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Régulation des neurones à RFRP-3 en fonction de la journée, du cycle estral et de l'âge et leur rôle dans la sécrétion de l'hormone lutéinisante chez les souris femelles

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Daily-, estral- and age- dependent regulation of RFRP-3 neurons and their role in luteinizing hormone secretion in female mice

Eleni Angelopoulou

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General Introduction

Based on:

Angelopoulou, E., Quignon, C., Kriegsfeld, L.J., Simonneaux, V., 2019. Functional Implications of RFRP-3 in the Central Control of Daily and Seasonal Rhythms in Reproduction. Front Endocrinol (Lausanne) 10. https://doi.org/10.3389/fendo.2019.00183

Simonneaux, V., Bahougne, T., **Angelopoulou, E.**, 2017. Daily rhythms count for female fertility. Best Pract. Res. Clin. Endocrinol. Metab. 31, 505–519. https://doi.org/10.1016/j.beem.2017.10.012

GENERAL INTRODUCTION

Adaptation of reproductive activity to environmental changes is essential for breeding success and offspring survival. In mammals, the reproductive system displays regular cycles of activation and inactivation, which are synchronized with seasonal and/or daily rhythms in environmental factors, notably light intensity and duration. Thus, most species adapt their breeding activity along the year to ensure that birth and weaning of the offspring occur at a time when resources are optimal. Additionally, female reproductive activity and the period of full oocyte maturation is highest at the beginning of the active phase, in order to improve breeding success. In reproductive physiology, it is therefore fundamental to delineate how geophysical signals are integrated in the hypothalamo-pituitary-gonadal axis, notably by the neurons expressing gonadotropin releasing hormone (GnRH). Several neurotransmitters have been reported to regulate GnRH neuronal activity, but recently two hypothalamic neuropeptides belonging to the superfamily of (Arg)(Phe)-amide peptides, RFRP-3 and kisspeptin (Kp), have emerged as critical for the integration of environmental cues within the reproductive axis. The goal of this thesis is to explore the temporal regulation of RFRP-3, and consider how it might combine with Kp to improve the synchronization of reproduction at different stages of the adult life.

1. Circadian rhythms are driven by the Suprachiasmatic nucleus

The rotation of the earth around its axis exposes all living organisms to 24-hour light-dark and temperature cycles. Through the course of evolution, most organisms; from bacteria to mammals; developed internal timekeeping systems in order to anticipate predictable daily changes in the environment. Thus, even in the absence of external cues (constant conditions of light, temperature, food, etc.) organisms exhibit behavioral and physiological cycles with a period close to, but usually not exactly 24 hours. In mammals, these cycles are driven by molecular oscillators in the suprachiasmatic nucleus (SCN) of the hypothalamus, the primary circadian pacemaker (Bollinger and Schibler, 2014). The SCN receives photic information from intrinsically photoreceptive retinal ganglion cells (ipRGCs) in the eyes,

which convert electrical signals into chemical ones, through the retinal hypothalamic tract in order to reset its molecular oscillators and synchronize the endogenous circadian clocks with the environment (Bollinger and Schibler, 2014; Hattar et al., 2002).

A plethora of studies established the SCN as the seat of the master clock. While SCN lesions render animals arrhythmic (Moore and Eichler, 1972; Stephan and Zucker, 1972), transplantation of fetal SCN can restore circadian rhythmicity (Ralph et al., 1990; Sawaki et al., 1984; Sollars et al., 1995; Sujino et al., 2003). Clock gene expression, firing activity, intracellular calcium concentration ([Ca2+]i) and glucose consumption change rhythmically in the SCN according to time of day (Green and Gillette, 1982; Inouye and Kawamura, 1979; Noguchi et al., 2017; Schwartz and Gainer, 1977). Dispersed SCN neurons maintain cell autonomous circadian rhythms of clock gene expression and $[Ca^{2+}]i$ levels, even though these rhythms are stronger in intact SCN slices (Noguchi et al., 2017). Importantly, SCN tissue in organotypic cultures can maintain structural coherence and circadian rhythms in gene expression and neuronal activity for months (Brancaccio et al., 2014; Patton et al., 2016; Yamaguchi et al., 2003).

A network of self-sustaining transcriptional and translational feedback loops (TTFLs) underlies the operating molecular mechanism for circadian oscillation within the SCN neurons (Figure 1). At circadian time 0 (CT0), corresponding to dawn, the positive regulators of the loop, CLOCK and BMAL1 form heterodimers that drive the transcription of the clock genes encoding the Period proteins; PER1 and PER2; and the Cryptochrome proteins; CRY1 and CRY2; via enhancer box (E-box) regulatory sequences. PER-CRY proteins form complexes that accumulate in the cytoplasm and enter the nucleus when they reach a threshold to attenuate transcriptional activity, at the end of the circadian day (CT12). During the circadian night (CT12-CT24), *Per* and *Cry* mRNA levels decrease and PER-CRY complexes degrade, therefore allowing the transcriptional cycle to reinitiate itself again after approximately 24 hours. The CLOCK-BMAL1 dimer also drives a complementary feedback loop that involves the transcription of nuclear receptor genes encoding REV-ERBα and REV-ERBβ, which suppress, and RORα and RORβ, which activate the *CLOCK* and *BMAL1* transcription (Hastings et al., 2018). Through the intertwined TTFLs of clock genes, the SCN

can generate robust circadian rhythms and achieve precise circadian timing. Brain regions outside the SCN and peripheral organs also contain such autonomous circadian oscillators, however, they lack strong intercellular coupling and direct signaling from the retina, so they depend on the SCN output in order to sustain circadian rhythmicity and entrain to the day/night cycle (Abe et al., 2002; Amir et al., 2004; Granados-Fuentes et al., 2004).

The circadian oscillation of clock genes in the SCN has been associated with circadian changes in SCN firing activity and neuropeptide synthesis. Multiple studies have demonstrated that the firing rate of SCN neurons increases during daytime and decrease at night, both in nocturnal and diurnal rodents, even under constant darkness (Green and Gillette, 1982; Inouye and Kawamura, 1979; Meijer et al., 1998; Sato and Kawamura, 1984; Welsh et al., 1995). Alterations in the molecular clock components change the circadian rhythms in behavior and in the SCN electrical activity, by changing clock gene-dependent molecular feedback loops (Liu et al., 1997; Meng et al., 2008) and clock gene deficient mice that are arrhythmic, lack circadian oscillations in the SCN firing activity (Albus et al., 2002). Altogether, these findings suggest that an intact molecular clock is necessary for the generation of circadian rhythms in the SCN electrical activity.

The vast majority of SCN neurons are GABAergic and co-express one or more neuropeptides (Buijs et al., 1995; Romijn et al., 1997). Based on anatomical connections and peptide expression, the SCN can be divided in two subregions: the core, receiving direct input from the retinal ipRGCs and mainly expressing vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP); and the shell, receiving input from limbic, hypothalamic and brainstem nuclei, and mainly expressing arginine vasopressin (AVP) (Abrahamson and Moore, 2001). VIP mRNA and protein expression display daily variations in the SCN, peaking during the middle of the dark period and decreasing during the light period (Dardente et al., 2004; Shinohara et al., 1999, 1993). Similarly, AVP mRNA and peptide release in the SCN exhibit daily variations, peaking around the middle of the light period and decreasing during the dark period (Dardente et al., 2004; Kalsbeek et al., 1995). Within the SCN, VIP acts primarily on VPAC2 receptors, the expression of which peaks during the subjective morning

(An et al., 2012), while AVP acts primarily on V1a receptors, the expression of which peaks during the dark period (Li et al., 2009).

The output of the SCN is organized in three major pathways: 1) one pathway runs dorsally and rostrally into the medial preoptic area (POA) and continues into the paraventricular nucleus of the thalamus, 2) a second pathway runs caudally to the retrochiasmatic area and the capsule of the ventromedial nucleus, 3) a third pathway that travels in an arc dorsally and caudally, giving off terminals along the course through the regions above the SCN such as the subparaventricular area and the paraventricular hypothalamic nucleus (PVN). A small part of these fibers continues dorsocaudally into the dorsomedial nucleus of the hypothalamus (DMH) where they terminate along its length (Saper et al., 2005). Via these output pathways the SCN drives numerous behavioral and physiological functions, among which is the reproductive activity (Williams and Kriegsfeld, 2012).

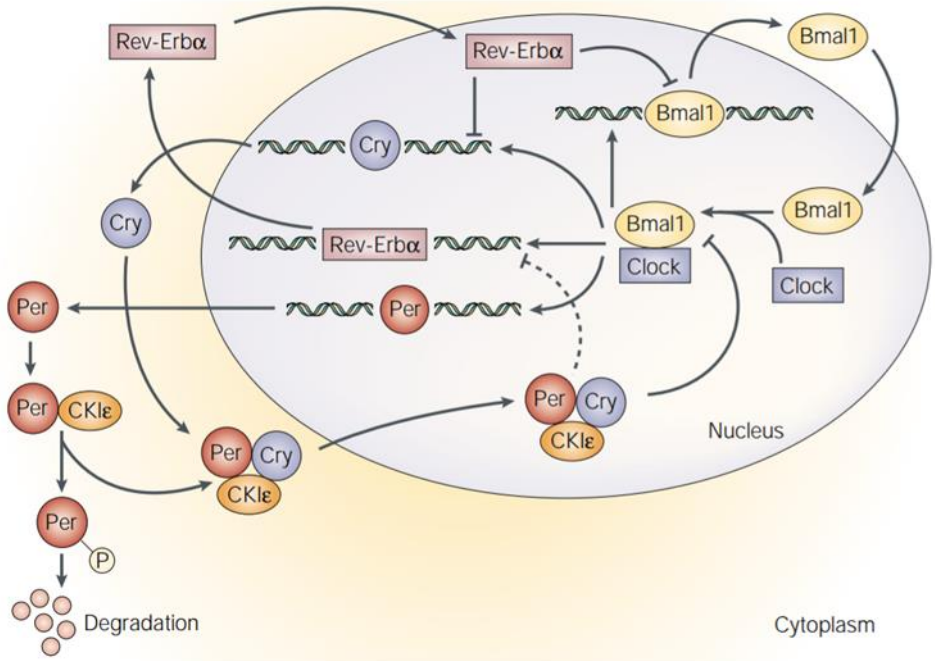

Figure 1: *Schematic representation of the molecular clock mechanism in SCN neurons. Transcriptional factors Clock and Bmal1 form heterodimers and bind to E-box sequences in the promoters of the Cry, Per and Rev–Erbα genes to activate transcription at the beginning of the circadian day. The Clock-Bmal1 heterodimer can also inhibit Bmal1 transcription. After transcription and translation, the Rev–Erbα protein enters the nucleus to suppress the transcription of Bmal1 and Cry genes. While Per proteins accumulate in the cytoplasm, they become phosphorylated (P) by casein kinase I ε (CKIε) and then degraded by ubiquitylation. Late in the subjective day, however, Cry accumulates in the cytoplasm, promoting the formation of CKIε/Per/Cry complexes, which enter the nucleus at the beginning of the subjective night. Once in the nucleus, Cry disrupts the Clock/Bmal1 transcriptional complex, resulting in the inhibition of Cry, Per and Rev–Erbα transcription, and the stimulation of Bmal1 transcription. The interacting positive and negative feedback loops of circadian genes ensure low levels of Per and Cry, and a high level of Bmal1 at the beginning of the new circadian day. Solid lines indicate direct regulation, and dashed lines indicate indirect regulation. Image from Fu and Lee (2003).*

2. Role of RFRP-3 in the central control of female reproduction

2.1 Functional organization of the Hypothalamo-Pituitary-Ovarian (HPO) axis

Mammalian reproduction is tightly controlled by a small set of neurons producing the neuropeptide GnRH. The GnRH cell bodies are concentrated in specific hypothalamic areas [the preoptic area, the vascular organ of the lamina terminalis and, in non-rodent species, the mediobasal hypothalamus] and project principally to the median eminence where they release GnRH in a pulsatile manner in the portal blood supply of the anterior pituitary (Marques et al., 2000). Within the anterior pituitary GnRH stimulates the secretion of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are released from the anterior pituitary into the general circulation to regulate gonadal gameto- and steroidogenesis respectively (Figure 2).

Mechanisms regulating the activity of GnRH neurons are thought to involve different upstream neuronal inputs. Glutamate and γ-aminobutyric acid fibers, located close to GnRH perikarya and axons, have been shown to stimulate and/or inhibit GnRH release (Morello et al., 1992; Ottem et al., 2002; Piet et al., 2018). Neuropeptide Y-containing fibers also contact a majority of GnRH neurons and predominantly exert an inhibitory effect on GnRH release (Klenke et al., 2010; Roa and Herbison, 2012). Recent studies, however, have highlighted an important role of two other hypothalamic neuropeptides, kisspeptin and RFRP-3, in the regulation of GnRH neuronal activity. Kisspeptin expressing neurons are located in two hypothalamic areas: the preoptic area, where they project to GnRH cell bodies to drive the GnRH surge in female mammals, and in the arcuate nucleus, where they project principally to GnRH fiber terminals in the median eminence to drive pulsatile GnRH release (Pinilla et al., 2012). RFRP-3 expressing neurons are mostly located in the DMH and project to various neuronal populations including GnRH and kisspeptin neurons, yet the effects of RFRP-3 on reproduction seem to vary according to species, sex, and environmental conditions (Henningsen et al., 2016a; Kriegsfeld et al., 2018; Leon and Tena-Sempere, 2015).

To maintain the reproductive axis within proper functioning limits, sex steroids produced by the gonads feed back to the pituitary and hypothalamus. In males, testosterone acts to suppress GnRH and the gonadotropins through negative feedback, whereas in females the feedback is more complex with estradiol (E2) having either positive or negative feedback effects depending on the stage of the ovarian cycle and its circulating concentration. Specifically, during the follicular phase of the ovulatory cycle, low concentrations of E2 exert negative feedback, whereas upon oocyte maturation, higher concentrations of E2 exert positive feedback, triggering a large release of GnRH in the anterior pituitary portal blood supply which, in turn, induces a surge of LH that initiates ovulation (Christian and Moenter, 2010). Contrary to early expectations, GnRH neurons do not appear to be directly responsive to E2 feedback as these cells do not express E2 receptors (ER)α and only express low levels of ERβ (Christian and Moenter, 2010; Leon and Tena-Sempere, 2015). Likewise, mice with a GnRH neuron-specific deletion of ERβ do not exhibit any gross reproductive dysfunction (Cheong et al., 2014). Therefore, the central structures integrating sex steroid feedback have to be upstream of GnRH neurons and evidence now indicates that kisspeptin neurons and, to a less and unclear extent, RFRP-3 neurons are relaying gonadal hormone feedback to the reproductive system (Kriegsfeld et al., 2006; Poling et al., 2012; Smith et al., 2005a, 2005b; Tumurbaatar et al., 2018).

Because reproduction is particularly energetically demanding, it is critical that intrinsic and extrinsic factors contribute to optimizing breeding success and offspring survival as much as possible. Therefore, the reproductive axis is sensitive to various signals such as metabolic activity, stress level, development stage, hormonal milieu, and geophysical cues. Thus, in female mammals, timing of the preovulatory LH surge is driven by daily signals in addition to positive E2 feedback. Additionally, in seasonal breeders, annual changes in daily light duration (photoperiod) synchronize reproduction with the time of the year (Henningsen et al., 2016a). Recent studies have highlighted the pivotal role of RFRP-3 neurons, as well as kisspeptin neurons, in relaying both daily and seasonal cues to the HPG axis, particularly to GnRH neurons.

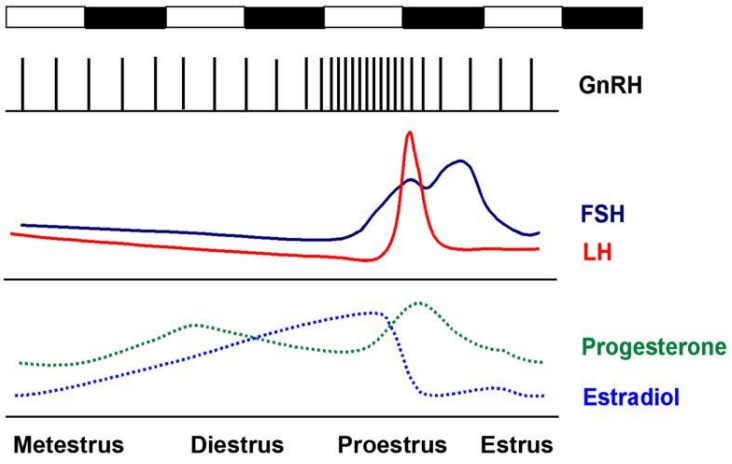

Figure 2: *Rhythmic hormone secretion during the rodent estrous cycle. In mice, ovulation occurs every 4–5 days. Metestrus and diestrus are characterized by low, but slowly increasing levels of estradiol. During the late afternoon of proestrus, elevated estradiol levels induce a burst of GnRH release from the hypothalamus, which triggers the preovulatory LH surge at approximately the start of the active (dark) period. Image from (Miller and Takahashi, 2014).*

2.2 The RFRP-3 system

The ortholog of RFRP-3 was originally discovered in birds, with Tsutsui et al. identifying a novel (Arg)(Phe) hypothalamic peptide that inhibited pituitary gonadotropin secretion from cultured quail pituitary (Tsutsui et al., 2000). Because this peptide selectively inhibited the gonadotropins, without altering other pituitary hormones, the authors named it gonadotropin-inhibitory hormone (GnIH). Subsequent findings indicated the GnIH receptor to be expressed in quail pituitary (Ubuka et al., 2012; Yin et al., 2005) and that *in vivo* GnIH administration decreases common α, LHβ, and FSHβ subunit expression (Ubuka et al., 2006; Yin et al., 2005). In birds, the GnIH precursor cDNA encodes one GnIH and two GnIH-related peptides (GnIH-RP1 and GnIH-RP2) (Molnár et al., 2011; Tsutsui et al., 2000). In mammals, the homologous gene encodes three peptides [RFamide-related peptides (RFRP)], with RFRP-1 and −3 both being RFamide peptides, while RFRP-2 is not (Tsutsui and Osugi, 2009). Since the initial discovery of these RFamide-related peptides in mammals, most findings in

reproductive biology have focused on RFRP-3 as the mammalian ortholog of GnIH. As described further below, studies across different mammalian species indicate a pronounced role for this neuropeptide in regulating reproductive function.

The receptor for GnIH/RFRP-3 is a G-protein coupled receptor (GPR), originally named OT7T022 (Hinuma et al., 2000), but now more commonly referred to by the name of the receptor for which it was found to be identical, the formerly-orphaned GPR147. Around the same time as this discovery, two receptors for another RFamide-peptide, neuropeptide FF, were identified and called NPFFR1 and NPFFR2 (Bonini et al., 2000). NPFFR1 was found to be identical to GPR147, whereas NPFFR2 was identical to another GPR, GPR74. GPR147 has a high affinity for GnIH/RFRP-3, whereas NPFF exhibits potent agonistic activity at GPR74 (Bonini et al., 2000; Liu et al., 2001; Yin et al., 2005; Yoshida et al., 2003). Together, these findings revealed GPR147/NPFFR1 as the GnIH/RFRP-3 receptor. GPR147 most-commonly couples to an inhibitory G protein (Gαi), with GnIH/RFRP-3 suppressing cAMP activity (Hinuma et al., 2000; Shimizu and Bédécarrats, 2010). However, in some instances, GPR147 is coupled to Gαs or Gαq proteins (Gouardères et al., 2007) and this differential coupling may account for the reported disparity in the effects of RFRP-3.

As indicated above, in most rodents, RFRP-3 perikarya are restricted to the DMH (Henningsen et al., 2016a; Kriegsfeld et al., 2018; Tsutsui and Ubuka, 2018), although, in rats, a significant number of cells are also observed in the region between the DMH and the ventromedial nucleus of the hypothalamus (VMH) (Hinuma et al., 2000; Legagneux et al., 2009) (Figure 3). In mammals, RFRP-3-immunoreactive (-ir) fiber projections are extensively scattered throughout the diencephalon, mesencephalon and limbic structures (Henningsen et al., 2016b; Kriegsfeld et al., 2006; Smith et al., 2008; Yano et al., 2003), providing divergent neural pathways to broadly influence neurophysiology and behavior.

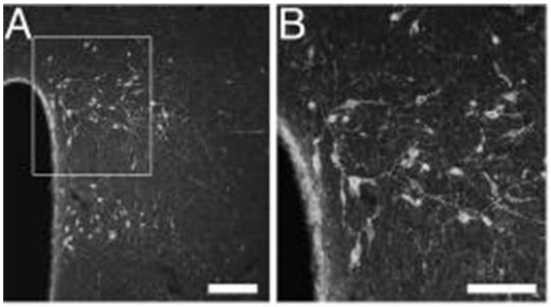

Figure 3: *GnIH/RFRP-3 cell bodies are tightly clustered in the dorsal and ventral regions of the DMH in Syrian hamsters (Scale bar: 200 µm). The box in the top image outlines the cells bodies shown at high power. Image from (Kriegsfeld et al., 2006).*

2.3 Evidence for a role of RFRP-3 in the central control reproduction

RFRP-3 acts both directly and indirectly to influence GnRH cell function. For example, RFRP-3 cell fibers form close contacts with GnRH cells (Figure 4) and about a third of GnRH cells express GPR147, pointing to direct actions of RFRP-3 on the GnRH system (Rizwan et al., 2012; Ubuka et al., 2012, 2009a, 2009b). Likewise, RFRP-3 inhibits cellular activity in about 40% of GnRH cells *in vitro* (Ducret et al., 2009; Wu et al., 2009). RFRP-3 may also act to suppress GnRH cellular activity via kisspeptin cells, as RFRP-3 cell projections form close connections with kisspeptin neurons in mice, sheep and monkeys (Poling et al., 2013; Qi et al., 2009; Ubuka et al., 2009a), with a small percentage of kisspeptin cells in the anteroventral periventricular nucleus (AVPV) and ~25% of kisspeptin cells in the arcuate nucleus, expressing GPR147 in mice (Poling et al., 2013; Rizwan et al., 2012).

Generally, RFRP-3 inhibits gonadotrophin synthesis and/or secretion across mammals, including humans (Clarke et al., 2008; George et al., 2017; Henningsen et al., 2017; Johnson et al., 2007; Kriegsfeld et al., 2006; Tsutsui and Ubuka, 2018). In some cases, however, RFRP-3 stimulates gonadotropin secretion, with differences observed based on sex, season or reproductive status (Table 1) (Figure 5 and 6). For example, in male Syrian hamsters (*Mesocricetus auratus*), RFRP-3 increases GnRH neuronal activity (i.e., increases *c-Fos* expression) and increases gonadotropin and testosterone release (Ancel et al., 2012). This

pattern differs from that observed in female Syrian hamsters, where RFRP-3 suppresses LH if administered around the time of the LH surge (Henningsen et al., 2017; Kriegsfeld et al., 2006). Similarly, in male mice (*Mus musculus*), RFRP-3 stimulates LH secretion, at least in part via actions on kisspeptin as the stimulatory effect of RFRP-3 is diminished in kisspeptin receptor knockout mice (Ancel et al., 2017). In female mice, as in Syrian hamsters, RFRP-3 inhibits LH when estradiol concentrations are high around the time of the LH surge, but is without effect during diestrus or in ovariectomized females with low estradiol concentrations when provided exogenously (Ancel et al., 2017). Finally, in male Siberian hamsters (*Phodopus sungorus*), RFRP-3 stimulates LH secretion in short-day, reproductively-inhibited hamsters, but inhibits LH secretion in long-day, reproductively-competent animals (Ubuka et al., 2012). Together, these findings confirm a role of RFRP-3 in the central control of reproduction, but its effects are dependent on species, sex, reproductive status and hormone concentrations, most likely due to the specific G-protein to which GPR147 is coupled. Surprisingly, however, GPR147/NPFFR1 female null mice exhibit moderate reproductive phenotypes with larger litter, and increased arcuate kisspeptin synthesis, higher serum FSH concentrations, and augmented LH responses to GnRH (León et al., 2014). The disparate results in the effects of GPR147/NPFFR1 inactivation and exogenous administration of RFRP-3 probably are explained by compensatory mechanisms by other RF-amide systems.

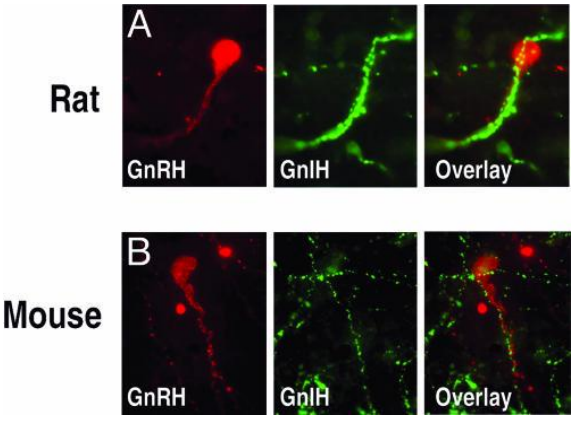

Figure 4: *GnIH fibers contact GnRH neurons in rats and mice. Images are shown as GnRH (red) alone and GnIH fibers (green) alone, followed by their respective overlays, taken at ×1,000 at the light level. Image from Kriegsfeld et al. (2006).*

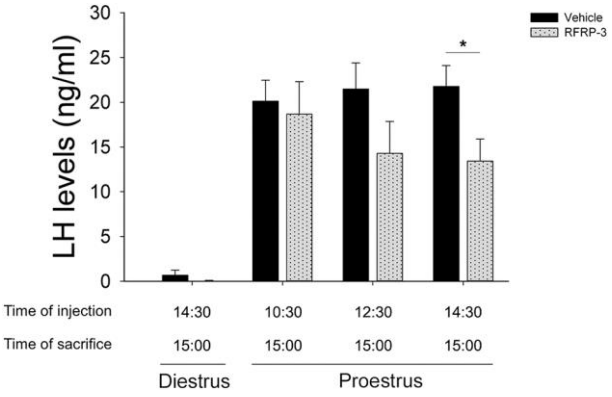

Figure 5: *Effects of intracerebroventricular RFRP-3 administration at different times of the day and estrous stages in long day-adapted female Syrian hamsters. In female hamsters, an injection of vehicle (4 µL Ringer's solution) or RFRP-3 (1500 ng in 4 µL Ringer's solution) was given in diestrus (14:30), as well as at 3 different time points on the day of proestrus (morning, 10:30; midday, 12:30; and just before the surge in LH, 14:30). Circulating LH was measured at 15:00 in diestrus and proestrus (time of the putative LH surge); data represent the mean level of LH ± standard error of mean (n = 7 in proestrus, n = 6 in diestrus); *P < 0.05 indicates a statistically significant effect of RFRP-3 when compared with vehicle. Image from (Henningsen et al., 2017).*

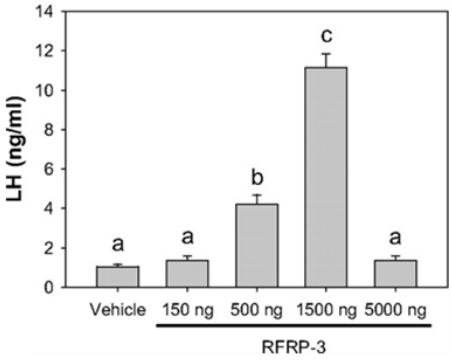

Figure 6: *Intracerebroventricular injection of RFRP-3 stimulates LH secretion in the male Syrian hamster. Centrally administered hamster RFRP-3 (150–5000 ng, icv) dose dependently increased LH secretion after 30 min. Data represent the mean ± SEM (n = 6/group). Bars with differing letters differ significantly (P < 0.05 by one way ANOVA followed by Tukey's analysis). Image from (Ancel et al., 2012).*

Table 1: Overview of the in vivo and in vitro effects of RFRP-3 on gonadotropin secretion in different species.

