ME, as well as their interaction with glial cells, inhibiting the secretion of GnRH into the portal vessels and hence promoting the end of the breeding season (Guh *et al*., 2019; Ikegami and Yoshimura, 2013; Nishiwaki-Ohkawa and Yoshimura, 2016). In addition, there is evidence to suggest that triiodothyronine also stimulates the release of kisspeptin and RFRP-3. This would suppose an additional regulative layer in which melatonin indirectly activates the classic stimulant and inhibitory pathways that regulate the activity of the reproductive axis, modulating not only the end, but also the beginning of the breeding season (Hut *et al*., 2014; Sáenz de Miera *et al*., 2014).

Considering the information stated above, and despite being considered a circadian pacemaker, it is clear that the SCN is an essential part of the circuit that regulates seasonal and infradian-like behaviors. Supporting this, it has been shown that the lesion of the SCN inhibits the testicular regression in hamsters exposed to short-days, a characteristic photoperiodic response of this species. Testicles of these animals start recrudescence after the surgery regardless of the lighting conditions and soon attain full size and adequate secretion of testosterone and spermatogenesis (Rusak and Morin, 1976). A similar effect is observed in pinealectomized hamsters and, in this model, the injection of melatonin inhibits the recrudescence, suggesting that the lesion of the SCN impairs photoperiodic responses because it interrupts the secretion of melatonin. This interpretation, however, fails to explain the fact that testicular recrudescence in SCN-lesioned hamsters is not inhibited by melatonin administration (Rusak, 1980). This implies that, in addition to the SCN-pineal pathway, the circadian system modulates seasonal sexual physiology by regulating other neuroendocrine targets, probably those described in the rat in previous paragraphs, including the neural pathways that links the SCN with the gonads through the peripheral nervous system.

In the case of females, the anovulatory state is interrupted by exposure to a 14:10 photoperiod. In such conditions, the estrous cycle repeats every 96 hours presenting the onset of sexual receptivity and ovulation at the end of each one. The experiments by Alleva and colleagues (1971) demonstrated that, under continuous dim illumination, the estrous cycle of the hamster is not abolished as it occurs in non-seasonal rodents. Its period is the quadruple of the circadian period of locomotor activity, not a deviation directly related to 96 hours, and sexual receptivity, gonadotropin secretion and ovulation still stablish close phase relationships with the onset of estrus, demonstrating its endogenous nature. Despite this, a reduction in the number of four-day cycles and an increase in desynchronized ovulation was observed in the animals submitted to constant illumination. In addition, pharmacological treatments that elongate circadian rhythms also elongates the estrous cycle by multiples of 24 hours, suggesting that the mechanism that sets the time of gonadotropin release depends on a circadian pacemaker as in rats (Alleva *et al*., 1968; Morin *et al*., 1977). In concordance, LH surges can also be evoked every 24 hours in ovariectomized hamsters by the administration of a high dose of estradiol and this is inhibited by pentobarbital injections or the deafferentation of the hypothalamic region containing the SCN (Norman and Spies, 1974; Norman *et al*., 1972; 1973). Finally, bilateral lesion of the SCN inhibit estrous cyclicity and ovulation, providing direct evidence of the participation of this nucleus in the regulation of reproduction in a long-day breeder (Meyer-Bernstein *et al*., 1999; Shander and Barraclough, 1980; Stetson and Watson-Witmyre, 1976).

Short-day breeders also rely on the SCN to display photoperiodic responses. In the sheep, bilateral lesion of the SCN impairs the secretion of melatonin but not the estrous cycle nor ovulation. The regularity of the cycles, however, is abnormal since spontaneous cycles are interrupted by periods of acyclicity and ovarian activity extends far into the non-breeding season, a result that is similar to the case of the hamster testes (Przekop and Domanski, 1980; Scott *et al*., 1995). It is difficult to conclude if the participation of the SCN in the regulation of reproduction in this species is restricted to the regulation of melatonin secretion, but there is evidence that suggests an alternative role. It has been shown that the seasonal circuit that drives reproduction in the sheep is only sensitive to the thyroid hormones for a short period during the late breeding season. According to this model, an endogenous mechanism is responsible for a seasonal change in the sensitivity of the neuroendocrine axis and the SCN is a good candidate to be part of it due to its connections with the visual system and with the rest of such axis (Thrun *et al*., 1997). In order to further explore this topic, experimental evidence assessing the changes in sensitivity to thyroid hormones in animals bearing lesions or deafferentation of the SCN is needed.