In vivo:

Species	Sex and status	Effect of GnIH/RFRP-3 administration	Reference
Human	Female - post-menopause	iv infusion: Inhibits LH secretion	(George et al., 2017)
Mouse	Female - Proestrus Female - Diestrus Female - OVX Female - OVX+E2	icv administration: Inhibits LH secretion No effect Inhibits LH secretion No effect	(Ancel et al., 2017)
Mouse	Male - intact/CAST	icv administration: Stimulates LH secretion	(Ancel et al., 2017)
Rat	Female - intact/OVX	icv and ip administration: Inhibits LH secretion	(Kriegsfeld et al., 2006; Pineda et al., 2010)
Rat	Male - intact/CAST	icv and ip administration: Inhibits LH secretion	(Johnson et al., 2007; Pineda et al., 2010)
Syrian Hamster	Female - OVX	icv and ip administration: Inhibits LH secretion	(Henningsen et al., 2017; Kriegsfeld et al., 2006)
Syrian Hamster	Male	icv administration: Stimulates LH secretion	(Ancel et al., 2012)
Siberian hamster	Male in LP Male in SP	icv administration: Inhibits LH secretion Stimulates LH secretion	(Ubuka et al., 2012)
Sheep	Female - OVX	iv administration: Inhibits LH secretion or no effect	(Clarke et al., 2008) (Decourt et al., 2016)
Goldfish		ip administration: Inhibits LH secretion	(Zhang et al., 2010)

In vitro:

Species	Culture	Effect of incubation with GnIH/RFRP-3	Reference
Bird	Quail anterior pituitary	Inhibits LH and FSH secretion	(Tsutsui et al., 2000)
Cow	Bovine anterior pituitary cells	Suppresses LH secretion	(Kadokawa et al., 2009)
Sheep (OVX)	Ovine pituitary cells	Inhibits LH and FSH secretion	(Clarke et al., 2008; Sari et al., 2009)

2.4 Evidence for a role of RFRP-3 in seasonal rhythms of reproduction

The marked changes in environmental factors throughout the year require species to display adaptation of their behavior and physiology to these predictive seasonal changes in order to survive. Notably, many mammalian species synchronize their reproductive activity with one particular time of the year so that depending on the duration of female gestation, offspring are born at the most favorable period of the year, usually in spring when temperature, humidity and food availability are optimal (Bronson, 1988). Thus, two categories of breeders are described depending on the mating period: long-day (LD) breeders like rodents with a few weeks of gestation and short-day (SD) breeders like sheep, goats, or deer, with a few month of gestation (Goldman, 2001).

Since the 60's, it has been known that the pineal hormone melatonin is a major signal for the synchronization of reproduction with the seasons. Indeed, melatonin synthesis and release occurs only during the night and, therefore, the nocturnal production of melatonin is longer in the short days (SD) in autumn/winter as compared to long days (LD) in spring/summer (Hastings et al., 1985). Hoffman and Reiter were the first to demonstrate that the elimination of this neuroendocrine calendar by pinealectomy abolishes the reproductive response of Syrian hamsters to the photoperiod signal (Hoffman and Reiter, 1965). It was later established through timed melatonin infusion experiments that the duration of circulating melatonin, and not its concentration or phase, is the crucial variable triggering photoperiodic adaptations in all seasonal species (Bartness et al., 1993; Goldman,

2001). Intriguingly, although the mechanism is unknown, the same photoperiodic melatonin signal has an opposite reproductive effect on LD and SD breeders.

In early studies, it was shown in seasonal quail and rodents that GnIH and RFRP-3 (Kriegsfeld et al., 2006; Tsutsui et al., 2000), respectively, are synthesized in hypothalamic neurons and are able to alter LH release, altogether indicating that this peptide may be involved in the seasonal regulation of reproduction. The first studies on quail and sparrow reported seasonal variation in GnIH synthesis that correlated with seasonal changes in reproduction (Bentley et al., 2003; Ubuka et al., 2005). Additionally, melatonin administration, in pinealectomized and enucleated (pineal gland and eyes removed to eliminate all sources of melatonin) quail, was shown to act directly on GnIH neurons to inhibit GnIH synthesis in a dose-dependent manner (Ubuka et al., 2006).

Subsequently, it was found that, in seasonal rodents, the number of RFRP-3 neurons in the dorso/ventromedial part of the DMH displayed marked photoperiodic changes (Revel et al., 2008). Indeed RFRP-3 synthesis was higher in LD-adapted, sexually active animals as compared to SD-adapted sexually inactive male Syrian and Siberian hamsters (Mason et al., 2010; Revel et al., 2008). Like in birds, although in an opposite manner, seasonal variation in RFRP-3 synthesis depends on melatonin, since pinealectomy increases and injection of melatonin decreases, the number of RFRP-3 expressing neurons in hamsters (Revel et al., 2008; Ubuka et al., 2012). Additionally, expression of GPR147 in various hypothalamic areas (Henningsen et al., 2016b) and the number of GnRH cell bodies receiving RFRP-3 fiber contacts (Smith et al., 2008; Ubuka et al., 2012) were increased in LD hamsters.

In male LD-adapted Syrian hamsters, an acute injection of RFRP-3 was found to increase LH, FSH and testosterone secretion. Furthermore, a chronic central infusion of RFRP-3 in SD-adapted, sexually inhibited male Syrian hamsters restored gonadal activity to that of hamsters kept in LD conditions (Ancel et al., 2012). Intriguingly, despite an acute inhibitory effect of RFRP-3 on the preovulatory LH surge in LD-adapted female Syrian hamsters, a chronic central infusion in sexually inactive SD-adapted females fully restored reproductive activity, as observed in male hamsters (Henningsen et al., 2017). Even more complexity was revealed following studies in closely-related male Siberian hamsters, where the effect of

RFRP3 on LH secretion depended on photoperiod, with RFRP-3 being stimulatory in SD-adapted and inhibitory in LD-adapted animals (Ubuka et al., 2012). However, administration of different doses of RFRP-3 had no effect on the reproductive status of photo-inhibited Djungarian hamsters of either sex (Cázarez-Márquez et al., 2019). In ewes, initial studies reported RFRP-3 to inhibit gonadotropin secretion (Clarke et al., 2008; Sari et al., 2009). However, a more recent study using different protocols of RFRP-3 administration could not find any effect on LH secretion in ewes (Decourt et al., 2016).

Therefore, although the melatonin-dependent photoperiodic regulation of RFRP-3 neurons is well conserved among seasonal species, the role of RFRP-3 in the seasonal regulation of reproduction is not straightforward and appears to be species dependent. Data so far, however, are insufficient to conclude whether RFRP-3 is responsible for the LD or SD breeding activity in seasonal species.

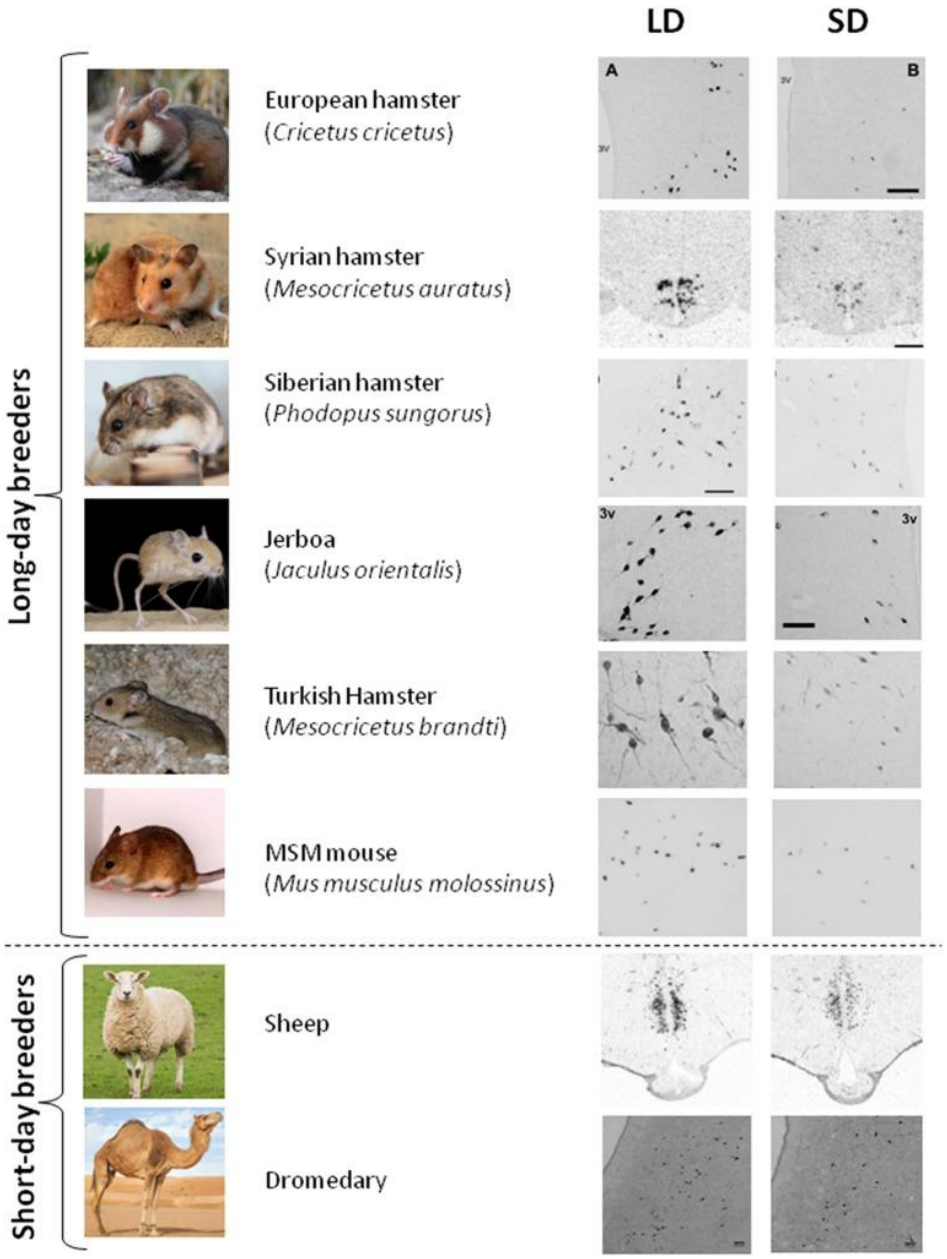

Figure 7: *RFRP-3 synthesis in the medial hypothalamus exhibits a conserved seasonal pattern. RFRP-3 expression, attested by the number of neurons or the level of Rfrp mRNA, is higher in the long-day (LD) condition as compared to the short-day condition (SD). Data are shown for LD breeders (European hamster, Syrian hamster, Siberian hamster, Jerboa, Turkish hasmter, and MSM mouse) as well as for SD breeders (sheep, dromadary). Image from:*

(Angelopoulou et al., 2019) and adapted from (Sáenz de Miera et al., 2014) (European hamster), (Revel et al., 2008) (Syrian hamster), (Talbi et al., 2016a) (Jerboa),(Piekarski et al., 2014) (Turkish hamster), (Miera et al., 2020) (MSM mouse), (Lomet et al., 2018)(sheep), (Ainani et al., 2020) (Dromedary), with appropriate permissions obtained from the copyright holders.

2.5 Potential roles of RFRP-3 in the pituitary and gonads

In addition to its actions on GnRH neurons, RFRP-3 may alter gonadotropin synthesis and secretion also via the pituitary, although findings are disparate across studies and species. For example, RFRP-3 projections have been shown to project to the outer layer of the median eminence [hamsters (Gibson et al., 2008), sheep (Clarke et al., 2008), macaque (Ubuka et al., 2009a), and humans (Ubuka et al., 2009b)]. In contrast, using peripheral injections of fluorogold to label hypophysiotropic cells, RFRP-3 cells were not labeled in rats (Rizwan et al., 2009). In other studies, RFRP-3 terminal fibers in the median eminence are sparse or absent [mice (Ukena and Tsutsui, 2001); brushtail possum (Harbid et al., 2013); macaque (Smith et al., 2010)]. Although results are thus equivocal regarding projections to the median eminence across species, GPR147 is expressed in the pituitary of hamsters (Gibson et al., 2008) and humans (Ubuka et al., 2009b) and RFRP-3 has been shown to inhibit gonadotropin release in cultured pituitaries from sheep (Sari et al., 2009), cattle (Kadokawa et al., 2009), and rat (Pineda et al., 2010). In ewes, RFRP-3 is detected in hypophyseal portal blood and exogenous RFRP-3 has been reported to significantly reduce the GnRH-induced LH response (Smith et al., 2012). In another study, however, peripheral administration of RFRP-3 in ewes was unable to inhibit pulsatile LH secretion or the E2-induced LH surge (Decourt et al., 2016), raising the question of whether or not RFRP-3 acts on pituitary gonadotropes despite being detectable in portal blood.

In addition to potential actions at the level of the pituitary, RFRP-3 also appears to be produced and act locally at other places, to regulate gonadal function. Early work discovered that GnIH is synthesized in ovarian granulosa cells and in the testicular interstitial layer and seminiferous tubules of birds (Bentley et al., 2008). Moreover, in birds, GnIH application decreases testosterone release from gonadotropin-stimulated testes *in*

vitro, pointing to a functional role for gonadal GnIH (McGuire et al., 2011). Later, it was shown that RFRP-3 is synthesized in the gonads of all mammals studied to date (Bentley et al., 2017), including humans (Oishi et al., 2012), non-human primates (McGuire and Bentley, 2010), Syrian hamsters (Zhao et al., 2010), mice (Oishi et al., 2012; Singh et al., 2011), rats (T et al., 2015), ewe (Li et al., 2014), and pigs (Fang et al., 2014). Across species, the gonads synthesize RFRP-3 and GPR147 (Bentley et al., 2017, 2008; McGuire and Bentley, 2010; Oishi et al., 2012; Singh et al., 2011). In mice, testicular RFRP-3 synthesis increases during reproductive senescence, possibly contributing to aging-related decrements in testicular functioning (Anjum et al., 2012). In human granulosa cell cultures, RFRP-3 inhibits gonadotropin-induced intracellular cAMP accumulation and progesterone secretion (Oishi et al., 2012). Finally, RFRP-3 and GPR147 are synthesized in ovarian granulosa cells and antral follicles during proestrus and estrus and in luteal cells during diestrus in mice (Singh et al., 2011), suggesting participation in follicular development and atresia. Together, these findings suggest that GnIH/RFRP-3 is commonly synthesized in the gonads across species and may act locally to fine-tune gonadotropin-regulated gonadal functioning.

2.6 Potential role of RFRP-3 in reproduction through metabolic activity and stress regulation

Although RFRP-3 is consistently reported to regulate reproductive axis function, the effect on GnRH neuronal activity and gonadotropin secretion is highly dependent on species, sex and environmental conditions (Ancel et al., 2017; Henningsen et al., 2016b). Determining the exact mechanism of RFRP-3 action is further complicated by increasing evidence indicating that RFRP-3 is a pleiotropic peptide involved in functions other than reproduction, notably metabolic activity and the stress response (Kriegsfeld et al., 2018; Schneider et al., 2017; Takayanagi and Onaka, 2010). Because reproduction is modulated by energy state and stress conditions, it is possible that RFRP-3, at least in part, indirectly regulates reproduction via metabolic- and stress-regulated mechanisms. Food intake and metabolic activity, for example, display major circadian and seasonal changes in mammals, which may interfere with reproductive cycles. Indeed, metabolic alterations such as food

restriction or obesity are known to impair reproduction. As RFRP-3 increases food intake in various species, possibly through actions on orexigenic NPY neurons (Cázarez-Márquez et al., 2020; Johnson et al., 2007; Talbi et al., 2016b), and food restriction decreases RFRP-3 synthesis in rats and sheep (Li et al., 2014; M et al., 2014), it is possible that RFRP-3 may also impact reproductive activity indirectly via metabolic pathways (Wahab et al., 2015). Likewise, a number of studies report that acute or chronic stress increases RFRP-3 synthesis via increased levels of glucocorticoids (Clarke et al., 2016; Kirby et al., 2009; Yang et al., 2017) and this stress-induced increase in RFRP-3 is associated with an inhibition of LH secretion (Kirby et al., 2009). Finally, *Rfrp* gene silencing completely rescues stress-induced infertility in female rats (Geraghty et al., 2015), strengthening the implication that stress can influence reproductive function via the RFRP system. In summary, although there is much more to learn, findings to date provided clear evidence for a role for RFRP-3 in the daily and seasonal regulation of reproduction. Whether RFRP-3 effectuates its influence through direct actions on the reproductive axis, and/or indirectly via actions on intermediate systems (e.g., stress or metabolic systems), requires further examination. The advent and application of new experimental tools and animal models to more precisely dissect the roles of this neuropeptide will help to further clarify the specific role of RFRP-3 in the LH surge/ovulation and the neural pathways by which melatonin inevitably influences RFRP3 cell activity.

3. RFRP-3 contributes to the daily rhythm of reproduction in female rodents

3.1 Daily and ovarian rhythms in female reproduction

Successful female reproduction requires the activation of specific neuronal and hormonal pathways in order to synchronize ovulation with maximal locomotor activity and an optimal arousal state. Female mammals display rhythms of different, recurrent time scales that range from minutes (pulsatile GnRH release) to hours/days (LH surge), days/weeks (ovarian cycle) or even months (seasonal reproduction).

Ovarian activity displays regular cycles (~28 days in women and 4–5 days in rodents) driven by changes in circulating levels of the pituitary gonadotropins LH and FSH. During the first stage of the ovulatory cycle (follicular phase in humans, metestrus-diestrus in rodents), FSH secretion gradually increases, promoting ovarian follicular development. In turn, maturing follicles secrete increasing concentrations of E2. The second stage of the reproductive cycle (luteal phase in women; proestrus-estrous in rodents) is immediately preceded by a pronounced and transient rise in LH secretion (surge) that initiates the release of mature oocyte(s) from ovarian follicles (Figure 8). The generation of the LH surge requires high circulating levels of E2, indicative of follicle maturation, as well as a daily signal, ensuring that ovulation occurs at the right arousal time to optimize breeding success. Indeed, the LH surge occurs at a specific time of day, corresponding to the end of the inactive phase, thus in late afternoon in nocturnal rodents (e.g., mice, rats, hamsters) and early morning in diurnal species (e.g., Nile grass rat, humans) (Kerdelhué et al., 2002; Simonneaux and Bahougne, 2015).

Exploring the pathways by which the circadian clock synchronizes GnRH neuronal activity and upstream modulatory systems is essential to fully understand the mechanisms of female reproduction. Indeed, circadian disruption has been associated with various abnormalities in fertility and reproduction. Early studies in the 50's demonstrated that chemical blocking of neural clock output alters the LH surge in female rats (Everett and Sawyer, 1950, 1949) and hamsters (Stetson and Watson-Whitmyre, 1977). Furthermore, SCN lesions cause anovulation in female rats, presumably resulting from the loss of diurnal

variation in the sensitivity of the reproductive axis to E2 positive feedback (Brown-Grant and Raisman, 1977) and stimulatory input from the SCN (Palm et al., 1999). Indeed, female mice deficient for the clock gene, *Clock*, exhibit abnormal estrous cycles, do not have a detectable LH surge on the day of proestrus, and generally fail to carry pregnancies to term (Miller et al., 2004). Similarly, women with single-nucleotide polymorphisms in the circadian clock gene *ARNTL* exhibit more miscarriages than those without such mutation (Kovanen et al., 2010).

It appears that the circadian signal is sent to the reproductive system each day, but its impact is masked by low circulating E2. Thus, in female rodents provided with chronic, proestrus-like concentrations of E2, daily LH surges are observed for several consecutive days, revealing the circadian mechanism underlying surge generation (Christian et al., 2005; Legan and Karsch, 1975; Norman et al., 1973) (Figure 5). Altogether, these findings, largely obtained in female rodents, indicate that the timing of the preovulatory LH surge is strictly time-gated by a combination of daily and ovarian signals. Although the daily signal is communicated each day by the SCN to the GnRH/LH pathway, E2 secretion from mature oocytes needs to reach a certain threshold in order to exert positive feedback on the HPG axis and allow the generation of the LH surge.

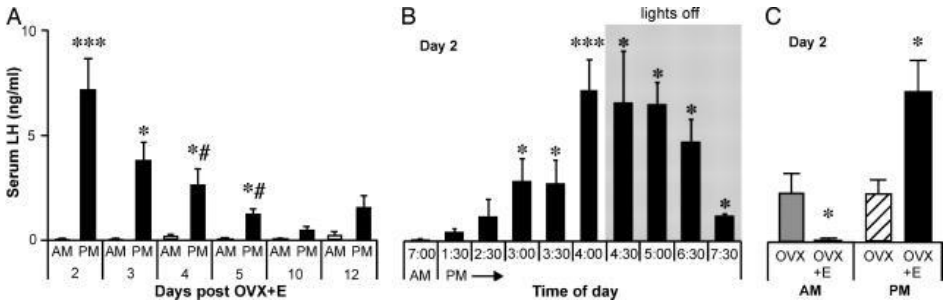

Figure 8: *Induction of daily LH surges by estradiol in mice. (A) Bars represent serum LH concentrations (mean ± SEM) with open bars showing samples obtained at 7 a.m., and filled bars showing samples obtained at 4 p.m., from 2 to 12 days after mice were ovariectomized with an estradiol implant (OVX+E). (B) Serum LH levels (mean ± SEM) sampled in OVX+E mice at various times on day 2 after OVX+E. Gray shading indicates time during which lights were off. LH surge reliably begins ≈1.5 h before lights off (4:30 p.m.). (C) Serum LH levels show no diurnal*

*difference in OVX mice, and estradiol induces negative feedback in the a.m. and positive feedback in the p.m. A, a.m. same-day control; B, 7 a.m. control; C, OVX control. *, P < 0.05; ***, P < 0.001; #, P < 0.01 vs. day 2 p.m. Image from: (Christian et al., 2005).*

3.2 Mechanisms regulating the circadian-estrogen sensitive preovulatory LH surge

Both two principal SCN neurotransmitters, VIP and AVP, are thought to be implicated in relaying daily cues to GnRH neurons and therefore controlling the timing of the preovulatory LH surge. VIP content in the rat SCN displays daily variation, decreasing during the light period and increasing during the dark period. This daily variation is abolished under constant darkness, suggesting that VIP is implicated in the transmission of photic information (Shinohara et al., 1993). Furthermore, the daily rhythm of VIP in the SCN appears sex-dependent since *VIP* mRNA levels peak during the light phase in female rats, but during the dark phase in male rats (Krajnak et al., 1998a). The observation that a central blockade of VIP signaling decreases the LH surge in female rats indicates a role of this peptide in female reproduction (Harney et al., 1996; van der Beek et al., 1999). Indeed, ~45% of the GnRH cells are innervated by VIP-containing fiber terminals and unilateral thermal lesions of the majority of VIP cells in the SCN results in a 50% decrease of VIP nerve contacts on GnRH cell bodies on the lesioned side, compared to the intact side of the brain (van der Beek et al., 1993). Furthermore, the use of anterograde tracing demonstrated a direct connection between the SCN and GnRH neurons (Van der Beek et al., 1997). Interestingly, there is a sex-dependent difference in the VIP-GnRH pathway, with the number of VIP terminals onto GnRH neurons, and the percentage of GnRH neurons contacted by VIP fibers, being higher in females compared to males (Horvath et al., 1998). About 40% of GnRH neurons express the VIP2 receptor (Smith et al., 2000) and exogenous VIP application to brain slices increases GnRH neuron action potential firing and intracellular calcium (Christian and Moenter, 2008; Piet et al., 2016), supporting the idea that VIP may provide a direct excitatory signal from the SCN to the GnRH system.

AVP expression exhibits both daily and circadian variation in the SCN, peaking during the latter part of the light period and dropping during the dark period (Cagampang et al., 1994). AVP release in the SCN vicinity has been found to peak during midday, while minimum release occurs at midnight (Kalsbeek et al., 1995). Unlike VIP, no sex-dependent differences in *AVP* gene expression are found in the SCN (Krajnak et al., 1998a). Increasing evidence indicates that the rhythm in SCN AVP release is critical for the daily timing of the preovulatory LH surge. Indeed, central administration of AVP in OVX, E2-treated rats, bearing complete SCN lesions, is sufficient to trigger a LH surge (Palm et al., 1999). However, the ability of AVP to trigger the surge is time-dependent, with administration during the latter half of the light period, but not the first half, being effective (Palm et al., 2001). Moreover, central administration of a V1a receptor antagonist decreases LH surge amplitude in rats (Funabashi et al., 1999). Finally, in *Clock* mutant female mice, central injections of AVP can restore a preovulatory-like LH surge (Miller et al., 2006). Unlike VIP, SCN AVP neurons appear to regulate the GnRH/LH surge indirectly via kisspeptin neurons located in the preoptic area (AVPV in rodents), a highly sex-dimorphic brain area (Adachi et al., 2007; Smith et al., 2006). Thus, in female rodents, AVPV kisspeptin neurons receive direct SCN-derived AVP inputs and express the V1a receptors (Vida et al., 2010; Williams et al., 2011), and direct application of AVP to brain slices increases neuronal firing and intracellular calcium concentrations in AVPV kisspeptin cells (Piet et al., 2015). Importantly, AVPV kisspeptin neurons display ERα, and E2 not only potently stimulates kisspeptin synthesis (Adachi et al., 2007; Smith et al., 2006, 2005a), but is also required for the AVP-induced activation of kisspeptin cells (Piet et al., 2015). Finally, activation of AVPV kisspeptin neurons coincides with the time of LH surge, during the sleep/wake transition in proestrus or in OVX E2-treated female rodents, but does not display daily rhythms during diestrus or in OVX animals (Chassard et al., 2015; Henningsen et al., 2017; Robertson et al., 2009; Williams et al., 2011).

Therefore, data primarily obtained in female rodents indicate that both SCN-derived VIP fibers acting directly on GnRH neurons, and AVP fibers acting indirectly via preoptic kisspeptin neurons, are involved in the timing of the preovulatory LH surge. In addition to

this mechanism of surge control, RFRP-3 neurons may also be part of the pathway relaying daily time cues from the SCN to GnRH neurons in order to time the preovulatory LH surge, as described further below.

3.3 Evidence for a role of RFRP-3 neurons in the daily timing of the LH surge

The hypothesis for a role of RFRP-3 neurons in the daily timing of the LH surge begins with the observation of a daily rhythm in RFRP-3 neuronal activity, with a lower number of RFRP-3 neurons expressing c-FOS coincident with the timing of the LH surge in female Syrian hamsters (Gibson et al., 2008; Henningsen et al., 2017) and mice (Poling et al., 2017). Equivocal findings are reported regarding the association between the RFRP-3 cell activation state and the number of *Rfrp* expressing neurons, with daily variation in RFRP-3 neuronal activity being associated (Gibson et al., 2008) or not (Henningsen et al., 2017; Poling et al., 2017), with corresponding changes in the number of *Rfrp* expressing cells. In ewes, *Rfrp* expression is decreased during the preovulatory period, but no activation data were reported (Clarke et al., 2012). The role of RFRP-3 neurons in relaying circadian information to GnRH neurons is further supported by an experimental protocol where female hamsters kept under constant light conditions split their locomotor activity and exhibit two daily LH surges. In these conditions, the left and right SCN oscillate in antiphase and RFRP-3 neurons are active asymmetrically in opposition to GnRH neuron activation (Gibson et al., 2008).

A recent study in female Syrian hamster demonstrated that AVP- and VIP-ergic fibers from the SCN form close appositions with RFRP-3 neurons and that a central injection of VIP decreases RFRP-3 neuronal activity in a time-dependent manner, being effective in the afternoon, but not in the morning, while central AVP had no significant effect (Russo et al., 2015). It is yet unclear, however, whether the action of VIP on RFRP-3 neurons is direct or indirect, since <10% of RFRP-3 neurons appear to express the *VPAC1* or *VPAC2* receptors (Russo et al., 2015). Altogether, these findings suggest a SCN-derived VIP daily regulation of RFRP-3 neuronal activity, at least in Syrian hamsters. Additionally, there is evidence in

female rodents that RFRP-3 neurons, similar to kisspeptin neurons (Chassard et al., 2015), are able to keep track of time intrinsically, expressing the clock protein PER1 with a peak at ZT12 (Russo et al., 2015).

Unlike kisspeptin cells, it is likely that high circulating levels of E2 are not required for the daily rhythm in RFRP-3 neurons as daily rhythms in RFRP-3/c-FOS are similar during diestrus and proestrus in Syrian hamsters (Henningsen et al., 2017). Although another report indicates that daily variation is abolished in OVX hamsters and restored in OXV+E2 animals (Gibson et al., 2008), in this study different time points were investigated and a different protocol was used, which might account for the disparity between both findings.

A number of studies are consistent with an inhibitory action of RFRP-3 on LH secretion in female mammals (Anderson et al., 2009; Kriegsfeld et al., 2006). In Syrian hamsters (Henningsen et al., 2017) and mice (Ancel et al., 2017), central RFRP administration decreases LH secretion when given around the time of the preovulatory LH surge, whereas it has no effect when given at other time points when LH secretion is low (early day of proestrus or diestrus). Therefore, decreased activity of RFRP-3 neurons in late afternoon, possibly mediated by an SCN VIP-ergic signal, associated with the inhibitory effect of RFRP-3 on LH secretion, indicates that tonic RFRP-3 inhibitory input is lifted at the time of the preovulatory LH surge (Figure 9).

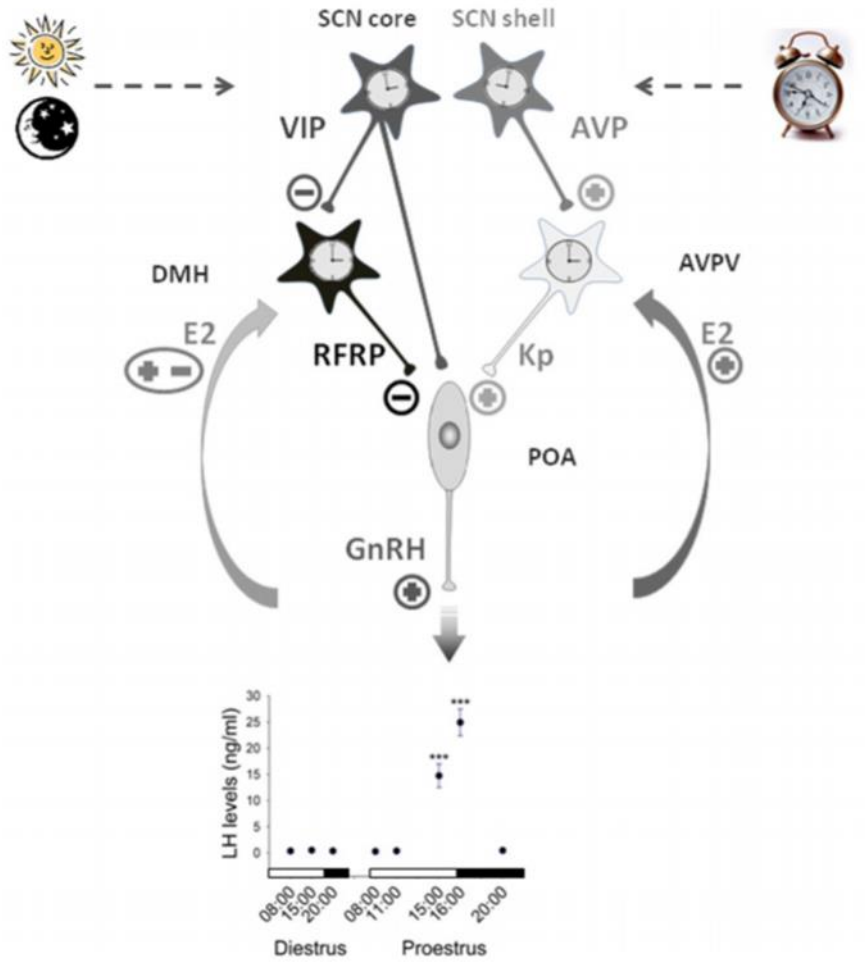

Figure 9: *Working model illustrating the contribution of RFRP-3 neurons in the central control of the daily gating of the preovulatory LH surge in female rodents. Neurons of the suprachiasmatic nuclei (SCN) synthesizing vasopressin (AVP) and vasoactive intestinal peptide (VIP) exhibit daily variation controlled by an intrinsic circadian clock and the daily change in light input. The SCN VIP output times the activity of GnRH neurons either directly and/or indirectly via the RFRP3 neurons located in the dorsomedial hypothalamus (DMH), which further inhibit GnRH neurons at the light/dark transition. The SCN AVP output activates GnRH neurons through the stimulation of neurons located in the anteroventral periventricular nuclei (AVPV) and releasing the potent stimulatory peptide kisspeptin. Additionally, kisspeptin neurons receive a positive estradiol (E2) feedback on the day of proestrus while the effect of E2 on RFRP3 neurons is still unclear. This coordinated pathway is proposed to trigger a preovulatory GnRH/LH surge at the light/dark transition of the proestrus stage. Image from (Angelopoulou et al., 2019).*

3.4 The controversy of E2 feedback on RFRP-3 neurons

The possibility that RFRP-3 neurons, similar to kisspeptin neurons (Simonneaux, 2020), may be a central site for the E2 feedback has been widely studied. However, the results obtained in different species, sex and conditions are conflicting.

ERα are found in 40% and 25% of RFRP-3 neurons in female Syrian hamsters (Kriegsfeld et al., 2006) and mice (Molnár et al., 2011; Poling et al., 2012), respectively. Studies have reported that E2 treatment in OVX Syrian hamsters increases c-FOS expression in RFRP-3 neurons (Kriegsfeld et al., 2006) while others, in contrast, show that E2 treatment decreases the amount of *Rfrp* mRNA per cell and the total amount of *Rfrp* mRNA in both male and female mice (Poling et al., 2012). In female rats, RFRP-3 neuronal activity is reported to be higher during diestrus compared to proestrus and estrous (Jørgensen et al., 2014), suggesting a role for E2 in the activational state of RFRP-3 cells across the ovulatory cycle in this species. Finally, in female rats (Quennell et al., 2010), male (Revel et al., 2008) and female Syrian hamsters (Henningsen et al., 2017), and male Djungarian hamsters (Rasri-Klosen et al., 2017), gonadectomy with/without sex steroid replacement does not have a significant effect on RFRP-3 synthesis.

On the other hand, other experimental paradigms do (indirectly) suggest a possible influence of E2 on RFRP-3 neurons. For example, E2 treatment increases RFRP-3 synthesis in the hypothalamic mHypoA-55 rat cell line (Tumurbaatar et al., 2018). In Syrian hamsters, food-restriction increases the percentage of RFRP-3 cells expressing *c-Fos*, with increased ovarian steroids at the time of estrus abolishing the impact of food restriction on RFRP-3 cellular activation (Benton et al., 2018). Finally, in female rats, RFRP-3 synthesis varies according to reproductive stage, with increased levels at the time of puberty when the endogenous sex steroid levels are highest (Quennell et al., 2010).

3.5 Concluding remarks on the role of RFRP-3 in the daily timing of the LH surge in females

Female reproduction is cyclic and in female mammals, possibly including women although this is still controversial, daily time cues are integrated within the reproductive system to coordinate the LH surge and consequential ovulation with the best period of the day. The hypothalamic SCN clock plays a key role in conveying daily information to the reproductive system, and increasing evidence indicates that RFRP-3 neurons, in addition to kisspeptin neurons, are a key relay between the SCN clock and GnRH neurons. Recent data indicate that the SCN-derived VIP output drives RFRP-3 neuronal activity, but the mechanisms involved are still unclear. Furthermore, while numerous studies now agree on the critical role of kisspeptin in the timing of LH surge, the specific significance of RFRP-3 on the occurrence of the LH surge requires further investigation.

4. The issue of altered reproduction in circadian disruption

4.1 Concept of shiftwork

The modern 24 h-functioning society requires an increasing number of employees to work outside of the natural active period. According to the International Labor Organization (ILO; 1990), working in shifts is “a method of organization of working time in which workers succeed one another at the workplace so that the establishment can operate longer than the hours of work of individual workers”. Shift work and night work cover a multitude of realities: different time systems called 2 × 9, 3 × 8, 4 × 8, 5 × 8, 2 × 12 h, with variability resulting from different choices made by the employer's company.

In industrial countries, 20–30% men and 15–20% women experience shift work or work at night (Pati et al., 2001), and this is an expanding phenomenon with a particularly significant increase among women under 30 years. One difficulty to classify when work is done in shifted conditions comes from variable definitions of shift/night work, even within the European Union. Thus, in France, night work is defined as any work between 9 pm and 6 am; in Germany it is 2 h of the daily work between 11 pm and 6 am; in Italy it is a minimum of 7 consecutive hours including the timeframe between 0 am and 5 am; in Belgium it is work performed between 8 pm and 6 am; and in the United Kingdom, it is 3 h of the daily work between 11 pm and 6 am. Moreover, shift work can be defined by a number of periods, duration of the periods, shift structure (continuous or not), start and end time of work, and time between shifts.

4.2 Impact of shift work on health

An increasing number of studies report that shift work or night work is associated with increased risks of developing cardiovascular/metabolic/gastro-intestinal disorders, some types of cancer, and mental disorders including depression and anxiety (Boivin et al., 2007; Chen et al., 2010; Matheson et al., 2014). In 2007 shiftwork was reclassified from a possible to a probable human carcinogen (class 2A) by the International Agency for Research on Cancer.

Bøggild and Knutsson, 1999, who analyzed 17 studies (between 1949 and 1998), evaluated the excess risk at 40% for ischemic heart disease in shift/night workers compared to day workers (relative risk was ranging from 0.4 to 3.6, with a majority between 1 and 2). Ten years later, Frost et al. published a new review (from 16 epidemiological studies done between 1972 and 2008), which reported limited epidemiological evidence for a correlation between shift/night work and ischemic heart disease (Frost et al., 2009). More recently, a large meta-analysis (34 studies published between 1983 and 2011, including more than two million people) indicated that shift/night work is associated with a significant increase in myocardial infarction and coronary events with or without adjustment for other risk factors (Vyas et al., 2012). Since then, four other epidemiological studies have indicated an increased risk of coronary events and cardiovascular disease mortality after 5 years of shift/night work (Carreón et al., 2014; Gu et al., 2015; Hermansson et al., 2015; Park et al., 2015). Also, a causal link between shift/night work and weight gain/high body mass index is often reported, notably after 5 years, suggesting that shift/night work is a risk for type 2 diabetes (Pan et al., 2011). Indeed, a retrospective study on 6413 male shift/night workers showed an increased risk of impaired glucose tolerance (even for workers with normal and stable body weight) compared to day workers (Kubo et al., 2010). Similarly, a recent meta-analysis reported an increased risk of 1.09 between shift/night work and type 2 diabetes (Gan et al., 2015). Shift/night work is also often associated with chronic stress and a significant impact on cortisol (in humans) or corticosterone (in rodents) is well documented (Goichot et al., 1998; Gumenyuk et al., 2014; Kiessling et al., 2010; Manenschijn et al., 2011; Ulhôa et al., 2015; Weibel and Brandenberger, 2002). This is important because glucocorticoids play a major role in the circadian resynchronization of the central and peripheral clocks in a chronic jet-lag context (Kiessling et al., 2010).

Given the importance of the circadian system in the regulation of female reproduction, and given the fetal exposure to the maternal daily rhythms in temperature, hormones and metabolic cues, female shift workers may display reproductive dysregulations. Indeed a few studies have reported increased risk of irregular menstrual cycles, endometriosis, miscarriage, low birth weight or pre-term delivery in women in shift/night work conditions

(Gamble et al., 2013; Lawson et al., 2011; Rocheleau et al., 2012). Notably, an animal study showed that maternal circadian disruption during pregnancy may lead to fetal SCN clock desynchronization (Nováková et al., 2010), in accordance with the well-known fact that the functioning of fetal clocks depends on maternal hormones (Serón-Ferré et al., 2012; Torres-Farfan et al., 2011).

4.3 Modeling shift work in rodents

In order to better understand the mechanisms underlying the negative impact of shift/night work on health, it is necessary to develop a relevant animal model of circadian disruption. However, shift work is a very complex situation and therefore it is difficult to design animal model conditions that truly mimic human shift work, which is often associated with potential confounding factors (diet, social stress, sleep disturbance, use of psychostimulants). Furthermore, most studies are carried out on nocturnal animals (rats, mice, hamsters), while humans are diurnal. Apart from melatonin, whose secretion is always highest during the dark period, other hormones (cortisol/corticosterone, glucose, leptin, gonadotropins) and many biological functions (food intake, sleep/wake, cardiac functions, vigilance) have opposite rhythms between diurnal and nocturnal species. Moreover, for most of these studies only males are used to avoid an effect of the female reproductive cycles in the measurement of the circadian disturbances. Yet, animal studies are essential for understanding the cellular and molecular mechanisms underlying circadian perturbations. A recent review listed four relevant models that use altered timing of either food intake, activity, sleep or light exposure, or a combination of these (Opperhuizen et al., 2015).

Regarding female reproduction, very few animal studies have investigated alterations in fertility or LH surge timing after a shift in the light/dark cycle or photoperiod. One study in female Syrian hamsters reported that after a 3 h phase advance, the LH surge is not fully resynchronized to the new dark onset even after 3 days, but when they are submitted to a 3 h phase delay, the LH surge is synchronized more rapidly (Moline and Albers, 1988).

Furthermore, photoperiod lengthening was associated with similar shifts in locomotor activity and the LH surge in female hamsters (Moline et al., 1981). In mice, exposure to either regular phase advances or delays at the beginning and throughout pregnancy resulted in a significant decrease in pregnancy success (Summa et al., 2012) . Finally, an *in vitro* study reported that the ovarian clock was not fully resynchronized 6 days after a 6 h phase advance in PER2:LUCIFERASE mice (Yamazaki et al., 2000). Thus, despite the extensive research on the impact of circadian disruptions, the negative effects on the reproductive system have not been fully explored yet.

4.4 Concluding remarks on the effect of circadian disruption on reproduction

The female reproductive system displays changes in hormone secretion and ovulation in a cyclical and circadian manner. Although only few studies have been performed, both epidemiological investigations and animal studies indicate that circadian disruption, observed when the light/dark cycle is acutely (jet-lag) or chronically (shift work) shifted, may impair the timing of the reproductive cycles. Clearly, more investigations are required in order to determine whether and, if yes, how disruptions in the endogenous circadian timing system underlie reproductive deficiency.

5. Neuroendocrine control of reproductive senescence

5.1 Reproductive aging in females

In female mammals, reproductive activity encompasses three defined periods throughout development, the pre-pubertal period, the fertile period, and the post-menopausal unfertile period. In healthy women, puberty starts around 11 and 13 years, and menopause occurs between 45 and 53 years (Barros et al., 2019; Gold, 2011). Postponing childbirth until after the age of 35 has become a complex socio-economic phenomenon, which is increasingly evident in the last decades (Lampinen et al., 2009). Advanced maternal age is associated with a higher risk of miscarriage, preterm birth and genetic disorders of the foetus (Newburn-Cook and Onyskiw, 2005; Schmidt et al., 2012). Therefore, identifying the sequence of events preceding menopause and the mechanisms coordinating these events is of outmost importance.

In women, reproductive senescence is associated with exhaustion of primary follicles and loss of fecundity. Menopausal transition is characterized by menstrual cycle variability, wide fluctuations in reproductive hormones and eventually permanent loss of menstruation at the average age of 51 years (Santoro, 2005). In contrast, female rodents do not undergo exhaustion of the follicular pool (Mandl and Shelton, 1959), although they do demonstrate progressively irregular ovarian cycles. Reproductive aging in female rodents is marked by the onset of longer irregular estrous cycles (> 4-6 days) at the age of 8-12 months, followed by a period of constant estrous (CE) or persistent vaginal cornification at the age of 10-16 months. CE period is followed by a prolonged diestrus phase with intermittent ovulation known as repetitive pseudo-pregnancy (RPP), before reaching the anestrous stage at the age of 22-25 months (Cruz et al., 2017).

Female mammals exhibit age-dependent changes in the neuroendocrine mechanisms that control reproduction. In humans, menopausal transition is characterized by huge swings in estradiol and gonadotropin levels before reaching post menopause (Hall, 2004). The aging ovary stops responding to normal FSH signals and ceases to produce adequate levels of estrogen and progesterone, which serve as down-regulatory signals to the hypothalamus

and the pituitary. Thus, perimenopausal women exhibit increased FSH production followed by increased LH secretion, both of which are markers of reduced fertility (Fitzgerald et al., 1998). Similar to women, rodents display increased gonadotropin secretion at advanced ages (Belisle et al., 1990), even though they do not undergo depletion of the follicular pool and therefore maintain high estrogen levels (Chakraborty and Gore, 2004; Mandl and Shelton, 1959). While rodent ovarian activity progressively deteriorates (Cruz et al., 2017), the timing of the preovulatory LH surge is delayed and exhibits reduced amplitude during middle age (Nelson et al., 1982)). Despite the differences in the circulating levels of estrogen between women and female rodents during aging, the use of non-human animal models offers certain advantages in deciphering the mechanisms of reproductive decline; since the rodent HPG axis is highly conserved and estropause closely assimilates perimenopausal transition (Kermath and Gore, 2012).

5.2 Primary role of the aging hypothalamus in the induction of reproductive decline

Although for many years menopause has been attributed to ovarian failure due to the exhaustion of primary follicles, an alternative perspective is that menopausal transition is initiated by age-related alterations in the central nervous system; notably in the hypothalamus and the pituitary. However, given the extent of interactions and feedback loops between the different levels of the HPG axis, determining their relative contributions to the induction of reproductive senescence is complicated (Rubin, 2000).

Age-related alterations in pituitary physiology have been associated with reproductive decline. Epidemiological studies demonstrated that the pituitary volume decreases with age (Grams et al., 2010; Lurie et al., 1990), while the amount of unoccupied space in the pituitary fossa increases (Pecina et al., 2017). Middle-aged rodents that exhibit attenuated LH surges show decreased pituitary LH responsiveness to GnRH stimulation (Brito et al., 1994; Krieg et al., 1995). Pituitary gene expression of the gonadotropin subunits and GnRH receptors

also decreases in middle-aged rodents, with no significant difference in the gene expression of the ER and PR steroid hormone receptors (Zheng et al., 2007).

Despite the well-characterized changes in the aging ovary and pituitary, multiple studies suggest that age-related alterations in the hypothalamus precede the onset of reproductive decline. A recent epigenome-wide study demonstrated that global hypothalamic DNA methylation decreases during aging and identified changes in DNA methylation in genes encoding hormone signaling, glutamate signaling, melatonin and circadian pathways (Bacon et al., 2019). Transplantation studies showed that old rodent ovaries exhibit cyclic activity when transplanted in young females. However, young ovaries transplanted in old anestrous rodents cannot maintain regular cyclic activity (Peng and Huang, 1972). Interestingly, electrical stimulation of the hypothalamus successfully induces ovulation in old acyclic rodents (Clemens et al., 1969). A pharmacological study showed that drug administration that corrects hypothalamic deficiencies, temporarily restores estrous cyclicity in middle-aged rodents (Quadri et al., 1973).

Hypothalamic GnRH neurons, the driving force of the reproductive axis, undergo changes during senescence as well. Middle-aged rodents display a decreased number of GnRH cells and GnRH neuronal activity during the preovulatory GnRH/LH surge (Funabashi and Kimura, 1995; Lloyd et al., 1994; Miller et al., 1990; Yin et al., 2009). Whether these changes in the GnRH system are intrinsic or due to age-dependent alterations in the neural circuits that regulate GnRH activity, has not been deciphered yet.

Kisspeptin (Kp), one of the main stimulators of the GnRH system, also undergoes age-dependent changes. Postmenopausal women display an increased number and size of Kp neurons and expression of the Kp encoding gene, *Kiss1*, in the infundibular nucleus (Rometo et al., 2007). Interestingly, these phenotypes resemble the ones observed in ovariectomized primates (Eghlidi et al., 2010; Kim et al., 2009; Rometo et al., 2007). In middle-aged rodents by contrast, a decreased number of Kp cells and reduced levels of *Kiss1* mRNA expression in the AVPV during the preovulatory LH surge are observed (Lederman et al., 2010; Neal-Perry et al., 2009). Central administration of Kp in the POA restores the attenuated amplitude of the LH surge in middle-aged rodents (Neal-Perry et al., 2009). Therefore, age-

related alterations in the GnRH system may be driven in part by altered Kp signaling, along with changes in other regulatory systems extrinsic to GnRH neurons. Notably, given the reported action of RFRP-3 on the GnRH system, it is worth examining whether ageing has an impact on the RFRP-3 system.

5.3 Age-dependent alterations in the circadian system

During aging, the circadian regulation of many physiological and behavioral processes is progressively disturbed. Age-dependent alterations in circadian rhythms include decreased amplitude and period length, increased fragmentation and tendency to desynchronization (Carskadon et al., 1982; Martin et al., 1986; Rs et al., 1991; Shibata et al., 1994; van Gool et al., 1987). Earlier studies demonstrated that transplantation of fetal SCN tissue can restore age-related deficits in the circadian system (Cai and Wise, 1996; Van Reeth et al., 1994). Therefore, circadian disruptions during senescence have been associated with impairment in SCN function. Numerous studies examined the components of the SCN that could be affected by aging; including the input pathways to the SCN, the SCN molecular clock, the electrical properties of SCN neurons and the output pathways of the SCN towards the periphery (Buijink and Michel, 2020).

During aging the ability of the SCN to be entrained by light is compromised, due to major changes in the light transduction pathway towards the SCN (Lupi et al., 2012; Sutin et al., 1993). Notably, advanced age is associated with a loss in the number, density and dendritic arborization of the ipRGCs (Esquiva et al., 2017; Lax et al., 2019). Despite the well-characterized deficits in the light input pathway towards the SCN, multiple studies demonstrated that the SCN molecular clockwork is preserved during aging (Asai et al., 2001; Nakamura et al., 2011; Polidarová et al., 2017; Yamazaki et al., 2002). However, findings in the molecular clock components are not always consistent. Of note, circadian expression profiles of *Per1* and *Cry1* mRNA are maintained in the senescent SCN (Asai et al., 2001; Weinert et al., 2001), contrary to the expression of *Bmal1* that decreases with aging (Chang and Guarente, 2013; Kolker et al., 2003), while expression of *Clock* is reported to either

decrease or show no variation with age (Kolker et al., 2003; Weinert et al., 2001). Interestingly, while the expression of *Per2* remains rhythmic during senescence under normal lighting conditions, this rhythmicity is abolished under constant light or constant darkness (Nakamura et al., 2015; Polidarová et al., 2017). Electrophysiological studies in rodents revealed age-related changes in the SCN neuronal activity, including decreased amplitude of the SCN electrical activity rhythm, desynchronization of SCN neurons and aberrant SCN firing patterns (Watanabe et al., 1995; Satinoff et al., 1993; Farajnia et al., 2015, 2012; Nakamura et al., 2011). Interestingly, the activity rhythms in one of the main circadian outputs of the SCN, the subparaventricular zone (SPZ), are also decreased at advanced ages (Nakamura et al., 2011). Aging may also affect the synthesis of neuropeptides that act as synchronizers within the SCN and/or as output signals of the SCN. In humans, the SCN volume and total number of AVP cells decreases at advanced ages (Swaab et al., 1985). The daily rhythm of AVP synthesis in the human SCN is disrupted during senescence, showing loss of diurnal oscillations, reduced amplitude and reversed diurnal pattern (Hofman and Swaab, 1994). Senescent rodents exhibit no changes in the SCN volume and in the total SCN cell number (Roozendaal et al., 1987). However, the number of AVP (-31%) and VIP (-36%) neurons also decreases in the rodent SCN during aging (Roozendaal et al., 1987; Chee et al., 1988), and in middle-aged rodents rhythmicity of *VIP* mRNA levels, but not of *AVP* mRNA levels, is attenuated (Krajnak et al., 1998b).

In conclusion, while the molecular clock remains functional during aging, the amplitude of the SCN electrical rhythm and the circadian expression of neuropeptides are both impaired, probably resulting in a compromised SCN output signal. Altogether these findings suggest an age-related uncoupling between the molecular and the electrical clock components of the SCN. Therefore, other brain areas and organs might exhibit age-dependent deficits in their own endogenous clocks as well as receive a weaker systemic timing signal (Buijink and Michel, 2020).

5.4 Concluding remarks on the neuroendocrine control of reproductive aging

During aging both the reproductive and the circadian systems undergo changes. Age-related alterations in the SCN function could explain changes in behavioral and physiological functions during reproductive senescence, such as the altered sleep/wake cycles (Gómez-Santos et al., 2016; Jehan et al., 2015) and the alterations in gonadotropin secretion (Fitzgerald et al., 1998; Nelson et al., 1982). While aging admittedly compromises the SCN output signal, more research must be done in order to unravel the exact mechanism through which circadian control of the GnRH/LH surge becomes impaired during senescence.

6. Outline and scope of thesis

The aim of the present thesis is to investigate the effects of time of day, estrous stage and aging on RFRP-3 neurons and LH secretion in female mice, using neuroanatomical, electrophysiological and endocrine approaches. First, we examined whether mouse RFRP-3 neurons display daily rhythms of activity and whether there are daily- and/or estral-dependent changes in the density of AVP- and VIP-ergic fiber innervation on RFRP-3 neurons. In addition, we aimed to characterize the firing properties of RFRP-3 neurons during different time points of the day and estrous stages, and the effect of the circadian peptides, AVP and VIP, on RFRP-3 electrical activity during various time points of the day in proestrus and diestrus (Part II, chapters 2 & 3). Next, we tested the hypothesis that circadian disruptions in the light/dark cycle have a direct impact on fertility and breeding success given that ovulation and estrous cyclicity are under circadian control in female mammals. Therefore, we evaluated the effects of a single or chronic light/dark cycle phase shifts on the characteristics of the preovulatory LH surge, the estrous cyclicity and the gestational success (Part III, chapter 4).