Conclusion and perspectives

Sexual reproduction allows the continuity of the information contained in an individual over time, with the advantages of DNA recombination. This seems to facilitate the *Darwinian*

evolution by favoring the adaptation to unpredictable and hostile environments. In this sense, the study of the mechanisms that couple reproduction with the environment to increase fitness is one of the most encouraging fields in modern Biology. The classic studies by Everett and Sawyer dragged a great amount of attention to the neural circadian mechanisms that operate during the afternoon of proestrus to drive the pre-ovulatory surge of gonadotropins in coherence with the light-dark cycle. Unfortunately, most of the research efforts that have been made to understand the circadian regulation of ovulation have focused on this aspect only, neglecting the importance of the regulatory mechanism that occur during the rest of the stages of the estrous cycle. The use of ovariectomized rodents, primed or not with estrogens, as a model to study the function of the hypothalamic-pituitary-ovarian axis has also led to an oversimplified picture of the system that does not consider the wide variety of ovarian stimuli that impact on the brain and peripheral organs, indirectly affecting its own physiology. In addition, the estrous cycle, the development of the ovarian follicles and the secretion of FSH have been poorly studied from a circadian point of view.

This study was exploratory in nature and, in consequence, lead us to ask more questions than the ones it answered. This is important since its intention was (and still is) to stimulate further research that explains the observations raised. With the results presented in Chapter III and IV we can conclude that the SCN is the locus of the neural signals that are generated during the "critical window" of each stage of the estrous cycle. These signals are pivotal for the regulation of ovulation and depend, at least in part, on interneurons located in the ARC, which is also indispensable for ovulation during this period. Based on these results, we are now describing the electric activity of the POA, RP3V and the ARC along the 96 hours of the estrous cycle and its correlation with the activity of the SCN. We hope this will help to clarify the timed communication of these structures through the reciprocal neural connections that link them. The role of the neural signals of the SCN on the regulation of the expression of estradiol and kisspeptin receptors in the hypothalamus is also in process. Finally, the chemical nature of this communication will be analyzed to understand the neurotransmitter systems involved in such coordination. With this we sought to explain how the SCN prepares the hypothalamus to respond to the cyclic changes in the secretion of gonadal hormones. Hopefully, these experiments will contribute to our understanding of the subtle talk between the brain and the ovaries.

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List of Figures and Tables

Scientific Production

Published articles

1. **Carlos-Camilo Silva**, Montserrat Bolaños-Hurtado, Cinthia Juárez-Tapia, Angélica Flores, Isabel Arrieta-Cruz, María-Esther Cruz, Roberto Domínguez. (2020). *Unraveling the role of discrete areas of the rat brain in the regulation of ovulation through reversible inactivation by tetrodotoxin microinjections*. Journal of Visualized Experiments 163. doi: 10.3791/61493.

2. Esteban Olvera-Juárez, **Carlos-Camilo Silva**, Angélica Flores, Isabel Arrieta-Cruz, Luciano Mendoza-Garcés, Hilda Martínez-Coria, Héctor López-Valdés, Mario Cárdenas, Roberto Domínguez, Roger Gutiérrez-Juárez, María-Esther Cruz. (2020). *The content of gonadotropin-releasing hormone (GnRH), kisspeptin, and estrogen receptors (ERα/ERβ) in the anteromedial hypothalamus displays daily variations throughout the rat estrous cycle.* Cell and Tissue Research 381:451-460.

3. **Carlos-Camilo Silva**, Roberto Domínguez. (2019). *Clock control of mammalian reproductive cycles: Looking beyond the pre-ovulatory surge of gonadotropins.* Reviews in Endocrine and Metabolic Disorders 21:149-163.

4. **Carlos-Camilo Silva**, Georgina Daniela Cortés, Cintia Yolanda Javier, Angélica Flores, Roberto Domínguez. (2019). *A neural circadian signal essential for ovulation is generated in the suprachiasmatic nucleus during each stage of the oestrous cycle.* Experimental Physiology 105:258-269.

Presentation in international scientific meetings

1. **CC Silva**, E Olvera, A Flores, R Librado, I Arrieta, R Gutiérrez, R Domínguez, ME Cruz. Poster presentation: "Asymmetries on the estradiol receptors, kisspeptin and GnRH content between the left and right hypothalamus during the estrous cycle of the rat". Neuroscience, October 19-23, 2019. Chicago, Illinois, EUA.