In the last part of our study (Part IV), we investigated some endocrine and neuronal aspects of the complex physiological process that occurs in female mammals during transition to reproductive senescence. First, we performed an individual longitudinal analysis of LH secretion, by examining the timing and amplitude of the preovulatory LH surge, in order to establish a longitudinal marker of female reproductive capacity in rodents and evaluate reproductive robustness throughout adult life (chapter 5). Then, because it is still unknown whether aging in the GnRH neurons is intrinsic or due to alterations in the input from the SCN and/or in the intermediate RF-amide modulatory systems, we investigated whether there are age-dependent alterations in the RFRP-3 neuronal system and in the daily pattern of AVP- and VIP-ergic fiber input on RFRP-3 neurons and whether they correlate to changes in the LH production (chapter 6).

Abbreviations

ARC: arcuate nucleus

AVP: arginine-vasopressin

AVPV: anteroventral periventricular nucleus

[Ca2+]i intracellular calcium concentration

CRY: cryptochrome

CT: circadian time

DMH: dorsomedial hypothalamus

E2: estradiol

FSH: follicle-stimulating hormone

GnIH: gonadotropin inhibitory hormone

GnRH: gonadotropin releasing hormone

HPG axis: hypothalamo-pituitary-gonadal axis

HPO axis: hypothalamo-pituitary-ovarian axis

ipRGCs: intrinsically photoreceptive retinal ganglion cells

Kp: kisspeptin

LH: luteinizing hormone

ME: median eminence

NPY: neuropeptide Y

PER: period

POA: preoptic area

RFRP-3: (Arg)(Phe)-amide peptide 3

SCN: suprachiasmatic nucleus

TTFLs: transcriptional-translational feedback loops

VIP: vasoactive intestinal peptide

VMH: ventromedial hypothalamus

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PART II

REGULATION OF RFRP-3 NEURONS IN ADULT FEMALE MICE

Chapter 2

Daily and Estral Regulation of RFRP-3 Neurons in the Female Mice

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Abstract

Female reproductive success relies on proper integration of circadian- and ovarian- signals to the hypothalamic-pituitary-gonadal axis in order to synchronize the preovulatory LH surge at the end of the ovarian follicular stage with the onset of the main active period. In this study, we used a combination of neuroanatomical and electrophysiological approaches to assess whether the hypothalamic neurons expressing Arg-Phe amide-related peptide (RFRP-3), a gonadotropin inhibitory peptide, exhibit daily and estrous stage dependent variations in female mice. Furthermore, we investigated whether arginine vasopressin (AVP), a circadian peptide produced by the suprachiamatic nucleus regulates RFRP-3 neurons. The number of c-Fos–positive RFRP-3 immunoreactive neurons is significantly reduced at the day-to-night transition with no difference between diestrus and proestrus. Contrastingly, RFRP neuron firing rate is higher in proestrus as compared to diestrus, independently of the time of the day. AVP immunoreactive fibers contact RFRP neurons with the highest density observed during the late afternoon of diestrus and proestrus. Application of AVP increases RFRP neurons firing in the afternoon (ZT6-10) of diestrus, but not at the same time point of proestrus, indicating that AVP signaling on RFRP neurons may depend on circulating ovarian steroids. Together, these studies show that RFRP neurons integrate both daily and estrogenic signals, which downstream may help to properly time the preovulatory LH surge.

Introduction

Mammalian reproductive competence is controlled centrally by neuroendocrine mechanisms operating along the hypothalamic- pituitary- gonadal (HPG) axis. In female mammals, the HPG axis is submitted to a complex regulation leading to a time-controlled ovulation triggered by an acute and transient luteinizing hormone (LH) release from the pituitary gland. This LH surge is driven by a former gonadotropin-releasing hormone (GnRH) surge which depends on high circulating estrogen (E2) secreted by mature ovarian follicles (Close and Freeman, 1997; Moenter et al., 1992). The preovulatory GnRH/LH surge also relies on a functional circadian system which coordinates the timing of ovulation and sexual behavior, ensuring that these events occur when the animal is most likely to breed (Brown-Grant and Raisman, 1977; Everett and Sawyer, 1950; Miller et al., 2004). Thus, in female nocturnal rodents, like mice, the LH surge is generated exclusively at the transition between the inactive (light) and the active (dark) periods at the proestrus stage when oocytes reach maturation (Christian et al., 2005; Christian and Moenter, 2010; Everett and Sawyer, 1950). E2 does not feedback directly on GnRH neurons which lack E2 receptors (ERα) (Christian and Moenter, 2010; Leon and Tena-Sempere, 2015) but rather on two hypothalamic ERα-expressing neuropeptidergic systems synthesizing the stimulatory kisspeptin (Kp) (Smith et al., 2005) and the inhibitory (Arg)(Phe) amide-related peptide-3 (RFRP-3) (Kriegsfeld et al., 2006a; Poling et al., 2012). Kp and RFRP-3 neurons lie upstream of the GnRH neurons and are thought to be the converging site where daily and ovarian signals are conveyed to the HPG axis, therefore establishing the gated time window during which the preovulatory GnRH/LH surge occurs (Angelopoulou et al., 2019).

There are two hypothalamic Kp populations, one in the arcuate nucleus (ARC) and the other in the anteroventral periventricular nucleus (AVPV), which project to several hypothalamic regions and notably to GnRH neurons (Clarkson and Herbison, 2006; Yip et al., 2015). Many studies have shown that Kp administration stimulates GnRH neuron activity (Pielecka-Fortuna et al., 2008; Piet et al., 2018) and LH secretion in female mammals (Gottsch et al., 2004; Navarro et al., 2005; Smith et al., 2006). AVPV Kp neurons are considered pivotal for timing the preovulatory LH surge as they exhibit an increased neuronal activity and *Kiss1*

expression at the day/night transition at the time of the LH surge (Chassard et al., 2015; Henningsen et al., 2017; Poling et al., 2017; Robertson et al., 2009). RFRP-3 is the mammalian ortholog of the avian gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000; Ukena et al., 2002). RFRP-3 neurons are located in the dorsomedial hypothalamus (DMH) and project to multiple brain regions but most notably to GnRH neurons (Henningsen et al., 2016; Kriegsfeld et al., 2006a; Rizwan et al., 2012; Smith et al., 2008; Ubuka et al., 2012) and to Kp neurons (Poling et al., 2013; Rizwan et al., 2012) that express the RFRP-3 receptor, GPR147. Numerous studies have shown that RFRP-3 inhibits GnRH neuron activity and suppresses the elevated preovulatory LH release in female mammals (Ancel et al., 2017; Anderson et al., 2009; Clarke et al., 2008; Ducret et al., 2009; Henningsen et al., 2017; Johnson et al., 2007; Kriegsfeld et al., 2006a; Pineda et al., 2010; Tsutsui et al., 2000). Notably, in female rodents, RFRP-3 neuronal activity decreases at late day, at the time of the LH surge (Gibson et al., 2008; Henningsen et al., 2017; Poling et al., 2017).

Studies have investigated the mechanisms through which circadian cues are relayed to AVPV Kp and RFRP-3 neurons, notably from the biological clock located in the suprachiasmatic nucleus (SCN) (Chassard et al., 2015; Gibson et al., 2008; Henningsen et al., 2017). It is now well established that SCN-generated arginine vasopressin (AVP) fibers make appositions to AVPV Kp neurons which express V1a receptors (Vida et al., 2010; Williams et al., 2011), and that AVP activates Kp neurons in an E2-dependant manner (Piet et al., 2015). Contrastingly, not much attention has been given to the circadian regulation of the RFRP-3 neurons. One study in Syrian hamsters showed that AVP- and vasoactive intestinal peptide (VIP)- ergic fibers originating from the SCN form close appositions to RFRP-3 neurons (Russo et al., 2015).

The aim of the present study was to investigate how the RFRP-3 system is regulated by daily and estrogenic cues in the female C57BL/6J mice. In a first set of neuroanatomical experiments, we analyzed whether there are daytime- and estrous cycle stage-dependent changes in the RFRP-3 expression and neuronal activity, and in the number of AVP-ergic fiber projections on RFRP-3 neurons. In a second set of electrophysiological experiments, we investigated the effect of daytime and estrous status on the RFRP-3 electrical activity

and whether AVP regulates electrical activity of RFRP-3/ EYFP fluorescent protein labelled neurons in hypothalamic sections of transgenic RFRP/Cre female mice.

Materials and Methods

1. Animals

Adult female C57BL/6J mice (Charles River, France) and heterozygous RFRP-Cre mice on a C57BL/6J background (Mamgain et al., 2020) were housed two or three per cage under a 12-hour light: 12-hour dark cycle (lights on at 7:00 am, given as zeitgeber 0 (ZT0)) with controlled temperature (22°C) and *ad libitum* access to food and water. All protocols were reviewed by the Regional Committee for Ethics in Animal Experimentation and approved by the French Ministry of Education and Research (authorization #8452-2017010613574177v2).

2. Monitoring of Estrous Cycle and LH Secretion

Female mice were studied at either proestrus or diestrus stage as assessed by vaginal smears and circulating LH. Vaginal smears were performed daily at ZT2 during at least two consecutive cycles and only mice showing regular 4-5 day cycles were sampled on diestrus (appearance of leucocytes) or proestrus (appearance of nucleated epithelial cells). From each mouse a 4 µl blood sample was collected from the tail tip at the moment of sacrifice and this sample was diluted in 116 µL phosphate buffer saline with 0,25% tween-20. The LH concentration was measured by ELISA using anti-bovine LHβ as capture antibody (monoclonal antibody, 518B7, NHPP, Torrance, California), rabbit anti-mouse LH as first antibody (polyclonal antibody, rabbit LH antiserum, AFP240580Rb, NHPP), goat anti-rabbit IgG as secondary antibody (D048701–2, Dako Cytomation, Polyclonal Goat Anti-Rabbit, Denmark) and mouse LH as standard (mLH, AFP-5306A, NHPP) as previously described (Bahougne et al., 2020).

3. Neuroanatomical Investigation of Daily and Estral Regulation of RFRP-3 Neurons

Tissue processing

Adult female mice at proestrus or diestrus stage were sacrificed by exposure to increasing concentration of CO_2 at six different time points (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20; n=5 per experimental point). After intracardiac blood puncture, mice were intracardially perfused with 10 mL phosphate buffer saline 0.1 M (PBS, pH 7.4) followed by 20 mL of periodate-lysine-paraformaldehyde fixative (formaldehyde 4%, $NaIO_4$ 10 mM and lysine 75 mM in 100 mM phosphate buffer, pH 7.3). The brains were collected, post-fixated in periodate-lysine-paraformaldehyde for 12 hours, washed out with PBS, dehydrated and embedded in polyethylene glycol as previously described (Klosen et al., 1993).

Twelve series of 12 µm-thick coronal brain sections were cut using a microtome throughout the DMH as presented in the Paxinos mouse brain atlas. For each mouse, one section in every twelve (i.e. 1 section every 144 µm giving 6-7 DMH-containing brain sections) was rehydrated and mounted on a SuperFrost Plus (Menzel-Glaser, Braunschweig, Germany) slide. For each immunolabeling, DMH-containing slices of all mice of different time points and estrous stages were processed at the same time in order to limit variations in the labeling background.

Double c-Fos/RFRP-3 and AVP/RFRP-3 immunohistochemistry

The number of c-Fos expressing RFRP-3 neurons and the density of AVP fibers surrounding RFRP-3 neurons were assessed by dual immunohistochemistry. Brain sections were first incubated either with a rabbit polyclonal antiserum raised against c-Fos (1:2000; Santa Cruz Biotechnology; RRID: AB_627251) or a rabbit polyclonal antiserum raised against neurophysin II, a cleavage product of prepro-vasopressin (1:15000; Sigma-Aldrich; RRID: AB_260747) diluted in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20, for 24 hours at room temperature. Brain sections were washed with PBS, incubated with biotinylated donkey antirabbit (1:2000; Jackson Labs; in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20) for 1 hour and then washed again with PBS. Immunoreactive signal was amplified by a treatment with the avidin biotin complex coupled

to peroxidase (1:250; Vector Laboratories) for 1 hour, then revealed using a solution of 0.5 mg/mL 3,3-diaminobenzidine (DAB; Sigma-Aldrich) diluted in water and 0.001% hydrogen peroxide urea (Sigma-Aldrich) for 30 minutes. Before performing the second immunolabelling, the antibodies were eluted with 2 x 15 minute washes in a solution of 100 mM glycine containing 0.3% Triton X-100 (pH 2.2). Brain sections were then incubated with a primary antibody directed against RFRP-3 (1:15000; rabbit anti-RFRP-3, University of Otago, NZ; RRID: AB_2877670; in a PBS buffer containing 10% donkey serum and 0.3% Tween 20) overnight at room temperature. The sections were then washed with PBS, incubated with biotinylated donkey anti-rabbit (1:2000; Jackson Labs; in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20) for 1 hour, and then washed again with PBS. The RFRP-3 signal was detected using streptavidin-peroxidase at 1/3000 (Roche) for 1 hour and revealed with Fast blue- BB (Sigma-Aldrich) for 7 minutes. Finally, sections were mounted with CC/mount (Sigma-Aldrich), dehydrated in toluene twice for 10 minutes, and mounted with Eukitt resin (Sigma-Aldrich).

Quantification of immunolabeled RFRP-3 cells and AVP-ergic fibers

Only five sections located at comparable levels of the DMH were taken into account for the analysis in order to limit differences stemming from rostrocaudal variations. The five sections were selected based on neuroanatomical markers such as the median eminence, the tuberoinfundibular sulcus and the pituitary stalk. Counting was done by the first author unaware of animal's identity. For each mouse, the total number of RFRP-3-immunoreactive (ir) neurons, the number of RFRP-3 neurons containing nuclear c-Fos, and the number of RFRP-3 neurons receiving direct AVP-fiber projections were counted manually. AVP-ergic appositions were defined as terminal fibers directly contacting RFRP-3 cell somas. For each mouse, the number of RFRP-3 neurons is given as the total number counted in 5 sections. The number of c-Fos expressing RFRP-3 neurons is given as a percentage of this total number of RFRP-3 neurons. Also, the number of RFRP-3 neurons with close AVP-fiber appositions is given as a percentage of the total number of RFRP-3 neurons.

The density of AVP-ir fibers was quantified in selected regions of interest (ROI) bilaterally in the DMH defined by neuroanatomical landmarks (such as the median eminence, the

tuberoinfundibular sulcus and the pituitary stalk), the DMH boundaries and the anatomical position of the RFRP-3 neurons. The density of AVP-fibers in the DMH was estimated by counting manually the number of points that a fiber crossed the intersections of a grid applied on the ROIs. For each mouse AVP-ir fiber density is given as total number of crossing points/total grid number of the ROIs.

4. Electrophysiological Investigation of Daily and Estral Regulation of RFRP-3 Neurons

In vivo labelling of RFRP-3 neurons

Labelling of RFRP neurons in adult female mice was performed using an adeno-associated virus containing a promoter upstream of a transcription-blocking cassette, followed by sequences encoding enhanced yellow fluorescent protein (pAAV5.EF1a.DIO.EYFP, 1×10^{13} pfu/mL, purchased from Addgene, catalog 27056-AAV5) in order to mediate the expression of EYFP exclusively in Cre-expressing RFRP neurons. Female RFRP-Cre mice were anaesthetized under a Zoletil (80 mg/kg) / Xylazine (10mg/kg) mixture and injected with Metacam (5 mg/kg) and Bupivacaine (2 mg/kg) for analgesia. Then mice were placed in a stereotaxic apparatus in order to perform a bilateral stereotaxic injection of the virus targeting the DMH area. The skull was exposed and a Hamilton syringe loaded with 1 µL of AAV-EYFP was lowered into the DMH area according to the atlas of Paxinos and Franklin coordinates (-1.6 mm posterior to bregma, -0.5 mm lateral to midline, and -5.3 mm ventral to dura.). Injection was performed at a rate of 100 nL/min followed by a 10-minute pause before removing the syringe. Mice were administered Metacam (2 mg/kg) dissolved in water for 3 days as a post-operative analgesic treatment. Preliminary tests indicated that the highest level of EYFP expression in RFRP-3 neurons was observed three weeks after the injections with the percentage of RFRP-3-ir neurons expressing EYFP estimated at 68.3 ± 2.5 % (n= 4 mice).

Brain slice preparation

Three weeks after the AAV injections, mice were anaesthetized with urethane (i.p. 1.9 g/Kg) at two estrous stages (diestrus or proestrus) and two different time points ZT4-ZT5 (*in vitro* recording at ZT6-ZT10) or ZT9-ZT10 (*in vitro* recording at ZT11-ZT14, time of the preovulatory LH surge at the proestrus stage). An intracardic perfusion was performed with oxygenated iced-cold sucrose-artificial cerebrospinal fluid containing 248 mM sucrose, 11 mM glucose, 26 mM NaHCO3, 2 mM KCl, 1.25 mM KH2PO4, 2 mM CaCl2, 1.3 mM MgSO4, 5 mM kynurenic acid). The solution was continuously bubbled with 5% CO2 and 95% O2. Brains were quickly removed and 300 µm thick coronal slices were cut at the level of the DMH using a vibratome (Leica VT1200S). Slices were then transferred in artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 26 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 2 mM CaCl2, 2 mM MgCl2, 10 mM glucose at room temperature for 1 hour before starting recordings.

RFRP cell-attached recordings

After recovery, slices were placed to the recording chamber under an upright microscope fitted for epifluorescence (Zeiss, Axioskop) and were continuously perfused with oxygenated aCSF (1.0-1.5 mL/min). EYFP/RFRP neurons were first visualized by brief fluorescent illumination. Spontaneous firing was recorded using the minimally invasive cell-attached loose patch configuration. Recording electrodes (3-5 MΩ) were pulled from borosilicate glass capillaries (Harvard apparatus) using a P1000 electrode puller (Sutter Instruments) and filled with aCSF. Spontaneous spikes were recorded in the voltage clamp mode using an Axopatch 200B amplifier (Axon Intruments). Signals were low-pass filtered (5 kHz) and acquired with Clampex software (Molecular devices). Current traces were digitized (10 kHz) and stored on the hard drive of a personal computer. All experiments were performed at room temperature (22–24 °C). The spontaneous firing of neurons was recorded in control aCSF for more than 5 minutes before bath application of AVP (1 µM in aCSF; Euromedex) for 3 minutes. Spontaneous spikes were detected using the threshold-

crossing method in pClamp 10 (Molecular device). Data calculated were the Mean Interspike Interval (ISI) and the Coefficient of Variation of ISI (CV = standard deviation/mean ISI) as an indicator of spike train regularity. Recordings in which the baseline firing was not stable were not included in the analysis.

5. Data Analysis

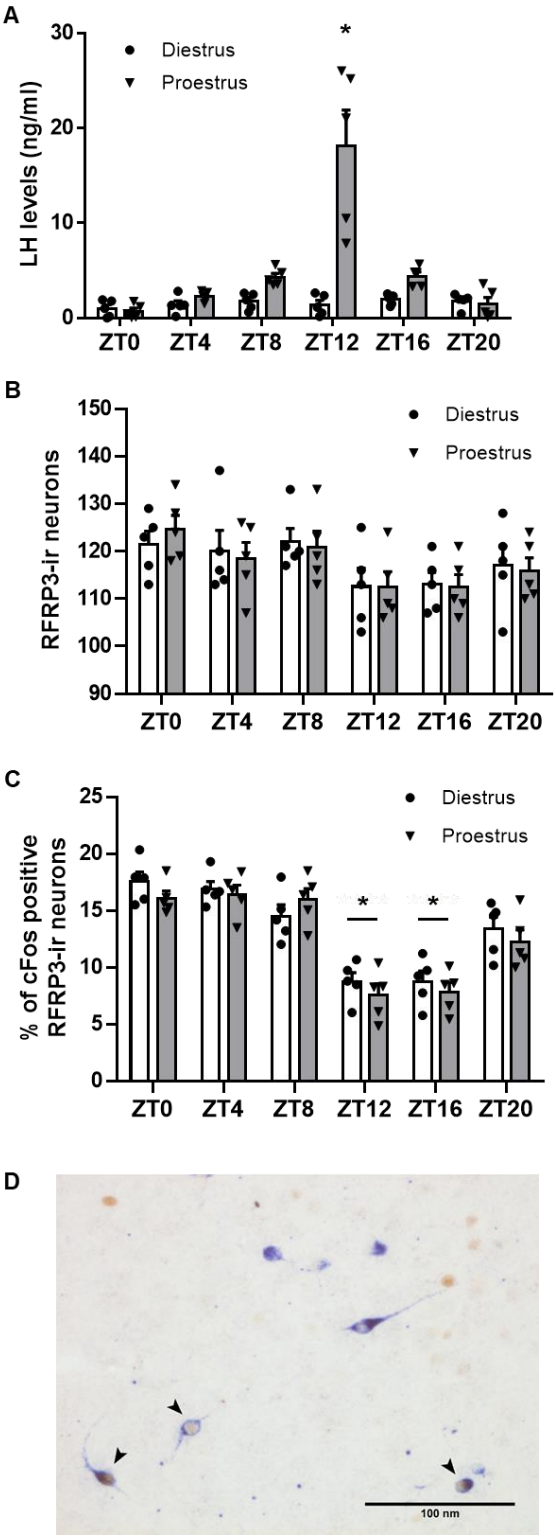
Statistical analysis was performed with GraphPad Prism 6. All data, including outliers, have been included in the analyses. All values are given as mean ± SEM. For neuroanatomical results, Two-way ANOVA was used to assess significant variations among different time points and estrous stages, followed by Sidak's multiple comparisons test. For the electrophysiological investigation, the firing pattern of individual RFRP neurons was determined with criteria previously used for Kp neurons: silent neurons displaying no spontaneous activity, tonic neurons exhibiting regular firing with the SD of their interspike intervals less than 100 msec, bursting neurons having more than 50% of their spikes occurring in bursts , and all other neurons classified as irregular (de Croft et al., 2012; Ducret et al., 2010). Two-way ANOVA was used to assess significant variations in the firing properties among different time points and estrous stages. For AVP experiments, the relative ISI (to a 5-minute baseline) was averaged in 1 minute-bin and drug effect was tested with repeated measures Two-way ANOVA (RM-ANOVA). Comparison of mean firing frequency between baseline and following AVP application was performed using a paired Student t-test. Differences were considered significant for $P < 0.05$.

Results

1. Neuroanatomical Investigation of Daily and Estral Regulation of RFRP Neurons in Female C57BL/6J Mice

This experiment aimed at investigating the occurrence of daily and estral variation in RFRP-3 expression, neuronal activity and inputs from AVP-ergic fibers in female mice. Mice sampled at different time points of the proestrus stage exhibited the expected surge in LH at ZT12, whereas mice sampled at the diestrus stage had constant low LH levels at all timepoints (Two-way ANOVA, *Estrous stage*: $F(1, 48) = 29.97$, $p < 0.0001$; *Daytime*: $F(5, 48) = 15.60$, $p < 0.0001$; Figure 1A).

The number of RFRP-3-immunostained neurons showed a significant daily variation (Two Way ANOVA, *Daytime*: $F(5, 48) = 3.518$, $p = 0.0086$; Figure 1B) in both proestrus and diestrus. We found no effect of estrous stage or the *Daytime * Estrous* stage interaction (Two Way ANOVA, *Estrous stage*: $F(1, 48) = 0.01923$, $p = 0.8903$; Interaction: $F(5, 48) = 0.1411$, $p = 0.9817$; Figure 1B). The percentage of c-Fos-positive RFRP-3 neurons was significantly reduced at the day-to-night transition (ZT12-ZT16), thus around the time of the LH surge, on the day of proestrus, but also at the same period on the day of diestrus (Two-way ANOVA, *Daytime*: $F(5, 48) = 39.56$, $p < 0.0001$; *Estrous stage*: $F(1, 48) = 1.413$, $p = 0.2403$; Figure 1C, Figure 1D).


A
LH levels (ng/ml)
30
20
10
0
Diestrus
Proestrus
*
ZT0
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RFRP3-ir neurons
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Figure 1. *RFRP-3 immunoreactivity and neuronal activity in the dorsomedial hypothalamus of female C57BL/6J mice sampled at different time points of the day of diestrus and proestrus. (A) LH blood concentrations; (B) number of RFRP-3-ir neurons in the DMH; (C) percentage of c-Fos–expressing RFRP-3-ir neurons; (D) Photograph where arrows show c-Fos–positive (brown) RFRP3-ir (blue) neurons at ZT0 (08:00 am) on the day of proestrus; scale bar = 100 µm. Data are presented as mean ± SEM of n = 5 mice for each experimental point. Two-way ANOVA was used to assess significant variations among different time points and estrous stages, followed by Sidak's multiple comparisons test. Daily differences with a value of P < 0.05 were considered as significant and are indicated by *. Cycle stage differences were insignificant (P > 0.05).*

Analysis of AVP-ir fiber density in the DMH showed a significant increase at ZT8, thus prior to the onset of the dark period, with no difference between proestrus and diestrus (Two-way ANOVA, *Daytime*: $F(5, 48) = 7.759$, $p < 0.0001$; *Estrous stage*: $F(1, 48) = 0.2844$, $p = 0.5963$; Figure 2A). Quantification of the percentage of RFRP-3 neurons receiving direct AVP-ir fiber appositions showed that the highest percentage is observed at ZT8-ZT12 and the lowest percentage at ZT0, with no difference between proestrus and diestrus (Two-way ANOVA, *Daytime*: $F(5, 48) = 7.673$, $p < 0.0001$; *Estrous stage*: $F(1, 48) = 1.467$, $p = 0.2318$; Figure 2B, Figure 2C).