2. **CC Silva**, GD Cortés, CY Javier, A Flores, R Domínguez. Poster presentation: "Transitory inhibition of the suprachiasmatic nucleus' electric activity by tetrodotoxin microinjection at 14:00 of every stage of the estrous cycle results on a blockade of ovulation in the rat". Neuroscience, November 03-07, 2018. San Diego, California, EUA.

3. **CC Silva**, C Javier, G Cortés, A Flores, R Domínguez. Poster presentation: "The suprachiasmatic nuclei are involved on the generation of neural signals required for the regulation of ovulation during each stage of the estrous cycle". 22nd International Symposium on Regulatory Peptides, September 22-25, 2018. Acapulco, Guerrero, México

4. **CC Silva**, CY Javier, GD Cortés, A Flores, R Domínguez. Poster presentation: "Tetrodotoxin-blockade of the suprachiasmatic nuclei disrupts ovulation. A study throughout the rat estrous cycle". XIV Latin American Symposium on Chronobiology, November 14-18, 2017. Valparaiso, Chile.

5. **CC Silva**, DP Benítez, JC Muñoz, GD Cortés, ME Cruz, A Flores, R Domínguez. Poster presentation: "The suprachiasmatic nuclei, asymmetries and the involvement on non-proestrous events in the regulation of ovulation". Neuroscience, October 17-21, 2015. Chicago, Illinois, EUA.

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Unraveling the Role of Discrete Areas of the Rat Brain in the Regulation of Ovulation through Reversible Inactivation by Tetrodotoxin Microinjections

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Abstract

Many experimental approaches have been used for studying the role of the brain in the regulation of ovulation. Examples include the lesion and deafferentation of neuronal groups, which are both invasive methods that permanently impair the integrity of the target area. These methods are accompanied by collateral effects that can affect the analysis of acute and temporal regulatory mechanisms. The stereotaxic implantation of guide cannulas aimed at specific brain regions, followed by a recovery period, allows researchers to microinject different drugs after the disappearance of the undesired effects of the surgery. Tetrodotoxin has been used to determine the roles of several brain areas in diverse physiological processes because it transiently inhibits the sodium-dependent action potentials, thus blocking all neural activity in the target region. This protocol combines this method with strategies for the assessment of the estrous cycle and ovulation to reveal the role of discrete brain regions in the regulation of ovulation at particular times of any given stage of the estrous cycle. Awake and unrestrained rats (*Rattus norvegicus*) were used to avoid the blocking effects that anesthetics and stress hormones exert on ovulation. This protocol can be easily adapted to other species, brain targets and pharmacological agents to study different physiological processes. Future improvements to this method include the design of a microinjection system using glass capillaries of small diameter instead of guide cannulas. This will reduce the amount of tissue damaged during the implantation and decrease the spread of the infused drugs outside the target area.

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REGULAR ARTICLE

The content of gonadotropin-releasing hormone (GnRH), kisspeptin, and estrogen receptors (ERα/ERβ) in the anteromedial hypothalamus displays daily variations throughout the rat estrous cycle

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Abstract

The content of gonadotropin-releasing hormone (GnRH), its mRNA, and estrogen receptor alpha (ERα) and beta (ERβ) in the hypothalamus varies throughout the estrous cycle. Furthermore, the abundance of these molecules displays asymmetry between the right and left side. In the present study, we investigated the changes in the content of ERα, ERβ, kisspeptin, and GnRH by western blot in the left and right anteromedial hypothalamus, at four different times during each stage of the rat estrous cycle. The serum levels of the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were also measured. ERα and ERβ levels changed depending on the stage of the estrous cycle, meanwhile that of kisspeptin was modified according to both the hour of the day and the stage of the cycle. Except in estrus day, ERβ was higher in the right hypothalamus, while ERα was similar in both sides. During both proestrus and estrus, the content of kisspeptin and GnRH was higher in the right hypothalamus. The highest levels of FSH and LH occurred at 17:00 h of proestrus. But at estrus, the highest FSH levels were observed at 08:00 h and the lowest at 17:00 h. Thus, the current results show that the content of ERα, ERβ, kisspeptin, and GnRH in the anteromedial hypothalamus are regulated as a function of the stage of the estrous cycle and the hour of the day. Furthermore, the content of these proteins is regularly higher in the right anteromedial hypothalamus, regardless of the stage of the cycle or time of the day.

Keywords GnRH · Estrogen receptor · Kisspeptin · Estrous cycle · Hypothalamic asymmetry

Introduction

Gonadotropin-releasing hormone (GnRH) is the main stimulating factor that regulates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both of which then regulate the development of ovarian follicles and ovulation (Herbison 2015). In the rodent brain, GnRH neurons are located in rostral areas of the encephalon as part of the

María-Esther Cruz is deceased. This paper is dedicated to her memory.

This article is dedicated to the memory of our beloved colleague, who recently passed away.

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Clock control of mammalian reproductive cycles: Looking beyond the pre-ovulatory surge of gonadotropins

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Abstract

Several aspects of the physiology and behavior of organisms are expressed rhythmically with a 24-h periodicity and hence called circadian rhythms. Such rhythms are thought to be an adaptive response that allows to anticipate cyclic events in the environment. In mammals, the circadian system is a hierarchically organized net of endogenous oscillators driven by the hypothalamic suprachiasmatic nucleus (SCN). This system is synchronized by the environment throughout afferent pathways and in turn it organizes the activity of tissues by means of humoral secretions and neuronal projections. It has been shown that reproductive cycles are regulated by the circadian system. In rodents, the lesion of the SCN results on alterations of the estrous cycle, sexual behavior, tonic and phasic secretion of gonadotropin releasing hormone (GnRH)/gonadotropins and in the failure of ovulation. Most of the studies regarding the circadian control of reproduction, in particular of ovulation, have only focused on the participation of the SCN in the triggering of the proestrus surge of gonadotropins. Here we review aspects of the evolution and organization of the circadian system with particular focus on its relationship with the reproductive cycle of laboratory rodents. Experimental evidence of circadian control of neuroendocrine events indispensable for ovulation that occur prior to proestrus are discussed. In order to offer a working model of the circadian regulation of reproduction, its participation on aspects ranging from gamete production, neuroendocrine regulation, sexual behavior, mating coordination, pregnancy and deliver of the product should be assessed experimentally.

Keywords Estrous cycle · Ovulation · Gonadotropins · Suprachiasmatic nucleus · Circadian rhythms

Acronyms

5-HT	Serotonin
ARC	Arcuate nucleus
AVP	Arginine-vasopressin
AVPv	Anteroventral periventricular area
Bmal1	Brain and muscle ARNT-like protein 1
Ccg's	Clock-Controlled genes
CG's	Clock genes
Clock (gene/protein)	Circadian locomotor output cycle kaput
Cry	Chryptochrome
DMH	Dorsomedial hypothalamus
ERα	Estrogen receptor α-isoform
ERβ	Estrogen receptor β-isoform
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
HPG	Hypothalamic-pituitary-gonadal axis
IGL	Intergeniculate leaflet
IML	Intermediolateral column
LH	Luteinizing hormone
NPY	Neuropeptide Y
Per	Period
POA	Preoptic area
RFRP-3	RFamide-related peptide-3
RHT	Retino-hipothalamic tract
SCN	Suprachiasmatic nucleus
VIP	Vasoactive intestinal polypeptide

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1 Adaptive significance of biological rhythms

Life oscillates because it evolved in an oscillating world. Since the beginning of their existence, living systems have

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Experimental Physiology WILEY

RESEARCH PAPER

A neural circadian signal essential for ovulation is generated in the suprachiasmatic nucleus during each stage of the oestrous cycle