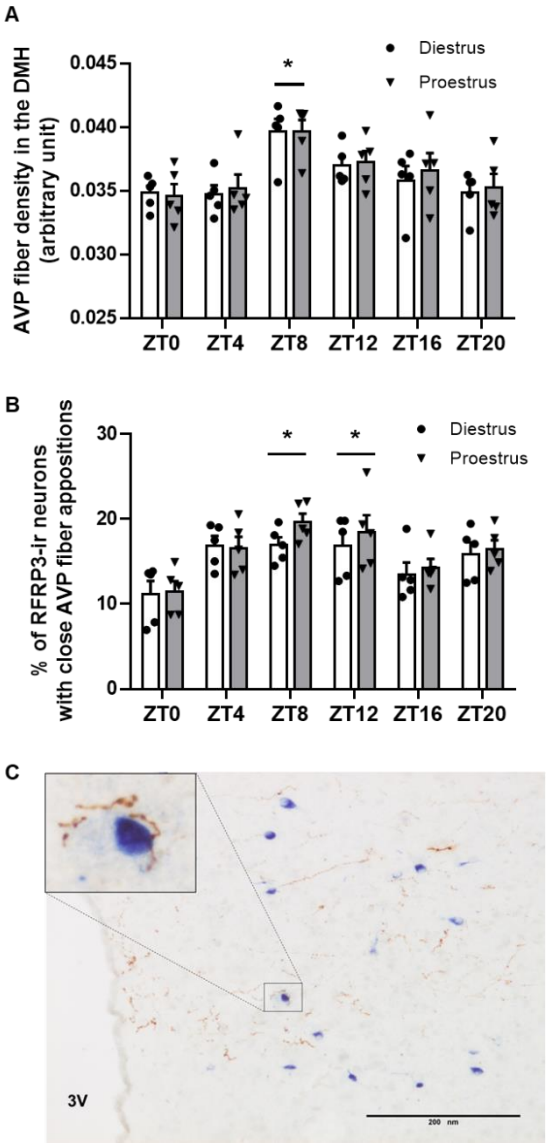

Figure 2. *Distribution of arginine vasopressin (AVP)-immunoreactive (ir) fibers in the dorsomedial hypothalamus of female C57BL/6J mice sampled at different time points of the day of diestrus and proestrus. (A) AVP-ir fiber density (arbitrary unit) in the DMH; (B) Percentage of RFRP-3-ir neurons with close AVP-ir fiber appositions; (C) Photograph*

*shows RFRP-3-ir neurons and AVP-containing fibers at ZT8 (16:00) on the day of proestrus; scale bar = 200 µm. Data are presented as mean ± SEM of n = 5 mice for each experimental point. Two-way ANOVA was used to assess significant variations among different time points and estrous stages, followed by Sidak's multiple comparisons test. Daily differences with a value of P < 0.05 were considered as significant and are indicated by *. Cycle stage differences were insignificant (P > 0.05).*

2. Electrophysiological Investigation of Daily and Estral Regulation of RFRP-3 Neuron Firing Activity in Female C57BL/6J Mice

This experiment aimed at recording the endogenous firing activity of RFRP-3 neurons at two time points of the diestrus and proestrus stages, and its regulation by AVP at these different experimental points. All recorded RFRP-3 neurons exhibited an irregular spontaneous firingpattern (Figure 3A). RFRP-3 neuron spontaneous firing rates were significantly higher in proestrus compared to diestrus (Two Way ANOVA, *Estrous stage*: F(1, 118) = 11.45, p = 0.0010) independently of the time of the day (Two Way ANOVA, *Daytime*: F(1, 118) = 0.1032, p = 0.7486; Figure 3A, Figure 3B). Similarly, on the day of proestrus, we recorded a lower InterSpikeInterval, indicating a shorter time between subsequent action potentials in RFRP-3 neurons (Two Way ANOVA, *Estrous stage*: F(1,118) = 6.288, p=0.0135), independently of the time of the day (Two Way ANOVA, *Daytime*: F(1,118) = 2.067, p = 0.1532, data not shown).

In contrast, the InterSpikeInterval-Coefficient of Variation (ISI-CV), indicator of firing regularity, was lower at ZT11-14 compared to ZT6-10 (Two Way ANOVA, *Daytime*: F(1, 118) = 5.021, p = 0.0269; Fig. 3c) independently of the estrous stage (Two Way ANOVA, *Estrous stage*: F (1, 118) = 0.00077, p = 0.9780; Figure 3C), thus indicating that RFRP-3 neurons have a more regular firing pattern at ZT11-14 compared to the earlier period.

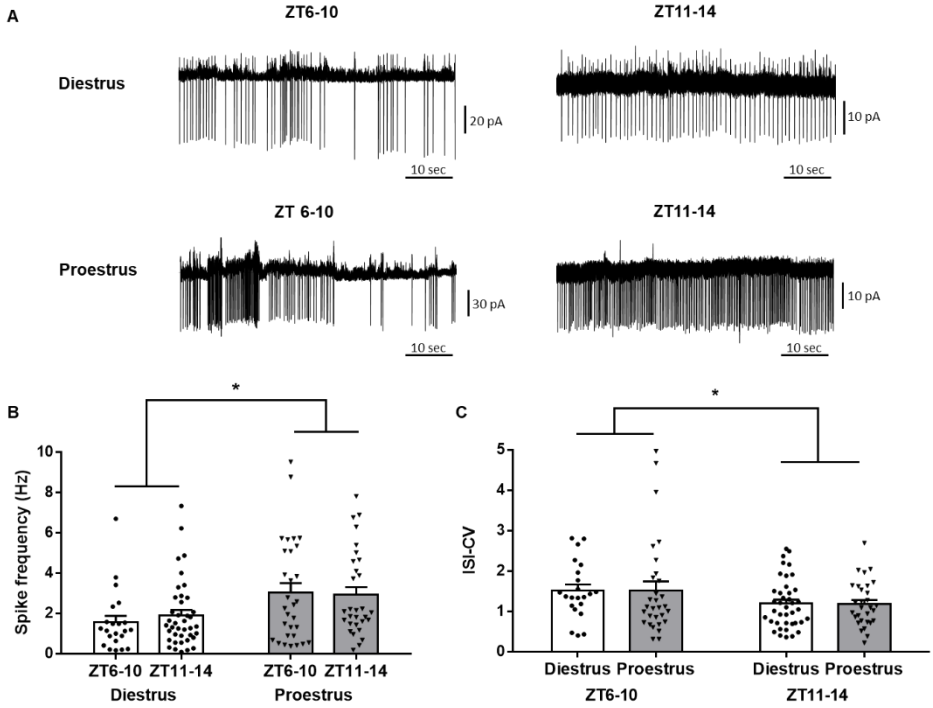

Figure 3. *Electrical firing characteristics of RFRP-3 neurons in the dorsomedial hypothalamus at two time points and stages of the estrous cycle in female C57BL/6J mice. (A) Representative traces of RFRP-3 neurons firing in the DMH at two time points (ZT6-10 and ZT11-14) during diestrus and proestrus; (B) Mean spike frequency of RFRP-3 neurons in the DMH at two time points during the day of diestrus (ZT6-10; n=22 neurons out of 7 mice and ZT11-14; n=42 neurons out of 10 mice) and proestrus (ZT6-10; n=30 neurons out of 7 mice and ZT11-14; n=30 neurons out of 9 mice); (C) InterSpike Interval CV (ISI-CV) of RFRP-3 neurons in the DMH at two time points during the day of diestrus and proestrus. Data are presented as mean ± SEM. Two-way ANOVA was used to assess significant variations in the firing properties among different time points and estrous stages. Differences among groups were considered significant for P < 0.05 and are indicated by *.*

In diestrus mice, bath application of AVP (1µM) at ZT 6-10 significantly decreased the ISI (RM-ANOVA for relative ISI, *Time*: F(12, 132) = 3,092, p = 0.007, n=6; Figure 4A, Figure 4B) and consequently increased the mean firing rate of RFRP-3 neurons (paired Student's t-test, p = 0.0199, n=6; Figure 4C), but had no effect at ZT11-ZT14. In proestrus mice, AVP (1µM) application had no effect on the ISI (RM-ANOVA, for relative ISI, *Time*: F(12, 108) = 1.023, p

= 0.4330, n=7; Figure 4D) and so neither on the mean firing rate of RFRP-3 neurons (paired Student's t-test, *Time*: p = 0.2840, n=7; Figure 4E) at either time point.

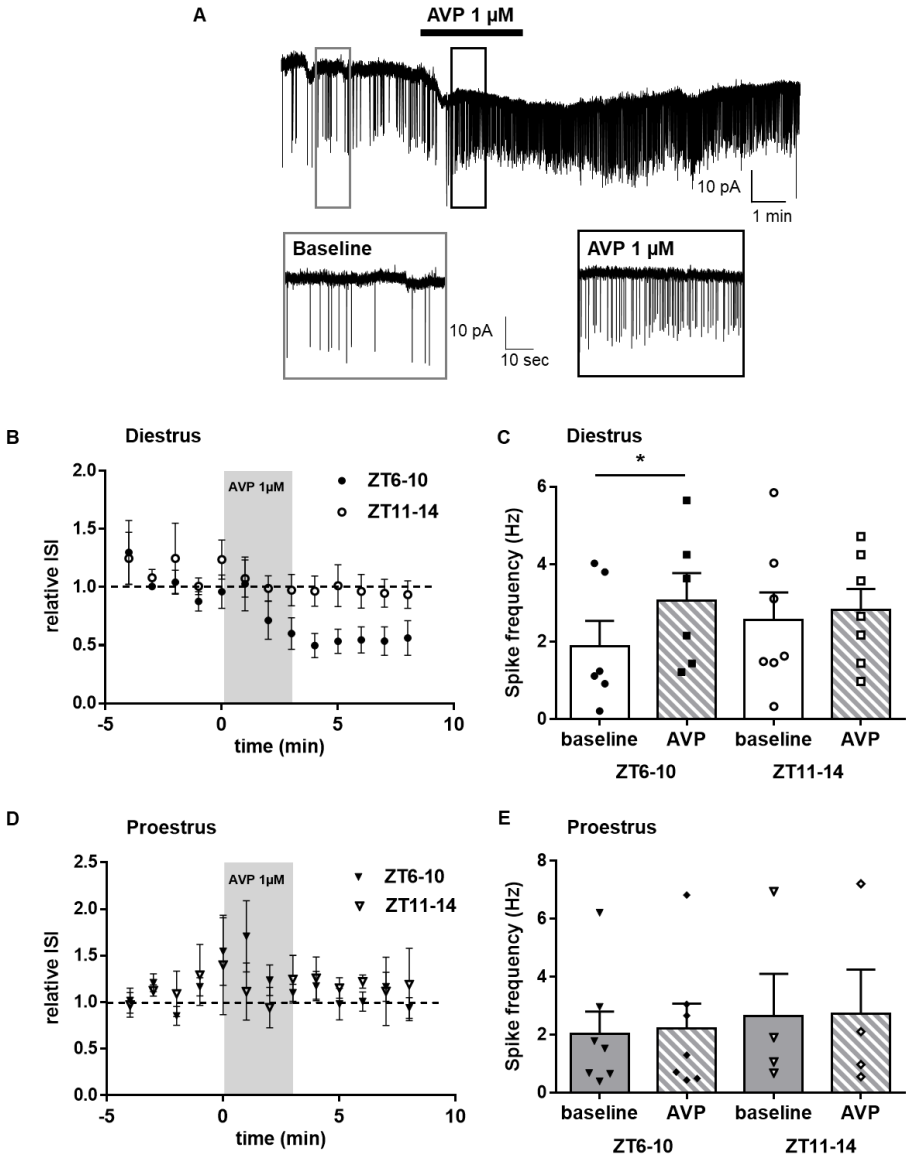

Figure 4. *Effect of AVP on RFRP-3 electrical activity in the dorsomedial hypothalamus at different time points and stages of the estrous cycle in female C57BL/6J mice. (A) Sample trace illustrating the effect of AVP on RFRP-3 neuron firing in Diestrus at ZT6-10. (B) Average time course of relative InterSpikeInterval (ISI) in response to AVP (1µM)*

*application of RFRP-3 neurons in the DMH at ZT 6-10 (n=6 neurons out of 5 mice) and ZT11-14 (n=7 neurons out of 3 mice) during the day of diestrus. (C) Summary bar graph of the AVP effect on mean spike frequency of RFRP-3 neurons in the DMH during the day of diestrus. (D) Average time course of relative InterSpikeInterval (ISI) in response to AVP (1µM) application of RFRP-3 neurons in the DMH at ZT 6-10 (n=7 neurons out of 5 mice) and ZT11-14 (n=4 neurons out of 3 mice) in the day of proestrus. (E) Summary bar graph of the AVP effect on mean spike frequency of RFRP-3 neurons in the DMH the day of proestrus. Data are presented as mean ± SEM. Repeated measures Two-way ANOVA was used to assess significant variations in the relative ISI among different time points in diestrus and proestrus. A paired Student t-test was used to compare RFRP mean firing frequency between baseline and after AVP application. Differences among groups were considered significant for P < 0.05 and are indicated by *.*

Discussion

Reproduction in female mammals displays regular daily and ovarian cycles driven by E2-sensitive neural circuits in the hypothalamus (Herbison, 2020; Pinilla et al., 2012; Simonneaux et al., 2017). In this study, we investigated in female mice whether RFRP-3 neurons, reported to regulate GnRH neuronal activity and LH secretion [12], act as an interface for SCN-derived AVP fibers and E2 feedback, in order to convey daily and estral cues to the HPG axis. Therefore, we examined whether the RFRP-3 system is under daily regulation and/or sensitive to estrogenic cues, as previously reported for Kp neurons (Pinilla et al., 2012; Simonneaux, 2020). Using a neuroanatomical approach, we report a decrease in RFRP-3 neuron activation coordinated with an increase in the number of RFRP3-ir cells with close AVP-ir fiber appositions around the day-to-night transition (ZT8-ZT16) with no difference between the diestrus and proestrus stages. Using electrophysiological analysis, we report that RFRP-3 neurons display an increased firing rate in proestrus as compared to diestrus and a more regular firing activity during the day-to-night transition (ZT11-14) as compared to midday (ZT6-10). Moreover, AVP affects the activity of RFRP-3 neurons in a time- and estrous stage-dependent way, with a stimulatory effect observed at midday of the diestrus day only. It is worth saying that in a preliminary experiment, we found a low density of VIP- containing fibers (another SCN-derived input on RFRP-3 neurons; (Dardente et al., 2004; Russo et al., 2015) in the DMH, and the VIP-ergic fiber input on RFRP-3 neurons

showed no daily or estral variation, while VIP was unable to alter the firing rate of RFRP-3 neurons (unpublished data).

In this study, we assessed RFRP-3-immunoreactivity and neuronal activity, both by c-Fos expression and by electrophysiological recordings, at different time points of diestrus and proestrus. We demonstrated that the number of RFRP-3 neurons changes over the day/night cycle, showing a moderate decrease during the beginning of the dark period in both diestrus and proestrus. Furthermore, we observed that RFRP-3 neuronal activation, as attested by c-Fos expression, decreases during the day-to-night transition of both proestrus and diestrus day, as previously reported in female Syrian hamsters (Gibson et al., 2008; Henningsen et al., 2017). Therefore, on proestrus, there is a coordinated decrease in RFRP-3 neuronal activity with a concurrent increase in Kp neuronal activity and LH surge (Chassard et al., 2015; Henningsen et al., 2017; Robertson et al., 2009) at the day-to-night transition, whereas on diestrus, only RFRP-3 neurons exhibit a daily variation. Thus, the daily pattern in RFRP-3 c-Fos activation appears independent to the stage of the estrous cycle, even though it has been reported that a subset of RFRP-3 neurons express ERα, and that E2 administration suppresses RFRP expression (Kriegsfeld et al., 2006b; Molnár et al., 2011; Poling et al., 2012). When investigating the electrical activity of RFRP-3 neurons in isolated hypothalamic sections, we observed that they exhibit different firing activities according to the time of the day and the estrous stage. Indeed, we calculated that the RFRP-3 neuron firing activity is more regular during the day-to-night transition compared to an earlier time period, in both estrous stages. However, RFRP-3 neurons fire action potentials at an about twice higher frequency in proestrus compared to diestrus with no time of the day difference.

Altogether, both neuroanatomical and electrophysiological results indicate that RFRP-3 neurons may be involved in the daily and estral regulation of the HPG axis. Yet, data acquired by means of immunohistochemistry and electrophysiology display some differences. Notably, we report a clear estrous stage difference in the intrinsic firing activity but not in c-Fos expression of RFRP-3 neurons. The inducible immediate early gene *c-fos* is a reporter of transcriptional activation widely used as a molecular marker of neural activity,

although the threshold of c-Fos induction differs among different neural populations. In some brain areas c-Fos is expressed after exposure to mild stimulus, in other brain structures a previous stimulus deprivation is necessary, and certain active neural populations cannot elicit sufficient c-Fos expression (Cullinan et al., 1995; Kaczmarek and Chaudhuri, 1997). Furthermore, c-Fos expression is triggered by different signals, among which an increase in calcium influx, but interestingly, calcium influx during spike activity cannot induce c-Fos expression (Luckman et al., 1994). Therefore, given our observation that RFRP-3 neurons are constantly spontaneously active, it is possible that the higher frequency observed at proestrus does not translate in a different c-Fos expression profile. However, it is important to note that c-Fos expression is not a reliable marker of neuronal activity, since its induction is not associated with firing rate but rather with the neuronal firing pattern (Guo et al., 2007).

Female reproduction relies on a synchronized circadian system to properly time the preovulatory LH surge as SCN lesions and genetic impairment of clock genes suppress the LH surge and lead to estrous acyclicity (Miller et al., 2004; Wiegand et al., 1980; Wiegand and Terasawa, 1982). A significant amount of evidence links the circadian peptide AVP with the SCN-driven daily regulation of the HPG axis. AVP central injections restore the LH surge in rodents with SCN lesions or clock gene mutation, while central administration of AVP receptor antagonists suppresses the LH surge (Funabashi et al., 1999; Miller et al., 2006; Palm et al., 1999). Neuroanatomical, pharmacological and electrophysiological data have proven that the SCN-derived AVP input on AVPV Kp neurons is critical for the LH surge induction (Piet et al., 2015; Vida et al., 2010; Williams et al., 2011). However, an earlier study in Syrian hamsters showed that SCN-derived AVP neurons also make close appositions with RFRP-3 neurons, although central administration of AVP had no effect on the RFRP-3 c-Fos expression (Russo et al., 2015). We confirmed, in female mice, that RFRP-3 neurons receive close AVP-ergic fiber appositions, and we furthermore reported a significant increase in the number of RFRP-3 neurons with close AVP-ergic fiber appositions at late afternoon, concurrently with an increase in the AVP-ergic fiber density in the DMH. Thus, prior to the light-dark transition, and in line with higher levels of AVP mRNA in the SCN (Dardente et al.,

2004), there is an increased input of AVP signaling on RFRP-3 neurons. Notably, the overall AVP-ergic fiber input to RFRP-3 neurons is similar in both diestrus and proestrus, which might reflect the E2 independent daily rhythms in SCN AVP synthesis (Mahoney et al., 2009). The presence of peptidergic fiber appositions on neuronal cell bodies does not prove that they make synapses or that there is a direct post-synaptic effect of the peptide. Therefore, in order to look for a functional role of AVP on RFRP-3 neurons, we examined its effect on RFRP-3 neuronal firing activity either at ZT6-10 or at ZT11-14 on diestrus and proestrus. We showed that AVP increases the RFRP-3 neuron firing frequency at midday and not at the day-to-night transition of diestrus, while it has no effect at both time points in proestrus. Our findings indicate that AVP may coordinate the RFRP-3 system in a specific time window under low E2 milieu (diestrus) and that E2 (at proestrus) may act in a suppressive manner by disabling the excitatory effect of AVP on RFRP-3 neurons. It is interesting to note that an earlier study also reported a role of E2 in the AVP-induced activation of AVPV Kp neurons (Piet et al., 2015). Future experiments should evaluate whether RFRP-3 neurons express V1aR receptors and whether there are circadian- and E2-dependent changes in the V1aR co-expression that could mediate the above mentioned changes. Comparing our results in mice, which report a stimulatory effect of AVP, but not VIP, on RFRP-3 neurons firing rate, to the earlier study in Syrian hamsters, which showed that VIP suppressed the number of c-Fos expressing RFRP-3 neurons in the evening while AVP had no effect on the RFRP-3 c-Fos expression, (Russo et al., 2015), it might be worth investigating whether there are species-dependent differences in the effect of circadian peptides on RFRP-3 neurons.

Conclusion

In conclusion, the current study adds to the evidence that RFRP-3 neurons are a converging site where daily and estrogenic signals are integrated and may be conveyed to the reproductive axis. Our current hypothesis is that the increased AVP input at late afternoon can induce an excitatory effect on the slow firing RFRP-3 neurons at diestrus, but is inefficient in further exciting the highly firing RFRP-3 neurons in proestrus. Given the

inhibitory effect of RFRP-3 on LH secretion (Kriegsfeld et al., 2006b; Pineda et al., 2010; Tsutsui et al., 2000), our findings support that RFRP-3 neurons facilitate the gating of the LH surge at the right time of the day and stage of the estrous cycle. However, given that RFRP-3 is now reported to also regulate food intake (Cázarez-Márquez et al., 2020) and stress response (Mamgain et al., 2020), we cannot exclude the possibility that RFRP-3 neurons may regulate female reproduction indirectly through metabolic- and stress-regulated mechanisms.

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Competing interests

The authors have no competing interests to declare.

Authors' contributions

E.A did the experimental work and wrote the manuscript; P.I. supervised and participated in the electrophysiological experiments; P.K. supervised and participated in the neuroanatomical experiments; G. A. provided the RFRP-Cre C57BL/6J mice and revised the

manuscript; A.K. supervised all the work and participated in the manuscript writing; V.S. designed the experiments, supervised all the work and participated in the manuscript writing.

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Chapter 3

Modulatory effect of vasoactive intestinal peptide on RFRP-3 neurons in female mice

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In preparation

Abstract

In mammals, female reproduction relies on the temporal control of interacting, estradiol-sensitive neural circuits that drive the hypothalamo-pituitary-gonadal (HPG) axis, in order to synchronize the preovulatory LH surge at the end of the ovarian follicular phase with the beginning of the active period. In this study, we used a combination of neuroanatomical and electrophysiological approaches to investigate whether vasoactive intestinal peptide (VIP) regulates Arg-Phe amide-related peptide (RFRP-3) neurons in a time- or estrous cycle stage-dependent manner in female mice. VIP-immunoreactive fibers made close appositions with RFRP-3 neurons, with the highest number of RFRP-3 neurons being contacted observed at the late afternoon and the lowest number observed at the day-to-nigh transition, with no difference between diestrus and proestrus. However, exogenous application of VIP on cultured hypothalamic sections did not change the RFRP neuron firing at the day-to-night transition neither in diestrus nor in proestrus. Together, these studies show that more RFRP-3 neurons are contacted by VIP fibers at late afternoon, but further experiments are required to decipher whether VIP regulates RFRP-3 neurons in the female mice.

Introduction

Female reproduction involves the integration of both daily timing and estrogenic signals in the hypothalamic- pituitary- gonadal (HPG) axis in order to generate a synchronized physiological output. Thus, in female rodents the preovulatory LH surge occurs at the proestrus stage when circulating estradiol (E2) levels are high and at a specific time window, i.e. at the beginning of the dark (active) period (Christian et al., 2005; Christian and Moenter, 2010; Everett and Sawyer, 1950). Although a hypothalamic population of neurons that release gonadotropin-releasing hormone (GnRH) drives the pituitary LH surge, these GnRH neurons lack E2 receptors (ERα) (Christian and Moenter, 2010; Leon and Tena-Sempere, 2015). Therefore, proper coordination of the HPG axis relies on two hypothalamic neuropeptidergic systems synthesizing the stimulatory kisspeptin (Kp) (Smith et al., 2005) and the inhibitory (Arg)(Phe) amide-related peptide-3 (RFRP-3) (Kriegsfeld et al., 2006; Poling et al., 2012), both of which operate upstream of the GnRH neurons and do express ERα.

In this study, we analysed the regulation of RFRP-3 neurons by VIP signals probably arising from the circadian clock located in the suprachiasmatic nucleus (SCN). RFRP-3 neurons are located in the dorsomedial hypothalamus (DMH) and project to GnRH (Henningsen et al., 2016; Kriegsfeld et al., 2006; Rizwan et al., 2012; Smith et al., 2008; Ubuka et al., 2012) and Kp neurons (Poling et al., 2013; Rizwan et al., 2012) that express the RFRP-3 receptor, GPR147. Central administration of RFRP-3 inhibits the GnRH neuron activity and suppresses the preovulatory LH release in female mammals (Ancel et al., 2017; Anderson et al., 2009; Clarke et al., 2008; Ducret et al., 2009; Henningsen et al., 2017; Johnson et al., 2007; Kriegsfeld et al., 2006; Pineda et al., 2010; Tsutsui et al., 2000). Furthermore, in female rodents, the RFRP-3 neuronal activity decreases during the day-to-night transition, thus at the time of the LH surge (Gibson et al., 2008; Henningsen et al., 2017; Poling et al., 2017).

The SCN conveys daily cues to GnRH neurons, either directly or indirectly through the Kp and RFRP-3 neurons. There is a monosynaptic connection between vasoactive intestinal peptide (VIP) neurons in the SCN and GnRH neurons (Beek et al., 1997; Horvath et al., 1998)

that express VIP_2 receptors (Smith et al., 2000). Moreover, VIP application activates GnRH neurons in a time- and E2-dependent manner (Christian and Moenter, 2008). Another pathway connects vasopressin (AVP) neurons in the SCN with the GnRH neurons via the preoptic Kp neurons that express V1a receptors (Vida et al., 2010; Williams et al., 2011). Indeed, AVP application activates Kp neurons in an E2-dependent manner (Piet et al., 2015). Recent studies have reported that RFRP-3 neurons also receive SCN peptidergic inputs. There is anatomical evidence for direct connections between SCN AVP neurons with RFRP-3 neurons in the female Syrian hamster (Russo et al., 2015) and mouse (Angelopoulou et al., 2021) and AVP application activates RFRP-3 neurons in a time-dependent manner in female mice (Angelopoulou et al., 2021).

The aim of the present study was to investigate the putative role of VIP in the temporal regulation of the RFRP-3 system in female C57BL/6J mice of different estrous cycle stages. In a first set of experiments, we analyzed whether there are time of the day- and estrous cycle stage-dependent changes in the number of VIP-ergic fiber projections on RFRP-3 neurons. In a second set of electrophysiological experiments, we investigated whether VIP regulates the electrical activity of RFRP-3/EYFP fluorescent protein labelled neurons in hypothalamic sections of transgenic RFRP/Cre female mice.

Materials and Methods

1. Animals

Adult female C57BL/6J mice (Charles River, France) and heterozygous RFRP-Cre mice on a C57BL/6J background (Mamgain et al., 2020) were kept two or three per cage under a 12-hour light: 12-hour dark cycle (lights on at 7:00 am, given as zeitgeber 0 (ZT0)) with controlled temperature (22°C) and *ad libitum* access to food and water. All protocols were reviewed by the Regional Committee for Ethics in Animal Experimentation and approved by the French Ministry of Education and Research (authorization #8452-2017010613574177v2).

2. Monitoring of estrous cycle and LH secretion

Female mice were studied at either proestrus or diestrus stage assessed by vaginal smears and circulating LH. Vaginal smears were performed daily at ZT2 during at least two consecutive cycles and only mice showing regular 4-5 day cycles were sampled on diestrus (appearance of leucocytes) or proestrus (appearance of nucleated epithelial cells). From each mouse a 4 µl blood sample was collected from the tail tip at the moment of sacrifice and this sample was diluted in 116 µL phosphate buffer saline with 0,25% tween-20. The LH concentration was measured by ELISA using anti-bovine LHβ as capture antibody (monoclonal antibody, 518B7, NHPP, Torrance, California), rabbit anti-mouse LH as first antibody (polyclonal antibody, rabbit LH antiserum, AFP240580Rb, NHPP), goat anti-rabbit IgG as secondary antibody (D048701–2, Dako Cytomation, Polyclonal Goat Anti-Rabbit, Denmark) and mouse LH as standard (mLH, AFP-5306A, NHPP) as previously described (Bahougne et al., 2020).

3. Neuroanatomical investigation of daily and estral regulation of RFRP-3 neurons

Tissue processing

Adult female mice at proestrus or diestrus stage were sacrificed by exposure to increasing concentration of CO_2 at six different time points (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20; n=5 per experimental point). After intracardiac blood puncture, mice were intracardially perfused with 10 mL phosphate buffer saline 0.1 M (PBS, pH 7.4) followed by 20 mL of periodate-lysine-paraformaldehyde fixative (formaldehyde 4%, $NaIO_4$ 10 mM and lysine 75 mM in 100 mM phosphate buffer, pH 7.3). The brains were collected, post-fixated in periodate-lysine-paraformaldehyde for 12 hours, washed out with PBS, dehydrated and embedded in polyethylene glycol as previously described (Klosen et al., 1993).

Twelve series of 12 µm-thick coronal brain sections were cut using a microtome throughout the DMH as presented in the Paxinos mouse brain atlas. For each mouse, one section in

every twelve (i.e. 1 section every 144 µm giving 6-7 DMH-containing brain sections) was rehydrated and mounted on a SuperFrost Plus (Menzel-Glaser, Braunschweig, Germany) slide. For each immunolabeling, DMH-containing slices of all mice of different time points and estrous stages were processed at the same time in order to limit variations in the labeling background.

Double VIP/RFRP-3 immunohistochemistry

The density of VIP fibers surrounding RFRP-3 neurons were assessed by dual immunohistochemistry. Brain sections were first incubated with a rabbit polyclonal antiserum raised against VIP (1:2000; Sigma-Aldrich Cat# HPA017324, RRID:AB_1858754) diluted in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20, for 24 hours at room temperature. Brain sections were washed with PBS, incubated with biotinylated donkey antirabbit (1:2000; Jackson Labs; in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20) for 1 hour and then washed again with PBS. Immunoreactive signal was amplified by a treatment with the avidin biotin complex coupled to peroxidase (1:250; Vector Laboratories) for 1 hour, then revealed using a solution of 0.5 mg/mL 3,3-diaminobenzidine (DAB; Sigma-Aldrich) diluted in water and 0.001% hydrogen peroxide urea (Sigma-Aldrich) for 5 minutes. Before performing the second immunolabelling, the antibodies were eluted with 2 x 15 minute washes in a solution of 100 mM glycine containing 0.3% Triton X-100 (pH 2.2). Brain sections were then incubated with a primary antibody directed against RFRP-3 (1:15000; rabbit anti-RFRP-3, University of Otago, NZ; RRID: AB_2877670; in a PBS buffer containing 10% donkey serum and 0.3% Tween 20) overnight at room temperature. The sections were then washed with PBS, incubated with biotinylated donkey anti-rabbit (1:2000; Jackson Labs; in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20) for 1 hour, and then washed again with PBS. The RFRP-3 signal was detected using streptavidin-peroxidase at 1/3000 (Roche) for 1 hour and revealed with Fast blue- BB (Sigma-Aldrich) for 7 minutes. Finally, sections were mounted with CC/mount (Sigma-Aldrich), dehydrated in toluene twice for 10 minutes, and mounted with Eukitt resin (Sigma-Aldrich).

Quantification of immunolabeled RFRP-3 cells and VIP-ergic fibers

In order to limit differences stemming from rostro-caudal variations only five sections located at comparable levels of the DMH were taken into account for the analysis. These five sections were selected based on neuroanatomical markers such as the median eminence, the tuberoinfundibular sulcus and the pituitary stalk. Counting was done by the first author unaware of animal's identity. For each mouse, the number of RFRP-3 neurons receiving direct VIP-fiber projections was counted manually. VIP-ergic appositions were defined as terminal fibers directly contacting RFRP-3 cell somas. For each mouse, the number of RFRP-3 neurons with close VIP-fiber appositions is given as a percentage of the total number of RFRP-3 neurons.

The density of VIP-ir fibers was quantified in selected regions of interest (ROI) bilaterally in the DMH defined by neuroanatomical landmarks (such as the median eminence, the tuberoinfundibular sulcus and the pituitary stalk), the DMH boundaries and the anatomical position of the RFRP-3 neurons. The density of VIP-fibers in the DMH was estimated by counting manually the number of points that a fiber crossed the intersections of a grid applied on the ROIs. For each mouse VIP-ir fiber density is given as total number of crossing points/total grid number of the ROIs.

4. Electrophysiological Investigation of the Effect of VIP on the Daily and Estral Regulation of RFRP-3 Neurons

In vivo labelling of RFRP-3 neurons

Labelling of RFRP neurons in adult female mice was performed using an adeno-associated virus containing a promoter upstream of a transcription-blocking cassette, followed by sequences encoding enhanced yellow fluorescent protein (pAAV5.EF1a.DIO.EYFP, 1 × 1013 pfu/mL, purchased from Addgene, catalog 27056-AAV5) in order to mediate the expression of EYFP exclusively in Cre-expressing RFRP neurons. Female RFRP-Cre mice were anaesthetized under a Zoletil (80 mg/kg) / Xylazine (10mg/kg) mixture and injected with

Metacam (5 mg/kg) and Bupivacaine (2 mg/kg) for analgesia. Then mice were placed in a stereotaxic apparatus in order to perform a bilateral stereotaxic injection of the virus targeting the DMH area. The skull was exposed and a Hamilton syringe loaded with 1 µL of AAV-EYFP was lowered into the DMH area according to the atlas of Paxinos and Franklin coordinates (-1.6 mm posterior to bregma, -0.5 mm lateral to midline, and -5.3 mm ventral to dura.). Injection was performed at a rate of 100 nL/min followed by a 10-minute pause before removing the syringe. Mice were administered Metacam (2 mg/kg) dissolved in water for 3 days as a post-operative analgesic treatment. Preliminary tests indicated that the highest level of EYFP expression in RFRP-3 neurons was observed three weeks after the injections with the percentage of RFRP-3-ir neurons expressing EYFP estimated at 68.3 ± 2.5 % (n= 4 mice).

Brain slice preparation

Three weeks after the AAV injections, mice were anaesthetized with urethane (i.p. 1.9 g/Kg) at two estrous stages (diestrus or proestrus) and two different time points ZT4-ZT5 (in vitro recording at ZT6-ZT10) or ZT9-ZT10 (in vitro recording at ZT11-ZT14, time of the preovulatory LH surge at the proestrus stage). An intracardic perfusion was performed with oxygenated iced-cold sucrose-artificial cerebrospinal fluid containing 248 mM sucrose, 11 mM glucose, 26 mM $NaHCO_3$, 2 mM KCl, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 1.3 mM $MgSO_4$, 5 mM kynurenic acid). The solution was continuously bubbled with 5% CO_2 and 95% O_2. Brains were quickly removed and 300 µm thick coronal slices were cut at the level of the DMH using a vibratome (Leica VT1200S). Slices were then transferred in artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 26 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose at room temperature for 1 hour before starting recordings.

RFRP cell-attached recordings

After recovery, slices were placed to the recording chamber under an upright microscope fitted for epifluorescence (Zeiss, Axioskop) and were continuously perfused with oxygenated aCSF (1.0-1.5 mL/min). EYFP/RFRP neurons were first visualized by brief fluorescent illumination. Spontaneous firing was recorded using the minimally invasive cell-attached loose patch configuration. Recording electrodes (3-5 MΩ) were pulled from borosilicate glass capillaries (Harvard apparatus) using a P1000 electrode puller (Sutter Instruments) and filled with aCSF. Spontaneous spikes were recorded in the voltage clamp mode using an Axopatch 200B amplifier (Axon Intruments). Signals were low-pass filtered (5 kHz) and acquired with Clampex software (Molecular devices). Current traces were digitized (10 kHz) and stored on the hard drive of a personal computer. All experiments were performed at room temperature (22–24 °C). The spontaneous firing of neurons was recorded in control aCSF for more than 5 minutes before bath application of VIP (1 µM in aCSF; Euromedex) for 3 minutes. Spontaneous spikes were detected using the threshold-crossing method in pClamp 10 (Molecular device). Data calculated were the Mean Interspike Interval (ISI) and the Coefficient of Variation of ISI (CV = standard deviation/mean ISI) as an indicator of spike train regularity. Recordings in which the baseline firing was not stable were not included in the analysis.

5. Data analysis

Statistical analysis was performed with GraphPad Prism 6. All values are given as mean ± SEM. For neuroanatomical results, Two-way ANOVA was used to assess significant variations among different time points and estrous stages, followed by Tukeys multiple comparisons test. For the electrophysiological investigation, the firing pattern of individual RFRP neurons was determined with criteria previously used for Kp neurons: silent neurons displaying no spontaneous activity, tonic neurons exhibiting regular firing with the SD of their interspike intervals less than 100 msec, bursting neurons having more than 50% of their spikes occurring in bursts, and all other neurons classified as irregular (de Croft et al.,

2012; Ducret et al., 2010). In the VIP experiments, the relative ISI (to a 5-minute baseline) was averaged in 1 minute-bin and drug effect was tested with repeated measures Two-way ANOVA (RM-ANOVA). Comparison of mean firing frequency between baseline and following VIP application was performed using a paired Student t-test. Differences were considered significant for $P < 0.05$.

Results

1. Neuroanatomical Investigation of Daily and Estral Regulation of VIP-ergic innervation on RFRP Neurons in Female C57BL/6J Mice

This experiment investigated the occurrence of daily and estral variations in VIP-ergic fiber inputs on RFRP3-ir neurons in female mice. The number of RFRP-3-immunostained neurons showed a significant daily variation (Two Way ANOVA, *Daytime*: $F(5, 48) = 2.990$, $p = 0.0198$; Figure 1A), with mean values being a little bit lower during the dark period in both proestrus and diestrus. We found no effect of estrous stage or the *Daytime * Estrous* stage interaction (Two Way ANOVA, *Estrous stage*: $F(1, 48) = 3.189$, $p = 0.0805$; Interaction: $F(5, 48) = 0.467$, $p = 0.7988$; Figure 1A). Analysis of VIP-ir fiber density in the DMH showed no daily changes neither in proestrus nor in diestrus (Two-way ANOVA, *Daytime*: $F(5, 48) = 0.7243$, $p = 0.6085$; *Estrous stage*: $F(1, 48) = 0.0026$, $p = 0.9595$; Figure 1B). Quantification of the percentage of RFRP-3 neurons receiving direct VIP-ir fiber appositions showed that the highest percentage was observed at ZT8 and the lowest percentage at ZT12, with no difference between proestrus and diestrus (Two-way ANOVA, *Daytime*: $F(5, 48) = 27.32$, $p < 0.0001$; *Estrous stage*: $F(1, 48) = 0.0345$, $p = 0.8534$; Figure 1C, Figure 1D).

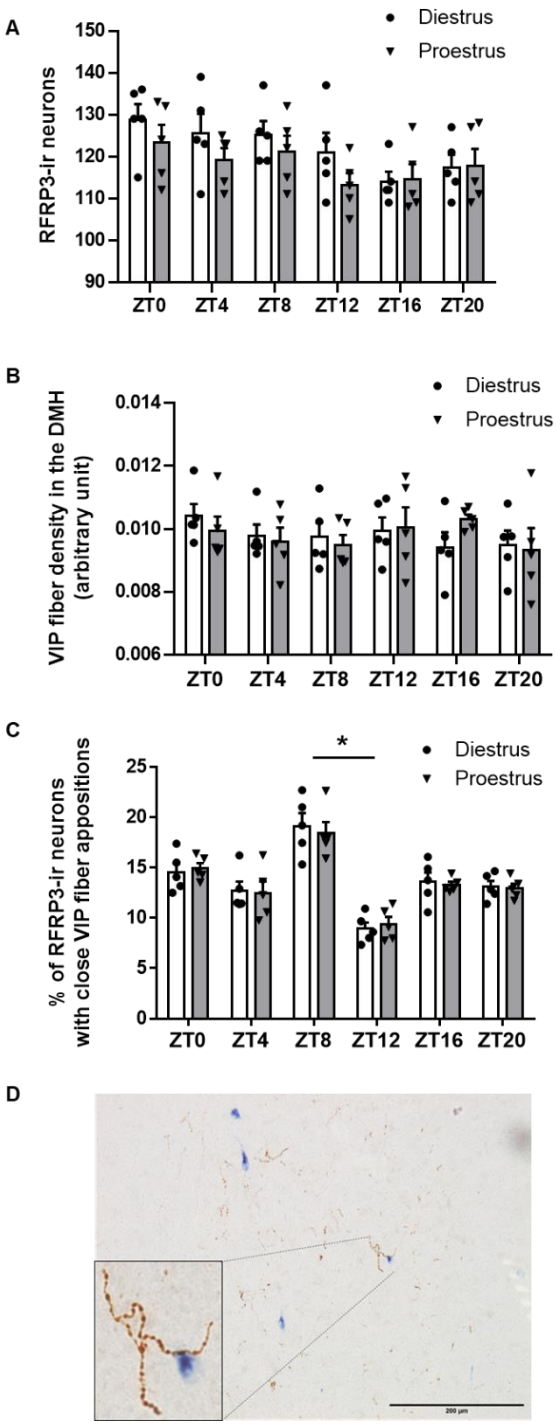
A
Diestrus
Proestrus
RFRP3-ir neurons
150
140
130
120
110
100
90
ZT0
ZT4
ZT8
ZT12
ZT16
ZT20
B
Diestrus
Proestrus
VIP fiber density in the DMH
(arbitrary unit)
0.014
0.012
0.010
0.008
0.006
ZT0
ZT4
ZT8
ZT12
ZT16
ZT20
C
*
Diestrus
Proestrus
% of RFRP3-ir neurons
with close VIP fiber appositions
25
20
15
10
5
0
ZT0
ZT4
ZT8
ZT12
ZT16
ZT20
D

Figure 1. *Vasoactive intestinal peptide (VIP)-immunoreactive (ir) fibers and RFRP-3-ir neurons in the dorsomedial hypothalamus of female C57BL/6J mice sampled at different time points of the day of diestrus and proestrus. (A) Number of RFRP-3-ir neurons in the DMH; (B) VIP-ir fiber density (arbitrary unit) in the DMH; (C) Percentage of RFRP-3-ir neurons with close VIP-ir fiber appositions; (D) Photograph shows RFRP-3-ir neurons and VIP-containing fibers at ZT8 (16:00) on the day of proestrus; scale bar = 200 µm. Data are presented as mean ± SEM of n = 5 mice for each experimental point. Two-way ANOVA was used to assess significant variations among different time points and estrous stages, followed by Sidak's multiple comparisons test. Daily differences with a value of P < 0.05 were considered as significant and are indicated by *. Cycle stage differences were insignificant (P > 0.05).*

2. Electrophysiological investigation of the regulation of RFRP-3 neuron firing activity by VIP in diestrus and proestrus in female C57BL/6J mice

In this experiment we assessed the regulation of RFRP-3 neuronal activity by VIP during the day-to-night transition, a period with the largest variation in the VIP-ergic input on RFRP-3 neurons, in both diestrus and proestrus. All recorded RFRP-3 neurons exhibited an irregular spontaneous firing pattern. Bath application of 1µM VIP had no effect on the ISI (RM-ANOVA, for relative ISI, *time*: F(13, 182) = 0.4826, p = 0.9323, n=10 for diestrus and n=6 for proestrus; Figure 2B) and on the mean firing rate of RFRP-3 neurons (paired Student's t-test, time, diestrus: p = 0.488, n=10; proestrus: p=0.747, n=6; Figure 2C) at either estrous cycle stage.

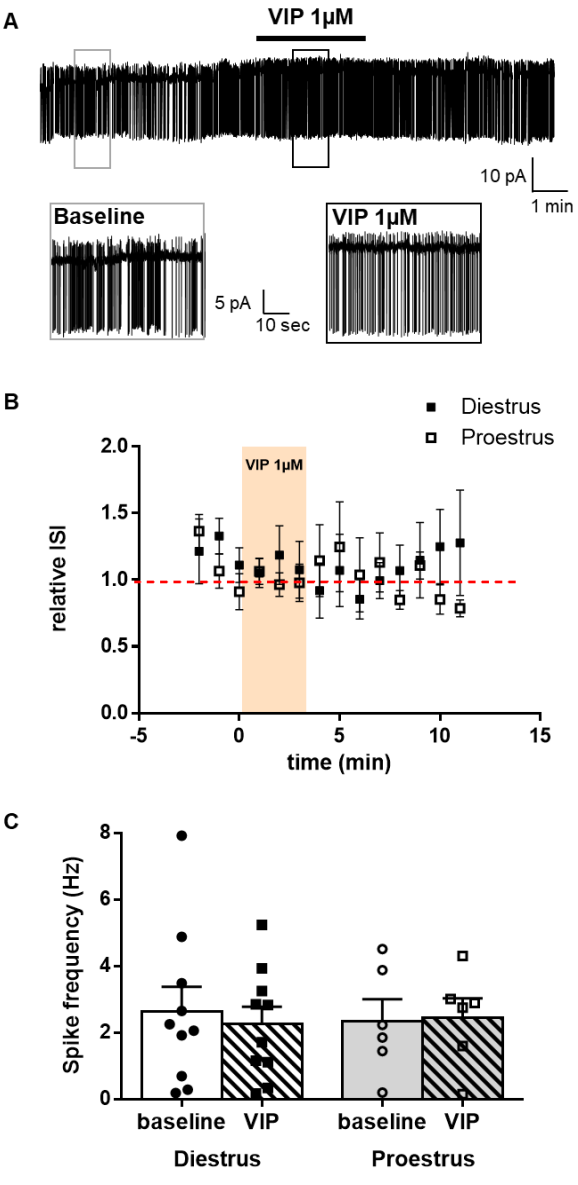

Figure 2. *Effect of VIP on RFRP-3 electrical activity in the dorsomedial hypothalamus at different stages of the estrous cycle during the day-to-night transition in female C57BL/6J mice. (A) Sample trace illustrating the effect of VIP on RFRP-3 neuron firing in Diestrus at ZT11-14. (B) Average time course of relative InterSpikeInterval (ISI) in response to VIP (1µM) application of RFRP-3 neurons in the DMH during the day of diestrus (n=10 neurons out of 7 mice) and during the day of proestrus (n=6 neurons out of 4 mice) at ZT11-14. (C) Summary bar graph of the VIP effect on mean spike frequency of RFRP-3 neurons in the DMH at ZT11-14 during the day of diestrus and proestrus.*

Data are presented as mean ± SEM. Repeated measures Two-way ANOVA was used to assess significant variations in the relative ISI among different time points in diestrus and proestrus. A paired Student t-test was used to compare RFRP mean firing frequency between baseline and after VIP application.

Discussion

In mammals, female reproduction exhibits daily and ovarian cycles driven by E2-sensitive mechanisms in the hypothalamus (Herbison, 2020; Pinilla et al., 2012; Simonneaux et al., 2017). In this study, we investigated in female mice whether RFRP-3 neurons, reported to regulate GnRH neuronal activity and LH release (Angelopoulou et al., 2019), act as an interface for VIP-ergic fibers and E2 feedback, in order to convey daily and ovarian signals to the HPG axis. Using a neuroanatomical approach, we report a decrease in the number of RFRP3-ir cells with close VIP-ir fiber appositions at the day-to-night transition, with no difference between the diestrus and proestrus stages. This change in VIP input occurs concomitantly with a decrease in RFRP-3 neuron activation and an increase in the number of RFRP3-ir cells with close AVP-ir fiber appositions as we showed previously (Angelopoulou et al., 2021). Surprisingly, however, we found no effect of VIP on the firing rate of RFRP-3 neurons at least at the investigated day-to-night transition (ZT11-14) in both diestrus and proestrus, using the electrophysiological analysis.

In female mammals, the SCN generates daily signals to trigger the LH surge at the beginning of the active phase. A significant amount of evidence links the circadian peptide VIP with the SCN-driven daily regulation of the HPG axis. Central administration of VIP suppresses LH secretion during the LH surge (Weick and Stobie, 1992) and VIP antiserum treatment causes a delay and reduction in the amplitude of the LH surge (van der Beek et al., 1999). Earlier studies demonstrated a monosynaptic connection between VIP-ergic SCN neurons and GnRH neurons (Beek et al., 1997; Horvath et al., 1998) that express the VIP receptor VIP_2 (Smith et al., 2000). Also, the majority of GnRH neurons that express c-Fos during the onset of the LH surge in proestrus receive VIP-containing fibers (van der Beek et al., 1994) and suppression of VIP synthesis in the SCN, through administration of antisense oligonucleotides, leads to significantly decreased GnRH neuron activation (Gerhold et al.,

2005; Harney et al., 1996). Moreover, electrophysiological studies showed that VIP increases GnRH neuron firing during the onset of the LH surge, but not during its peak, while administration of a VIP receptor antagonist decreased GnRH neuron firing (Christian and Moenter, 2008; Piet et al., 2016). Interestingly, VIP innervation on GnRH neurons is sexually dimorphic, with female rodents exhibiting a higher percentage of GnRH neurons contacted by VIP fibers (Horvath et al., 1998). Moreover, the percentage of GnRH neurons receiving VIP-ergic contacts increases following puberty (Kriegsfeld et al., 2002). Altogether, these studies suggest a direct effect of VIP on the GnRH system. Yet, GnRH neurons do not express ERα (Christian and Moenter, 2010; Wintermantel et al., 2006) and their responsiveness to VIP does not change across the estrous cycle (Piet et al., 2016). Therefore, VIP may also act indirectly on GnRH neurons, through ERα-expressing neural circuits that can convey both daily and estrogenic signals to the HPG axis.

An earlier study in female Syrian hamsters showed that SCN-derived VIP fibers make close appositions with RFRP-3 neurons and that central administration of VIP suppresses the RFRP-3 neuron activity (c-Fos expression) in the evening, but not in the morning. However, in that same study it was reported that the majority of RFRP-3 neurons do not express the VIP receptors (VPAC1 and VPAC2), therefore indicating that VIP may inhibit RFRP-3 neurons indirectly (Russo et al., 2015). We confirmed, in female mice, that RFRP-3 neurons receive close VIP-ergic fiber appositions, and we furthermore reported that the number of RFRP-3 neurons with close VIP-ergic fiber appositions increased during late afternoon and decreased during the day-to-night transition. Surprisingly, the daily patterns of VIP mRNA and protein expression in the SCN, which peak during the middle of the dark period and decrease during the light period (Dardente et al., 2004; Shinohara et al., 1999, 1993), were not reflected in daily variations in VIP fiber density in the DMH. The presence of daily variations in the number of RFRP-3 neurons with close VIP-ergic fiber appositions in spite of a lack of daily variations in the VIP fiber density in the DMH might be due to the change in the number of RFRP-3 neurons over the day/night cycle, which shows a moderate decrease during the dark period. On the other hand, our observation that the overall VIP-ergic fiber input on RFRP-3 neurons was similar in both diestrus and proestrus, is in agreement with

the E2-independent pattern of VIP gene expression in the SCN as observed in female rats (Krajnak et al., 1998).

Because the presence of peptidergic fiber appositions on neuronal cell bodies does not prove that they make synapses or that there is a direct post-synaptic effect of the peptide, we next, examined the effect of VIP on RFRP-3 neuronal firing activity. We tested the effect of VIP during the day-to-night transition as in this period we found the largest daily changes in the VIP input on RFRP-3 neurons. We found no effects of VIP on the firing rate of RFRP-3 neurons at this time point, neither in diestrus nor in proestrus. Future experiments should investigate the effect of VIP at other time points in order to evaluate whether there are daily changes in its effect on RFRP-3 neuron firing rates, since it was reported in female Syrian hamsters that administration of VIP suppresses c-Fos expression in RFRP-3 neurons in the evening but not in the morning (Russo et al., 2015). Alternatively, as shown in our previous study (Angelopoulou et al., 2021), there might also be a mismatch between the peptide effect on the RFRP-3 neuronal c-FOS expression and firing rate, as both phenomenon involve different signaling pathways. Finally, we cannot exclude species-dependent differences in the peptide effect on RFRP-3 neurons.

In conclusion, the current study demonstrates that there are daily variations in the VIP-ergic input on RFRP-3 neurons in female mice. Given that in Syrian hamsters most RFRP-3 neurons do not express VIP receptors, future experiments should address whether this is the case also in mice. Moreover, we showed that VIP does not alter the RFRP-3 neuron firing rates during the day-to-night transition. Thus, the effect of VIP should be examined at other time points in order to unravel whether this peptide has a functional role in the daily regulation of the RFRP-3 system. Based on the current results we hypothesize that VIP inhibits RFRP-3 neurons during the day-to-night transition, resulting in a decrease in the amount of cellular RFRP-3 protein expression.

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Competing interests

The authors have no competing interests to declare.

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PART III

EFFECT OF CIRCADIAN DISRUPTION ON FEMALE REPRODUCTION

Chapter 4

Impact of Circadian Disruption on Female Mice Reproductive Function

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Abstract

In female mammals, cycles in reproductive function depend both on the biological clock synchronized to the light/dark cycle and on a balance between the negative and positive feedbacks of estradiol, whose concentration varies during oocyte maturation. In women, studies report that chronodisruptive environments such as shiftwork may impair fertility and gestational success. The objective of this study was to explore the effects of shifted light/dark cycles on both the robustness of the estrous cycles and the timing of the preovulatory luteinizing hormone (LH) surge in female mice. When mice were exposed to a single 10-hour phase advance or 10-hour phase delay, the occurrence and timing of the LH surge and estrous cyclicity were recovered at the third estrous cycle. By contrast, when mice were exposed to chronic shifts (successive rotations of 10-hoursour phase advances for 3 days followed by 10-hour phase delays for 4 days), they exhibited a severely impaired reproductive activity. Most mice had no preovulatory LH surge at the beginning of the chronic shifts. Furthermore, the gestational success of mice exposed to chronic shifts was reduced, because the number of pups was 2 times lower in shifted than in control mice. In conclusion, this study reports that exposure of female mice to a single phase shift has minor reproductive effects, whereas exposure to chronically disrupted light/dark cycles markedly impairs the occurrence of the preovulatory LH surge, leading to reduced fertility.

Introduction

Reproductive activity in female mammals displays regular cycles (menstrual cycles in women, estrous cycles in rodents) driven by complex interactions between hypothalamic neuropeptides, pituitary gonadotropins (LH [luteinizing hormone] and FSH [follicle-stimulating hormone]), sex steroid hormones produced by the ovaries, and the circadian system. The final output of this regulatory process is to combine the production of a mature oocyte (ovulation) with a receptive reproductive tract that will ensure correct embryonic development (Simonneaux and Bahougne, 2015). During the first part of the reproductive cycle (follicular phase in women; metestrus–diestrus in rodents), the progressive increase in FSH contributes to the recruitment and development of ovarian follicles leading to a gradual increase in estradiol secretion. The second part of the reproductive cycle (luteal phase in women; proestrus–estrous in rodents) begins with a marked and transient surge of LH secretion which triggers ovulation a few hours later in rodents, and between 9 and 23 hours later in women. This preovulatory LH surge takes place approximately every 28 days in women and every 4 to 5 days in female rodents. Additionally, the LH surge usually occurs at the end of the resting period, thus at the late day/early night transition in nocturnal rodents (Bronson and Vom Saal, 1979; Moline et al., 1981; Smith et al., 1975) and the late night/early morning transition in diurnal rodents (McElhinny et al., 1999) and women (Cahill et al., 1998; Kerdelhué et al., 2002). Experimental studies in rodents have demonstrated that a functional circadian system is required for a proper reproductive activity. Ablation of the suprachiasmatic nuclei (SCN), where the master circadian clock is located (Brown-Grant and Raisman, 1977), or clock gene mutations (Miller et al., 2004) severely impair estrous cyclicity and fertility.