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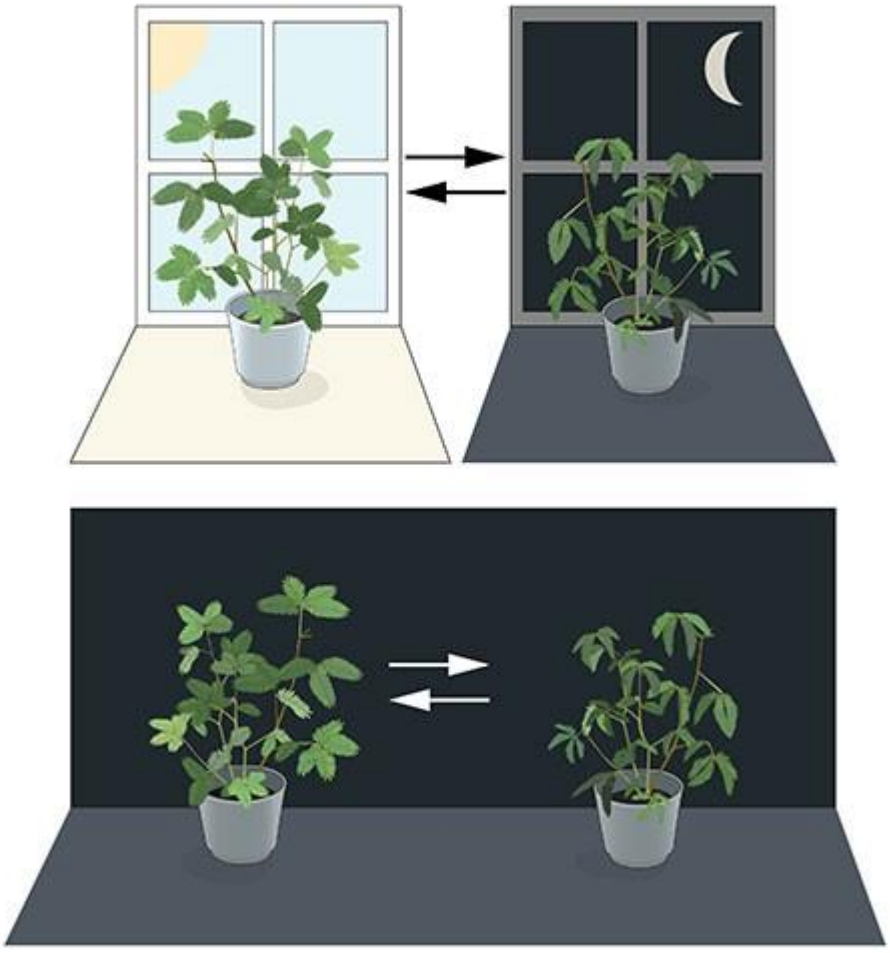
Abstract

Reproduction is a highly timed process that depends on both the reproductive and circadian systems. The core oscillator of the latter resides at the suprachiasmatic nuclei (SCN) and it is pivotal for the regulation of the pro-oestrus pre-ovulatory surge of gonadotropins in females. There is evidence to suggest that this system may be involved in the regulation of neuroendocrine events that are essential for ovulation and that occur prior to pro-oestrus. We explored this possibility by transiently inactivating the SCN. Female rats were implanted with guide cannulas aimed at the SCN. After recovery of the oestrous cycle, animals were injected with tetrodotoxin (TTX), artificial cerebrospinal fluid (ACSF) or saline solution while freely moving. Injections were performed at 14.00 h of each stage of the oestrous cycle. Animals were killed on the next predicted oestrus day, the number of ova shed was counted and intact rats at oestrus stage were used as absolute control. ACSF did not modify ovulation. Saline solution blocked ovulation in oestrus- and dioestrus-injected rats. Irrespectively of the stage of the oestrous cycle, TTX blocked ovulation. These results lead us to suggest that a neural circadian signal, pivotal for triggering the gonadotropin pre-ovulatory surge, arises from the SCN during the critical window of pro-oestrus. We also suggest that a similar signal, needed for the regulation of other events that are indispensable for proper regulation of ovulation, is also generated in this nucleus during the other stages of the cycle at a similar time.

KEYWORDS
oestrous cycle, ovulation, suprachiasmatic nucleus

1 INTRODUCTION

Perpetuity of all mammalian species relies on sexual reproduction, which implies the production and interaction of gametes. In the case of females, oocytes develop in association with somatic cells forming a functional unit known as the ovarian follicle. Follicular development is regulated by neural information that arrives at the ovary through its innervation and also by the secretion patterns of the two pituitary gonadotropins: follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Domínguez & Cruz-Morales, 2011). In the process of ovulation, mature oocytes capable of being fertilized are released from the follicles and deposited into the oviduct once every oestrous/menstrual cycle. In laboratory rodents, this process is gated by a surge-like secretion of LH and FSH that results from a timed increase in the firing and secretory activity of the hypothalamic neurons that synthesize the gonadotropin releasing hormone (GnRH)



"*…the beauty of science comes from the unexpected findings that change the focus of the things, that allows to link concepts and to understand what before couldn't be understood.*"

Michael Menaker