Nowadays, an increasing number of women work in conditions of disrupted light/dark cycles (almost 20% of female workers in industrial countries) that have overall negative impacts on health (Boivin and Boudreau, 2014; Pan et al., 2011; Pati et al., 2001). Some epidemiological studies have reported that shiftwork is associated with irregular menstrual cycles, lower fertility, and an increased risk of miscarriage (Gamble et al., 2013; Simonneaux et al., 2017; Simonneaux and Bahougne, 2015). Yet to date, only a few fundamental studies

in female rodents have evaluated the effect of circadian disruption on reproductive parameters (Finkelstein et al., 1978; Moline and Albers, 1988; Yoshinaka et al., 2017). In this context, the objective of this study was to assess the effects of single phase shifts (mimicking a large transmeridian shift or “jetlag”) or chronic shifts (mimicking shiftwork conditions) on the preovulatory LH surge and estrous cycle characteristics in individual adult female mice.

Material and Methods

Animals

Eight-week-old virgin C57BL/6J female mice were obtained from the Charles River Laboratories (Écully, France). Upon arrival, mice were placed in groups of 2 or 3 per cage and quarantined for 2 weeks. Mice were kept at 22°C on a 12-hour light/12-hour dark (12L/12D) schedule, with lights on at zeitgeber time 0 (ZT0) and lights off at ZT12. Water and food were given ad libitum unless otherwise specified. Two weeks before experimentation, mice were manipulated and habituated to regular vaginal smears and blood sampling.

Ethics approval

All experimental procedures were approved by the local ethics committee (CREMEAS) and the French National Ministry of Education and Research (authorization # 2015021011396804).

Experimental protocols

Mice were assigned to different experimental protocols.

Control group (n = 9).

Control group mice were kept under a stable 12L/12D cycle. After the habituation period, 2 consecutive estrous cycles were followed, and 4 µL of tail tip blood was sampled every hour from ZT8 to ZT16 on the day of proestrus for LH measurement.

Single phase advance (n = 9).

Mice were initially kept under a 12L/12D cycle. After a day of proestrus, light onset was advanced by 10 hours and the following estrous cycles were monitored during the next 3 cycles with 4 µL of tail tip blood sampled every hour on the day of proestrus from ZT8 to ZT16 for LH measurement.

Single phase delay (n = 9).

Mice were initially kept under a 12L/12D cycle. After a day of proestrus, light onset was delayed by 10 hours and the following estrous cycles were followed for 3 cycles with 4 µL of tail tip blood sampled every hour on the day of proestrus from ZT8 to ZT16 for LH measurement.

Chronic shifts (n = 9).

Mice were initially kept under a 12L/12D cycle and were monitored during 2 consecutive estrous cycles with 4 µL of tail tip blood sampled every hour during the second proestrus for LH measurement. The day following the second proestrus, mice were submitted to a 10-hour phase advance for 3 days followed by a 10-hour phase delay for 4 days, and this rotating condition was maintained for up to 9 months. This duration was chosen because we previously reported that both estrous cycle and timing of LH surge are stable up to 9 months in mice (Bahougne et al., 2020). During the shift, water was given ad libitum, but food was given only during the 12-hour dark phase to help mice resynchronize to the new 12L/12D schedule (Bartol-Munier et al., 2006). Two consecutive estrous cycles and LH secretion profiles at proestrus were monitored at different periods: at the beginning of the chronic shifts (the first 2 proestrus after the chronic shifts were started), at the sixth month of chronic shifts, then after 9 months just after cessation of the chronic shifts.

Fertility assessment after chronic shifts (n = 28).

Female mice were either kept under a stable 12L/12D or submitted to chronic shifts as stated above for 4 weeks (n = 14 for each condition, with 2 mice per cage). Thereafter all female mice were kept in a stable 12L/12D cycle and 1 male mouse (n = 14, kept under a stable 12L/12D cycle from birth) was added in each cage for 1 week. The mice were not manipulated during the whole procedure to avoid the negative impact of stress on fertility. The number of gestated mice and the number of pups per litter were measured in the following weeks.

Monitoring of estrous cycle and LH secretion

Estrous cycles and LH secretion were monitored as described previously (Bahougne et al., 2020). In C57BL/6J female mice kept under a stable 12L/12D schedule, estrous cycles monitored by vaginal smear exhibit a period of 4 to 5 days divided into 3 phases according to vaginal cytology: diestrus characterized by leukocytes and nucleated cells with leukocytic predominance; proestrus (during which the preovulatory LH surge occurs) characterized by oval nucleated epithelial cells with some cornified squamous epithelial cells; and estrus characterized by a predominance of cornified squamous epithelial cells (Nelson et al., 1982). Vaginal smears were performed twice each day, around ZT2 and ZT11, during at least 2 consecutive estrous cycles. When a preovulatory LH surge was expected (on proestrus day), a 4-µL blood sample was taken hourly from the tail tip (<1 mm) starting at 4 hours before (ZT8) until 4 hours after (ZT16) the light/dark transition. Each blood sample was immediately diluted in 116 µL of phosphate-buffered saline 1× + 0.25% Tween-20) and stored at –80°C until LH assay.

LH assay

LH concentrations were determined by a highly sensitive enzyme-linked immunosorbent assay (Steyn et al., 2013) using antibovine LHβ as capture antibody (518B7, NHPP, Torrance, CA, USA), rabbit antimouse LH as first antibody (AFP240580Rb, RRID:AB_2665533, NHPP), goat antirabbit immunoglobulin G as secondary antibody (D048701-2, Dako Cytomation, Denmark), and mouse LH as standard (AFP5306A, NHPP). Four given concentrations of LH (1, 10, 20, and 30 ng/mL) were used as quality controls. The intra-assay variation was 15% and the interassay variation was 12%.

Data analysis

To assess the effect of 1 shift (advance or delay) in the estrous cycle and LH surge, mice were monitored during 3 successive estrous cycles and were compared with each other and with the control group. To assess the effect of chronic shifts on the estrous cycle and LH surge, mice were monitored longitudinally before, at the beginning, at 6 months, and after

9 months just after the cessation of the chronic shifts, and these 3 periods were compared with the period before the chronic shifts.

For each group, the robustness of the estrous cycles was characterized by different parameters (Nelson et al., 1989): the number of acyclic mice (acyclic mice being those exhibiting 1 estrous cycle ≥10 days, except if this long cycle is followed by 1 consecutive cycle <10 days, or if it is associated with an LH surge); the mean duration (± standard error of the mean [SEM]) of the estrous cycles in days (after exclusion of acyclic mice); and the percentage of mice with irregular cycles (cycles being defined as irregular when the length of 2 consecutive cycles differs by more than 1 day).

The preovulatory LH surge occurrence, timing, and amplitude were assessed by measuring individual circulating LH for up to 3 consecutive proestrus. For each group, the pattern of LH secretion was characterized by different parameters: mean ± SEM circulating LH values (ng/mL) at different ZT; mean ± SEM ZT of the peak; mean ± SEM ZT of the beginning of the surge (estimated by LH value >4 ng/mL); and mean ± SEM peak amplitude (when data are available).

Statistical analysis was performed using GraphPad Prism 8 (San Diego, CA, USA). Assumptions were taken into account (all assumptions were verified). The D'Agostino and Pearson test was used to check normality. Homoscedasticity was verified with Bartlett's test. Statistical comparisons were made with an analysis of variance (ANOVA). Newman–Keuls post hoc tests were performed as appropriate to determine specific interactions. If assumptions did not hold, a nonparametric Kruskall–Wallis test was performed, and post hoc comparisons were made with the Dunn test. For occurrence of preovulatory LH surge, statistical comparisons were made with the chi-square test. The significance level was set at $P \leq .05$.

Results

Effect of a single 10-hour phase advance or 10-hour phase delay on the estrous cycle and preovulatory LH surge

Mice submitted to a single 10-hour phase advance or delay kept displaying estrous cycles, although the length of the second estrous cycle after the phase advance (5.9 ± 0.6 days) or delay (6.2 ± 0.7 days) was significantly longer than in the control group (4.6 ± 0.2 days) (Fig. 1A). The percentage of irregular mice (intraindividual variability) was higher in the phase advance (37% and 25% on the second and third cycle) and phase delay (22% on the second and third cycles) groups than in the control group (11%) (Fig. 1A). No mice were acyclic.

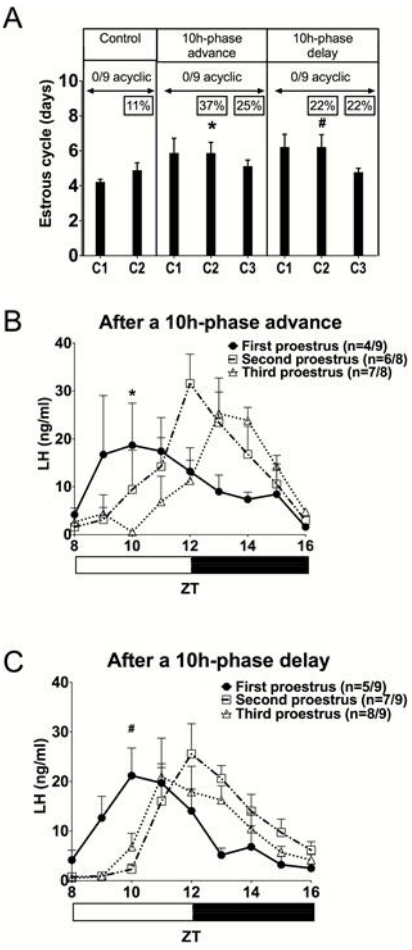

Figure 1. *Longitudinal monitoring of estrous cycles and preovulatory LH surges of C57BL/6J female mice kept in a stable 12 light/12 hours dark cycle (control) or exposed to a single 10-hour phase advance or a single 10-hour phase delay. (A) Monitoring of estrous cycle duration and mice regularity. Estrous cycle duration (black bars) is given as mean number of days ± SEM (n = 9 on each groups). Values in square boxes indicate the percentage of irregular mice. Values above the arrow indicate the number of acyclic mice. In the phase advance group, one mouse was followed only during the first estrus cycle because it was in permanent diestrus during 10 days before an LH surge was observed, followed by a 2-day cornified stage, making the experimental period too short to observe two successive estrous cycles. Duration of the second estrous cycle was significantly higher after the phase advance (*P = .019, Kruskall Wallis/Dunn test) and after the phase delay (#P = .044, Kruskall Wallis/Dunn test) than the control group. (B,C) Mean LH secretion between ZT8 and ZT16 during the first, second and third proestrus after a 10-hour phase advance (B) or a 10-hour phase delay (C). The ZT of the first preovulatory LH surge peak was significantly earlier than the ZT of the third preovulatory LH surge after the phase advance (*P = .0327; Newman–Keuls test, ANOVA test F (2, 12) = 4.609) or the phase delay (#P = .0167 Newman–Keuls test, ANOVA test F (2, 17) = 5.256). Only mice exhibiting an LH surge were included. Time is given in ZT (zeitgeber time), with ZT0 as time of light onset and ZT12 as time of light offset; white and black bars under the graphs indicate periods of light and darkness, respectively.*

The individual monitoring of LH secretion showed that after the acute shift, a significant number of mice did not exhibit an LH surge during the first cycle (5/9 after advance; 4/9 after delay) or even the second cycle (2/8 after advance; 2/9 after delay). At the third cycle, however, the number of mice exhibiting an LH surge was similar in the control (8/9), phase advance (7/8), and phase delay (8/9) groups (Figs. 1B and 1C, and Table 1). In the phase advance and delay groups, when LH surges were observed, the first LH surge was significantly earlier (ZT of the LH peak at 10.6 ± 0.9 hours for the phase advance group and 10.6 ± 0.4 hours for the phase delay group) than in the control group (ZT of the LH peak at 13 ± 0.3 hours) (Figs. 1B and 1C, and Table 1). Analysis of individual LH (Figs. 2A–C) values showed that mice in all 3 groups exhibited a comparable LH surge pattern at the second (control) or third (phase advance or delay) proestrus. Overall, at the third proestrus after a single phase advance or phase delay, the preovulatory LH surge characteristics (percentage of occurrence, ZT of the peak, and amplitude at the peak) were similar to those of the control group (Table 1).

Table 1. Characteristics of the preovulatory LH surge in c57bl/6j female mice kept in a stable 12-hour light/12-hour dark cycle (control), or during 3 estrous cycles after a 10-hour phase advance (phase advance) or a 10-hour phase delay (phase delay).

	Control	Phase advance			Phase delay		
	Second proestrus	First proestrus	Second proestrus	Third proestrus	First proestrus	Second proestrus	Third proestrus
LH surge occurrence (%) (n: number of mice with a surge)	89 (n = 8/9)	44 (n = 4/9)	75 (n = 6/8)	88 (n = 7/8)	55 (n = 5/9)	78 (n = 7/9)	89 (n = 8/9)
LH value (ng/mL) at the peak (n: number of mice with a visible peak)	28.3 ± 3.8 (n = 6/8)	30.7 ± 9.9 (n = 3/4)	42.3 ± 6.3 (n = 5/6)	34.7 ± 5 (n = 7/7)	28.8 ± 3 (n = 5/5)	35.5 ± 3.9 (n = 7/7)	34.5 ± 4.6 (n = 8/8)
ZT of the peak (n: number of mice with a visible peak)	13 ± 0.4 (n = 6/8)	10.6 ± 0.9[a] (n = 3/4)	11.8 ± 0.8 (n = 5/6)	13.1 ± 0.5[b] (n = 7/7)	10.6 ± 0.4[a] (n = 5/5)	12.4 ± 0.4 (n = 7/7)	12.3 ± 0.4[b] (n = 8/8)
ZT of LH surge onset (LH > 4 ng/mL)	11.9 ± 0.4	9.8 ± 1.4	11.2 ± 0.5	11.9 ± 0.6	9.2 ± 0.7	11.4 ± 0.4	11.3 ± 0.5

Only mice exhibiting an LH surge were included.

Abbreviations: LH, luteinizing hormone; ZT, zeitgeber time.

[a]The mean ZT of the LH surge peak was significantly earlier during the first proestrus after the phase advance and after the phase delay when compared to the control group (Newman-Keuls test, P = 0.0082; ANOVA test F (2, 11) = 7.674). [b]The mean ZT of the third preovulatory LH surge peak was significantly later when compared to the mean ZT of the first preovulatory LH surge after the phase advance (P = 0.0327; Newman-Keuls test, ANOVA = 4.609) or the phase delay (P = 0.0167 Newman–Keuls test, ANOVA test F (2, 17) = 5.256).

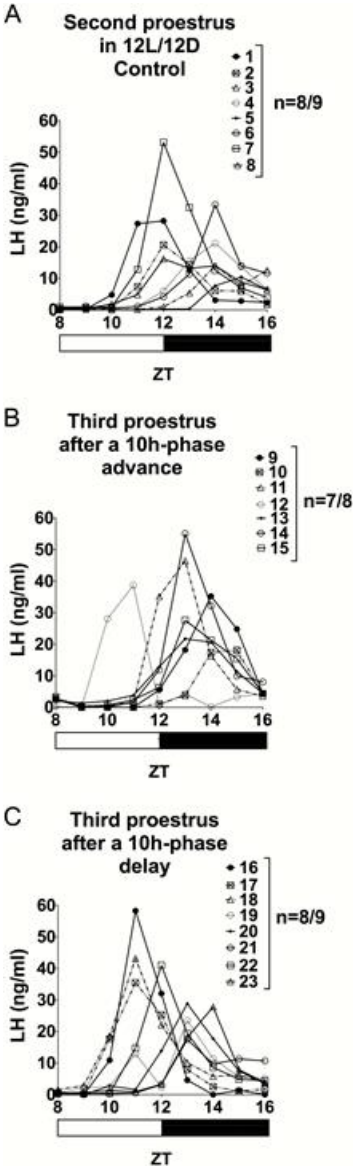

Figure 2. *Individual LH values at the preovulatory surge in C57BL/6J female mice (A) at their second proestrus in a stable 12-hour light/12-hour dark cycle (mice 1–8), (B) at their third proestrus after a 10-hour phase advance (mice 9–15), and (C) at their third proestrus after a 10-hour phase delay (mice 16–23). Only mice exhibiting an LH surge are shown. Time is given in ZT (zeitgeber time), with ZT0 as time of light onset and ZT12 as time of light offset; white and black bars under the graphs indicate periods of light and darkness, respectively.*

Effect of chronic shifts on estrous cycle and preovulatory LH surge

Nine mice were kept under a stable 12L/12D cycle and then submitted to chronic shifts of 10-hour phase advance for 3 days followed by 10-hour phase delay for 4 days. This phase advance and delay pattern was then repeated for up to 9 months. Before the shift, all mice exhibited estrous cycles, but once exposed to chronic shifts, 1 mouse became acyclic at the beginning of the shift, another 1 at 6 months in chronic shifts, and then 4 mice after 9 months of chronic shifts (Fig. 3A). The mean duration of 2 consecutive estrous cycles did not change significantly in the cycling mice exposed to various duration of chronic shifts (5.9 ± 0.7 and 5.4 ± 0.6 days at the beginning; 8.8 ± 1.7 and 5.5 ± 0.5 days at 6 months; and 6.3 ± 1.0 and 7.0 ± 1.3 days after 9 months of chronic shifts) compared with their estrous cycle duration before the chronic shifts (5.1 ± 0.2 days). However, prevalence of irregular mice increases (intraindividual variability) with time in chronic shifts (50% at the beginning, 63% at 6 months, and 75% after 9 months), compared with 22% irregular cycles before the chronic shifts (Fig. 3A). Overall, the number of acyclic mice and the number of irregular mice were significantly increased after 9 months of chronic shifts when compared with before the chronic shifts ($P < .05$, chi-square test).

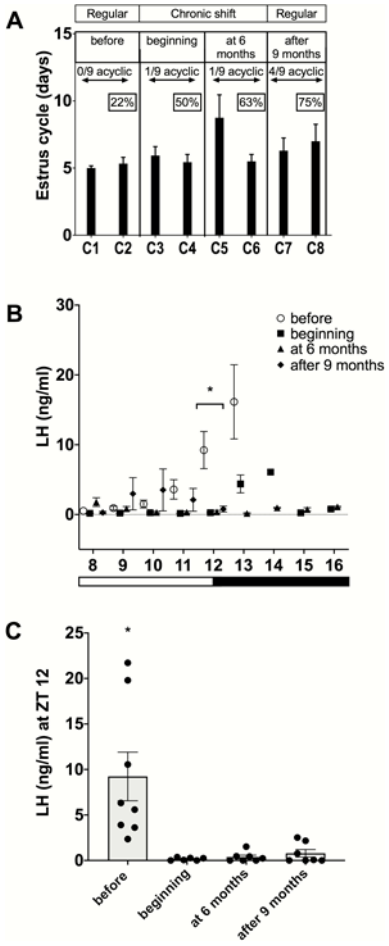

Figure 3. *Longitudinal monitorring of estrous cycles and LH secretion at proestrus in C57BL/6J female mice kept in a stable 12-hour light/12-hour dark cycle then exposed to chronic shifts (rotations of a 10-hour phase advance for 3 days followed by a 10-hour phase delay for 4 days) for up to 9 months. Mice were monitored individually before, at the beginning, at 6 months, and just after the 9-month shift period was stopped. (A) Monitoring of estrous cycle duration and regularity. Estrous cycle duration of cyclic mice (black bars) is given as mean number of days ± SEM (n = 5–9). Values in square boxes indicate the percentage of irregular mice. Values above the arrow indicate the number of acyclic mice (at each stage) and which were excluded from the analyses. The percentage of irregular mice and the number of acyclic mice was significantly higher (P = .0233, chi-square test) after 9 months of chronic shifts than before the chronic shifts. (B) Mean LH secretion between ZT8 and ZT16 during the second proestrus of mice monitored at the different stages. LH secretion at ZT12 before the shift was significantly higher than all other time points after chronic shifts (*P = .0008, Kruskall Wallis/Dunn test). Time is given in ZT (zeitgeber time) with ZT0 as time of light onset and ZT12 as time of light offset; white and black bar under the graph indicate period of light*

*and darkness, respectively. (C) Individual and mean LH values at ZT12 during the second proestrus of mice monitored at different stages. LH secretion at ZT12 was significantly higher before the chronic shifts than any other stages of shifts (*P = .0008, Kruskall–Wallis/Dunn test).*

The occurrence of a preovulatory LH surge was severely impaired at different stages of the chronic shifts compared with the previous regular 12L/12D cycle. Analysis of the mean LH values at the second proestrus of the different stages revealed a significant alteration of the LH secretion pattern at the beginning, at 6 months, or after 9 months of chronic shifts (Fig. 3B and Table 2). Notably, at the expected time of the LH surge, ZT12, LH values were very low at the beginning of the chronic shifts, at 6 months of chronic shifts, but also just after the 9-month chronic shifts were stopped (Figs. 3B and 3C). However, when LH secretion was followed in individual mice, an increase in LH value (>4 ng/mL) was sometimes observed at various ZT (ZT13 and ZT16 for 2/8 mice on the second proestrus after the beginning of chronic shifts; ZT2 for 1/8 mice on the first and second proestrus at 6 months of chronic shifts and ZT10 for 1/5 mice on the second proestrus after 9 months of chronic shifts (Table 2).

Table 2. LH secretion on the day of proestrus in C57BL/6J female mice kept in a stable 12-hour light/12-hour dark cycle (group: before), then exposed to chronic shifts (10-hour phase advance for 3 days followed by 10-hour phase delay for 4 days) and monitored just after the beginning of the chronic shifts (group: beginning), at 6 months under chronic shifts (group: at 6 months), or just at the end of the 9 months chronic shifts (group: after 9 months).

	Before	Beginning		At 6 months		After 9 months	
Chronic shifts	**Second proestrus**	**First proestrus**	**Second proestrus**	**First proestrus**	**Second proestrus**	**First proestrus**	**Second proestrus**
LH surge (> 4 ng/mL) occurrence (%) (n: number of mice with a surge)	78 (n = 7/9)	0^{a} (n = 0/8)	25 (n = 2/8)	12.5^{a} (n = 1/8)	12.5^{a} (n = 1/8)	0^{a} (n = 0/5)	20 (n = 1/5)
ZT (hours) of maximal LH secretion	12.3 ± 0.2	nd	14.5 ± 1.5	2	2	nd	10

The acyclic mice (1 at the start, 1 at 6 months, and 4 after 9 months of chronic shifts) were excluded from the analysis.

Abbreviations: LH, luteinizing hormone; nd, not detected; ZT, zeitgeber time.

[a]P < .05 (chi-square test) indicate that the occurrence of a preovulatory LH surge is significantly lower than before the chronic shifts started.

Of note, the chronic shifts had no significant impact on body weight, since the change in body weight of mice submitted to chronic shifts was similar to that of an age-comparable group of mice kept in regular light/dark cycle (data from (20)): before the shifts (22.6 ± 0.6 g vs 22.8 ± 0.2 g) at 6 months of shifts (30.3 ± 2.4 g vs 26.8 ± 0.8 g) and after 9 month of shifts (31.2 ± 2.1 g vs 31.1 ± 1.1 g).

Impact of chronic shifts on female mouse fertility

Female mice of similar age were either kept in regular 12L/12D cycles (control) or exposed for 4 weeks to chronic shifts of 10-hour phase advance for 3 days followed by 10-hour phase delay for 4 days (chronic shifts), and then released into a regular 12L/12D cycle together with a male mouse that stayed 1 week. Mice of the control group displayed a higher rate of gestation (78.6% [11/14] pregnant mice) than mice of the chronic shift group (57.1% [8/14] pregnant mice) and delivered larger litters (7.7 ± 0.6 pups per litter, n = 11) than the shifted mice (5.4 ± 0.8 pups per litter, n = 8) (Fig. 4). Altogether, female mice of the control group gave birth to a higher number of pups (85 pups in total) than the female mice of the chronic shift group (43 pups in total). Of note, a majority (10/11, 91%) of the mice of the control group gave birth between 20 and 24 days after mating, whereas only 38% (3/8) in the chronic shift group did (P = .01) (Fig. 4).

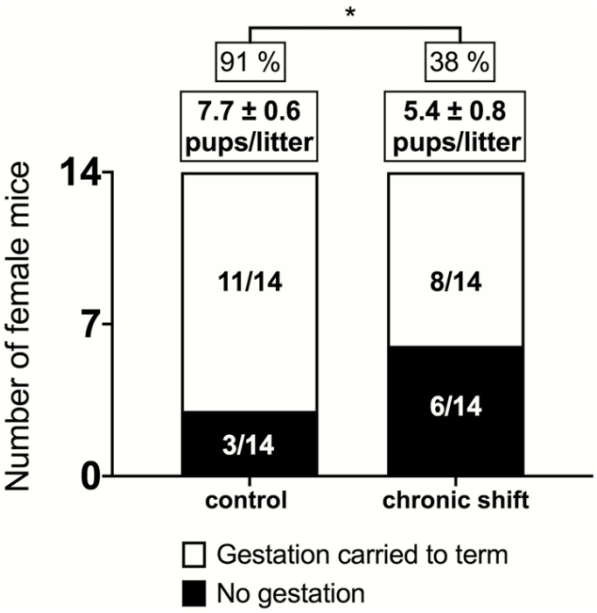

Figure 4. *Fertility parameters in control mice kept in a stable 12-hour light/12-hour dark cycle and mice exposed for 4 weeks to chronic shifts (rotations of a 10-hour phase advance for 3 days followed by a 10-hour phase delay for 4 days). Mice were exposed to either of the 2 schedules (n = 14 per group, 2 mice/cage) for 4 weeks, then all mice were released in a stable 12-hour light/12-hour dark cycle together with a male (1 male for 2 mice) for 1 week. Female mice were then kept undisturbed under the same 12-hour light/12-hour dark cycle for up to 40 days. The number of mice that carried (or not) pregnancy to term is indicated in the white (or black) part of the bars (P > .05, chi-square test). Values in upper square boxes indicate the percentage gestation carried to term 20 to 24 days after mating (P = .01, chi-square test). Values in lower square boxes indicate the number of pups per litter.*

Discussion

In the last 30 years, the number of shift workers has considerably increased particularly in the female population with 20% women working in shifted daily schedules (Boivin and Boudreau, 2014; Gamble et al., 2013; Opperhuizen et al., 2015; Pati et al., 2001; Simonneaux et al., 2017). Epidemiological studies indicate that shiftwork is associated with higher risks of developing cardiovascular, metabolic and gastrointestinal disorders, sleep disturbances, various types of cancer, notably breast cancer, and mental disorders including depression and anxiety disorders (Boivin and Boudreau, 2014; Gan et al., 2015; Menegaux

et al., 2013; Vyas et al., 2012). Strikingly, the impact of shiftwork on human fertility has barely been investigated (Gamble et al., 2013; Simonneaux et al., 2017). In women, some studies have reported that shiftwork is associated with more menstrual irregularities, longer time to get pregnant, and higher risks of miscarriage or low newborn weight. In general, no study actually assessed the impact of chronodisruptive conditions on ovarian cycles and the timing of the preovulatory LH surge, which are considered as robust indicators of fertility in both women and female rodents (Guermandi et al., 2001; Kolstad et al., 1999; Takasu et al., 2015). In order to tackle this issue, this study investigated the effects of acute or chronic phase shifts on both estrous cycles and the preovulatory LH surge in adult female mice.

We found that a single 10-hour phase advance or delay of the light/dark cycle induced a temporary increase in the estrous cycle length observed at the second cycle after the shift. However, at the first cycle after the 10-hour phase advance or delay, only half of the mice exhibited an LH surge, and when the LH surge was observed the ZT of the LH peak was significantly advanced by 2 hours when compared with control mice kept in a stable 12L/12D cycle. On the third proestrus after the phase shift, the LH surge frequency and the ZT and amplitude of the LH peak were no longer different from the control group. Therefore, a single 10-hour phase shift (whether advance or delay) induces a temporary alteration of the occurrence and the timing of the preovulatory LH surge, with a minor effect on the estrous cycle. Our results are in line with a previous study showing that exposure of female Syrian hamsters to a single 12-hour shift has minor effects on the estrous cycle, which returned to normal values within 2 to 4 days after the shift, thus faster than the locomotor activity rhythm which took 7 days to resynchronize (Finkelstein et al., 1978). Similarly, another study reported that exposure of young mice (2–6 months) to a moderate shift (3-hour delay of darkness onset for 2 days with a return to the former 12L/12D cycles for 5 days) had no effect on the estrous cycle (Takasu et al., 2015). Interestingly, 82% of middle aged mice (8–12 months) submitted to the same shift schedule exhibited irregular cycles, indicating that estrous cycle adaptation to shifted schedules is age dependent (Takasu et al., 2015). To our knowledge, only 2 studies have investigated the impact of a single phase shift on the preovulatory LH surge in female rodents. An early work reported that when

female Syrian hamsters are exposed to a 3-hour phase advance, the LH surge is not fully resynchronized to the new schedule after 3 days, while after a 3-hour phase delay the LH surge more rapidly resynchronizes to the dark onset (Moline and Albers, 1988). Another study showed that exposure of ovariectomized gonadotropin-releasing hormone green fluorescent protein mice to a 2-hour phase advance for 5 days leads to a 1.5-hour advance of the LH surge (Ca et al., 2005). Furthermore, some studies investigated the impact of acute phase shifts on the timing of ovulation determined by autopsy. In female rats, ovulation was delayed by 4 hours on the fourth day following a 5-hour phase delay, but had no effect after a 5-hour phase advance (McCormack, 1973). In female Syrian hamsters, ovulation was reported to take 15 to 60 days to resynchronize after a 6-hour phase advance (Alleva et al., 1968). Altogether, these data indicate that single phase shifts have no, or minor, effects on the overt estrous cycles, but lead to a more negative, although temporary, impact on the preovulatory LH surge.

Our study reported that exposure to a chronic rotation of 3 days 10-hour phase advance/4 days 10-hour phase delay has a minor impact on estrous cycle regularity and length. However, there was a tendency to irregular and longer estrous cycles after 9 months of chronic shifts with 4/9 mice becoming acyclic. By contrast, such a chronic exposure strongly impaired the occurrence of the preovulatory LH surge at the beginning and during the whole exposure. Only 2/9 mice exhibited an increase in preovulatory LH but with a much lower amplitude and with an out of phase timing (different from the expected ZT12/13). We previously demonstrated that female mice maintained under a stable 12L/12D cycle keep their estrous cycle and preovulatory LH surge unchanged for up to 9 months (Bahougne et al., 2020). Therefore, the dramatic alteration in the LH surge occurrence and timing observed under chronic shifts is not due to aging. One recent study reported that female mice submitted to 10-hour phase advance/10-hour phase delay shifts with 3, 6, or 12 days of rotation intervals exhibit a higher rate of irregular estrous cycles than control mice, with the 6-day rotation interval being the less severe protocol (Yoshinaka et al., 2017). Although representing a different paradigm, the impact of different day lengths has also been investigated on female rodent estrous cycles. Thus, increasing the duration of the day to 28

hours (instead of 24 hours) increases estrous cycle length (Kaiser, 1967) while adjusting the light/dark schedule to the endogenous circadian rhythm helps restoring estrous cycle regularity and fertility in CRY mutant mice (Takasu et al., 2015). To our knowledge, our study is the first demonstrating the severe impact of chronic shifts on LH secretion. Notably, studies on aging female mice reported that after 12 months of age, the timing and amplitude of the preovulatory LH surge are impaired (Akema et al., 1985; Bahougne et al., 2020; DePaolo and Chappel, 1986; Matt et al., 1998; Nass et al., 1984; Wise, 1982), thus indicating that exposure to chronodisruptive conditions may induce a premature aging of the reproductive system, as recently reported in women under shiftwork (Stock et al., 2019).

Our investigation of the gestational outcome of mice exposed to chronic shifts for 4 weeks showed that shifted mice had a reduced success of gestation with a 2 times lower number of pups than control mice. Further, the shifted mice possibly had a delayed ovulation since a majority of them gave birth 5 days later that the majority of the control mice. Other studies have investigated the effect of chronodisruptive conditions on gestation although with the phase shifts applied after mating. A chronic 6-hour phase advance or delay every 5 days during pregnancy decreases the full-term gestational success in both conditions with a larger impact of the chronic phase advances (Summa et al., 2012). In rats, by contrast, successive 12-hour phase advances and delays during gestation did not alter the gestational outcome (Varcoe et al., 2011).

Altogether our results indicate that single phase shifts have a moderate impact on female reproduction, whereas chronic phase shifts lead to a severely impaired preovulatory LH surge, which in turn may reduce fertility and gestational success. These observations confirm the requirement of a functional and well-synchronized circadian clock for proper female reproduction. Recent studies have indicated that the SCN clock forwards the daily signal to the reproductive system directly via a vasoactive intestinal peptidergic input onto the gonadotropin-releasing hormone neurons, or indirectly via a vasopressinergic input onto the preoptic kisspeptin neurons, which in turn drive the GnRH-induced LH surge (Simonneaux, 2020). Therefore, further studies should investigate how shifts in the

light/dark cycle alter peptidergic SCN outputs, as well as kisspeptin and/or GnRH neuronal activity. Interestingly, mutations of various clock genes induce similar negative impacts on estrous cycle regularity, LH surge occurrence, and gestational success, especially in middle-aged female mice (Miller et al., 2004; Pilorz and Steinlechner, 2008; Takasu et al., 2015). Further, selective ovary-specific knockout of the clock gene Bmal1 disrupts ovulation and embryo implantation, pointing to a role for the peripheral secondary circadian clocks in addition to the master SCN clock (Liu et al., 2014; Mereness et al., 2016). Notably, an in vitro study reported that after a 6-hour phase advance, the ovarian clock resynchronizes more slowly than the SCN clock (Yamazaki et al., 2000; Yoshikawa et al., 2009). Therefore, it might be interesting to delineate if and how chronic phase shifts applied to PER2/luciferase female mice misalign their SCN and reproductive peripheral clocks.

Acute or chronic shifts alter a number of other functions, notably glucocorticoid production and metabolic activity, which may indirectly impact reproduction. Thus, acute or chronic shifts alter the peak of glucocorticoid secretion (Goichot et al., 1998; Kiessling et al., 2010), which usually occurs around the same time of the LH surge in both rodents (Atkinson and Waddell, 1997) and women (Kerdelhué et al., 2002). In humans, one study reported a 2-fold increase in the hair cortisol level of male shift workers compared with daytime workers (Manenschijn et al., 2011). High glucocorticoid levels are reported to display negative impacts on fertility (Pivonello et al., 2016; Whirledge and Cidlowski, 2013) and glucocorticoid administration induces anestrus and alters the preovulatory GnRH/LH surge in mice through the inhibition of the preoptic kisspeptin neurons (Breen and Mellon, 2014; Luo et al., 2016; Takumi et al., 2012; Whirledge and Cidlowski, 2013). One of the major and well-recognized effects of chronic circadian disruption is the increased risk of developing metabolic syndrome (Barclay et al., 2012; Tucker et al., 2012), a pathology known to impair reproduction (Clark et al., 1998; Norman and Clark, 1998). This strong interaction between metabolism and reproduction is notably illustrated by the ability of exogenous leptin to rescue puberty and fertility in ob/ob mice (Mounzih et al., 1997). In our study, however, chronic phase shifts had no impact on mouse body weight, suggesting that the observed reproductive alteration is not due to major metabolic change.

In conclusion, our study reports that female mice exposure to a unique shift causes a moderate and temporary shift of the LH surge whereas exposure to chronic shifts induces a severe, rapid, and long-lasting alteration of the preovulatory LH surge leading to reduced fertility. Notably, the estrous cycles appeared moderately altered, thus indicating that fertility assessment should use LH secretion rather that estrous, and probably menstrual, cycle regularity. Our results, obtained in a rodent model of shiftwork, should prompt us to conduct similar experimental investigations in women exposed to chronodisruptive conditions, notably to chronic shift work.

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PART IV

EFFECT OF AGING ON FEMALE REPRODUCTION

Chapter 5

Individual evaluation of luteinizing hormone in aged C57BL/6 J female mice

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Abstract

In female mammals, reproductive senescence is a complex process involving progressive ovarian dysfunction associated with an altered central control of the hypothalamic-pituitary axis. The objective of this study was to compare the longitudinal change in preovulatory luteinizing hormone (LH) secretion as well as estrous cycle in individual C57BL/6 J female mice at 3, 6, 9 and 12 months. Amplitude and timing of LH secretion at the surge were similar from 3 to 9 months but were altered in 12-month old mice with a significant decrease of more than 50% of peak LH value and a 2 h delay in the occurrence of the LH surge as compared to younger mice. The analysis of two to three successive LH surges at 3, 6, 9 and 12 months showed low and similar intra-individual variability at all ages. The estrous cycle length and intra/inter variability were stable over the age. This study shows that female mice in regular environmental conditions display stable LH surge timing and amplitude up to 9 months, but at 12 months, the LH surge is delayed with a reduced amplitude, however without overt modification in the estrous cycles. Analysis of individual preovulatory LH secretion and estrous cycle indicates that mice can be followed up to 9 months to investigate the detrimental effects of various parameters on mouse reproductive activity.

Introduction

Reproductive activity in female mammals displays regular cycles driven by a complex interaction of hypothalamic neuropeptides, pituitary gonadotropins (luteinizing (LH) and follicle stimulating (FSH) hormones), sex steroid hormones and the circadian system (Simonneaux and Bahougne, 2015). The final output of this regulatory process is to combine the production of a mature oocyte (ovulation) with a prepared reproductive tract which will ensure embryo development and offspring survival. At the beginning of the reproductive cycle, FSH promotes oocyte maturation associated with a progressive increase in estradiol (E2) production until the occurrence of a massive and transient increase in LH secretion which triggers ovulation (Kerdelhué et al., 2002; Legan and Karsch, 1975). The timing of the preovulatory LH surge, and consequent ovulation, is tightly controlled since it requires both elevated circulating E2 and a daily signal (Christian et al., 2005) which gates the LH surge at the end of the resting period, thus end of the day in nocturnal species and end of the night in diurnal species, including humans (Kerdelhué et al., 2002; Mahoney et al., 2004). In female rodents, it has been demonstrated that the daily timing of the LH surge is driven by a pathway including the master biological clock localized in the suprachiasmatic nuclei (SCN), the kisspeptin neurons and the GnRH neurons (Simonneaux and Bahougne, 2015).

Throughout adult life, a number of events can alter female reproductive capacity. It is therefore critical to perform longitudinal analysis to follow the long-term effect of negative events such as metabolic alteration, stress, circadian disruption or sickness. In rodents, a relevant measurable longitudinal marker of female reproductive activity is the change in vaginal cytology (McLean et al., 2012; Nelson et al., 1982) allowing to measure the length and regularity of the various estrous stages for several weeks or months in a single individual. However, with the recent development of a micro LH assay (Steyn et al., 2013), it is now possible to perform similar individual longitudinal analysis of LH secretion, notably the timing and amplitude of the preovulatory LH surge. Therefore, the objective of this study was to follow individual LH secretion on the day of proestrus in female mice of different ages, 3 to 12 months, in order to follow reproductive robustness throughout the adult life.

Material and methods

Animals

Eight week-old virgin C57BL/6 J female mice were obtained from the Charles River laboratory. Upon arrival, mice were placed 3 per cage, quarantined for 2 weeks and then manipulated every day for at least 2 weeks for habituation before experimentation. Mice were kept until the age of 12 months at 22–25 °C with food and water available ad libitum on a 12 h light / 12 h dark schedule, with lights on at zeitgeber time 0 (ZT0) and lights off at ZT12. The health of the mice was followed regularly and their cages and water bottles were changed and autoclaved weekly. All experimental procedures were approved by the local ethical committee (CREMEAS) and the French National Ministry of Education and Research (authorization # 2015021011396804).

Analysis of LH secretion and estrous cycles

LH secretion was followed on the day of proestrus in individual mice belonging to one of the 4 groups of age: 3 months (n = 9), 6 months (n = 6), 9 months (n = 9) or 12 months (n = 9). Estrous cycles were followed in post-pubertal mice by vaginal smears performed according to (Nelson et al., 1982). In C57BL/6 J female, estrous cycles exhibit a period of 4–5 days and are divided into three phases according to the vaginal cytology: diestrus (D) characterized by leukocytic and nuclei cells with leukocytic predominance, proestrus (P, during which preovulatory LH surge occurs) characterized by oval nucleated epithelial cells with some cornified squamous epithelial cells, and estrus (E) characterized by a predominance of cornified squamous epithelial cells. A fourth stage of metestrus, sometimes described as a transition between the estrus and diestrus phases (Nelson et al., 1982) was not considered here because of the difficulty to objectively assess this phase and was combined with diestrus (because of the leucocytic predominance). Vaginal smears were performed two times every day, between 08 h00 and 14 h00, during at least 2 consecutive estrous cycles.

A preliminary investigation on 3 month-old mice showed that all LH peak occurred between ZT8 and ZT16, with LH values <1 ng/ml outside this time window (data not shown). This timing is in agreement with previous studies in rodents (Akema et al., 1985; Czieselsky et al., 2016; Wise, 1982) and illustrates the synchronization of the LH surge to the light/dark cycle by the master hypothalamic clock (Simonneaux and Bahougne, 2015). On the day of proestrus, a 4 µL blood sample withdrawn from the tail tip (< 1 mm) was taken every hour from ZT8 to ZT16, thus 4 h before and after lights off, at a time when the preovulatory surge is expected. On the following two or three proestrus stages, another series of blood sampling was made at the same time in order to estimate LH secretion reproducibility in the same mouse.

Each blood sample was immediately diluted in 116 µL of PBST (10% of 10X phosphate buffered saline, 0.25% of Tween-20 in milliQ H2O) and stored at −80 °C until LH assay.

LH assay

LH concentration was determined using a highly sensitive Enzyme-Linked Immunosorbent Assay (ELISA) (Steyn et al., 2013) using anti-bovine LHβ as capture antibody (monoclonal antibody, 518B7, NHPP, Torrance, California), rabbit anti-mouse LH as first antibody (polyclonal antibody, Rabbit LH antiserum, AFP240580Rb, NHPP, Torrance, California), goat anti-rabbit IgG as secondary antibody (D048701–2, Dako Cytomation, Polyclonal Goat Anti-Rabbit, Denmark) and mouse LH as standard (mLH, AFP-5306A, NHPP, Torrance, California). Four given concentrations of LH (1, 10, 20 and 30 ng/ml) were used as quality controls. The intra-assay variation was 11% and the inter-assay variation was 13%.

Data analysis

The occurrence of a LH surge was found in about 70–80% of the mouse expected to be on proestrus, whatever the age group, and only the mice with a detected LH secretion (> 4 ng/ml) were included in the analyses. To estimate the effect of age on the timing, amplitude, and reproducibility of the preovulatory LH surge, circulating LH of each mouse (either 3, 6, 9 or 12 months) was followed for up to three consecutive proestrus from ZT8 to ZT16. For each mouse at a given age, the LH values at the 2 to 3 consecutive surges were combined.

For each age group, LH values were given either for each individual or as mean ± standard error of the mean (SEM) of 4 to 8 mice of the same age. Further, for each age group, the mean (± SEM) ZT of the peak, ZT of the beginning of the surge (estimated by a LH value >4 ng/ml) and peak amplitude were calculated from each individual values. Intra individual variability of the ZT of the LH peak was calculated for each age group as mean ± SEM.

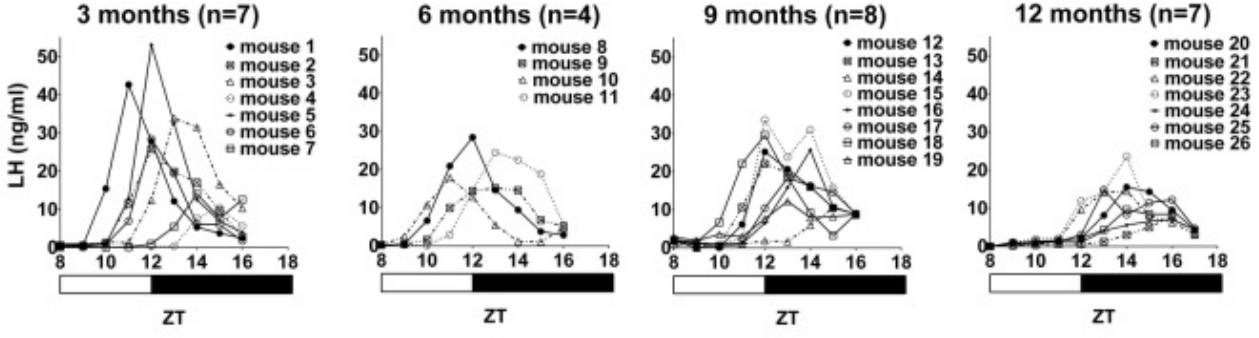
To estimate the longitudinal effect of age on the estrous cycle, each mouse was followed for at least two consecutive cycles at 3, 6, 9 or 12 months. For each age, the characteristics (duration and estrus/diestrus ratio) of the first observed estrus cycle were given as the mean ± SEM. The intra-individual variability was estimated by defining a cycle as irregular when the length of the two consecutive estrous cycles differs by more than one day and was given as the percentage of irregular mice for each group of age.

Statistical analysis and figures were realized using GraphPad Prism 6 (San Diego, USA). Assumptions were taken into account. The D'Agostino and Pearson test was used to check normality. Homoscedasticity was verified with the Bartlett's test. Statistical comparisons were made with an analysis of variance (ANOVA). Newman Keuls post-hoc tests were performed as appropriate to determine specific interactions. The significance level was set at $p \leq 0.05$.

Results

Evolution of the individual preovulatory LH surge characteristics and estrous cycle with age

Preovulatory LH surges were observed in C57BL/6 J mice from 3 up to 12 months with significant differences in phase and amplitude appearing at 12 months (Fig. 1). Analysis of LH values at the surge in individual mouse shows that the mean maximal LH peak value decreases from 29.8 ± 5.7 ng/ml at 3 months to 13.3 ± 2.2 ng/ml at 12 months, with the maximal LH peak value at 3 months significantly higher as compared to 12 months ($p < 0.05$, ANOVA test $F(3, 22) = 3.324$). The mean individual ZT of LH peak is similar at 3 (12.7 ± 0.5 h), 6 (12.3 ± 0.5 h) and 9 (12.9 ± 0.4 h) months but is significantly delayed at 12 months (14.4 ± 0.4 h) ($p < 0.05$, ANOVA test $F(3, 22) = 3.961$). In line with this observation, the ZT at which the LH starts to increase over 4 ng/ml is significantly delayed by 1.2 to 2 h at 12 months as compared to earlier ages ($p < 0.05$, ANOVA test $F(3, 22) = 3.622$). Analysis of the mean ± SEM of LH values in mice of the same group of age (Fig. 2) shows that the maximum LH values at the ZT of the peak are 21.3 ± 7 ng/ml (at ZT12) at 3 months, 19.4 ± 2.9 ng/ml (at ZT12) at 6 months, 17.1 ± 4.2 ng/ml (at ZT12) at 9 months, and 11.5 ± 2.6 ng/ml (at ZT14) at 12 months (with a significant difference between 3 and 12 months ($p < 0.05$), and the ZT of the peak significantly different at 12 months as compared to earlier ages ($p < 0.05$, ANOVA test $F(7, 154) = 16.68$). Notably, the comparative analysis between individual and mean values at different ages, although delivering an overall similar message, shows some differences with higher maximal LH concentration (from 2 to 8 ng/ml) and later ZT of the peak (from 0.4 to 0.9 h) when considering individual as compared to mean values.



LH value (ng/ml) at the peak	29.8 ± 5.7	21.5 ± 3	22.1± 2.9	13.3 ± 2.2 (*)
ZT (h) of LH peak	12.7 ± 0.5	12.3 ± 0.5	12.9 ± 0.4	14.4 ± 0.4 (*)
ZT (h) of LH > 4 ng/ml	11.4 ± 0.5	11.0 ± 0.4	11.8 ± 0.4	13.0 ± 0.4 (#)

Figure 1: *Individual preovulatory LH surge on proestrus days in C57BL/6 J female mice at 3 (mouse 1 to 7, n = 7), 6 (mouse 8 to 11, n = 4), 9 (mouse 12 to 19, n = 8) or 12 (mouse 20 to 26, n = 7) months. At each age, LH value was measured by ELISA in 4 µL blood sampled hourly from 4 h before lights off (ZT 8) up to 4 h after lights off (ZT 16). Values below the graphs are mean ± SEM of the 3, 6, 9 and 12-month old female mice. Maximal LH value is significantly higher at 3 months than at 12 months (* p < 0.05, 95% CI of difference; 7.743 to 26.08); ZT at the peak is significantly different at 12 months as compared to 3, 6 and 9 months (+ p < 0.05, ANOVA test F(3, 22) = 3.961); ZT at the starting of the LH surge (LH > 4 ng/ml) is significantly different at 12 months as compared to 3, 6 and 9 months (# p < 0.05, ANOVA test F(3, 22) = 3.622). ZT: zeitgeber time.*

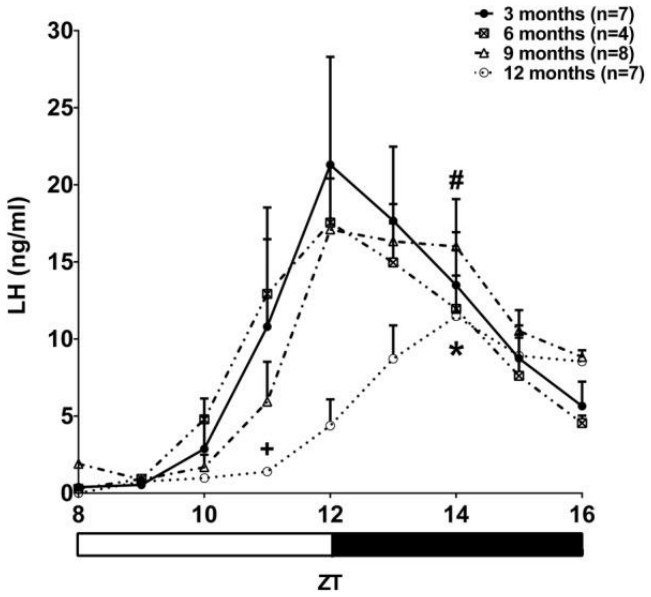


Figure 2: *Mean LH secretion at the day of proestrus in 3 to 12 month-old C57BL/6 J female mice. At each age, LH value was measured by ELISA in 4 µL blood sampled hourly from 4 h before lights off (ZT 8) up to 4 h after lights off (ZT 16). LH values are given as mean ± SEM (n = 4 to 8 mice). ZT of LH peak at 12 months is significantly different (* p < 0.05, ANOVA test F(3, 22) = 3.961) from ZT of LH peak at other ages; LH peak value at 12 months is significantly different (# p < 0.05, 95% CI of difference; 7.743 to 26.08) from LH peak value at 3 months; LH value at ZT 11 is significantly lower at 12 months (+ p < 0.05, 95% CI of difference; 0.2312 to 18.57) as compared to 3 months. ZT: zeitgeber time*

All mice exhibited estrous cycles from 3 up to 12 months. The estrus cycle duration was stable at 3, 6 and 9 months, lasting 4.2 ± 0.1, 4.3 ± 0.3 and 4.6 ± 0.3 days respectively (with low inter-individual variability: 11, 16, and 16%, mice displaying irregular cycles, respectively). At 12 months, the mean cycle duration and inter-individual variability appeared higher with a mean duration of 6.6 ± 1.7 days (one mouse having a duration cycle of 15 days) and 40% mice exhibited irregular cycles. There was no statistical difference however in the estrous cycle duration among the different groups of age. The percentage of mice with an estrus cycle duration of 4–5 days was 94, 91.5, 81.5 and 71.5% at 3, 6, 9 and 12 months respectively. The estrus/diestrus length ratio was stable at 3, 6, 9 months, with values of 1.3 ± 0.2, 1.1 ± 0.1, 1.4 ± 0.3 respectively and increased at 2 ± 0.6 at 12 months, yet with no statistical difference among the four groups of ages.

Intra-individual variability in the preovulatory LH surge and estrous cycle with age

The longitudinal analysis of individual LH secretion over several cycles allows estimating intra-individual variability at different ages. At 3 months, the comparison of consecutive preovulatory LH surges in 3 representative individual mice shows that intra-individual variability in the ZT of the LH peak over 2 or 3 consecutive surges is less than 2 h with a mean variation of 0.9 ± 0.4 h (Fig. 3A). Notably, the intra-individual variability in the ZT of the LH peak remains low in 6 month-old (1.2 ± 0.6 h, Fig. 3B) and 12 month-old (0.5 ± 0.4 h, Fig. 3C) mice.

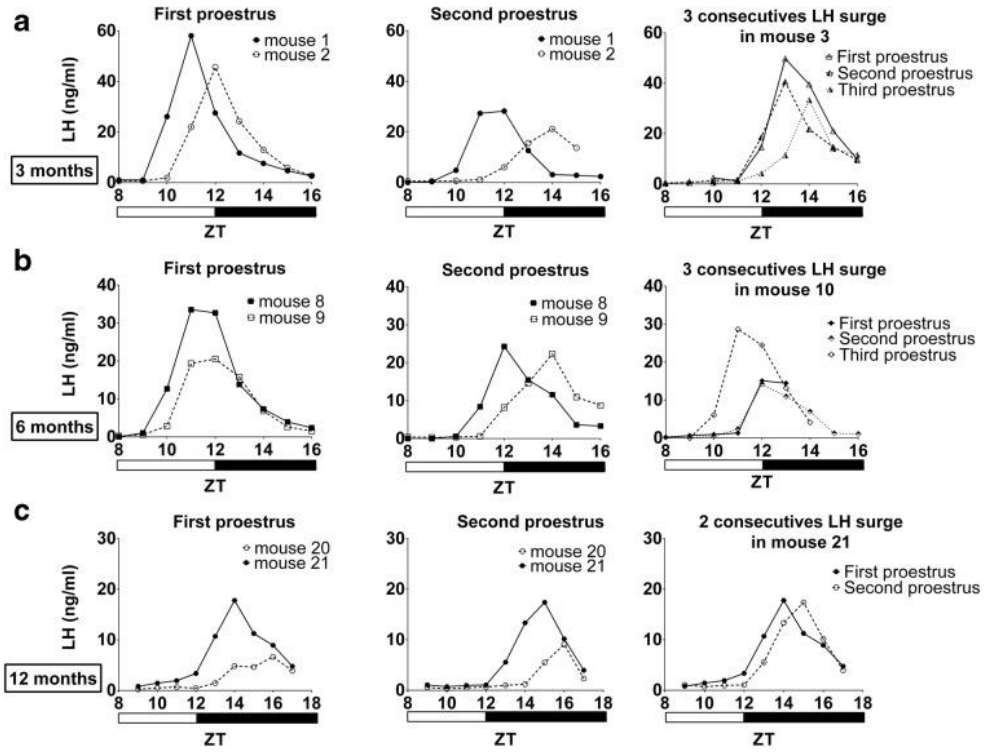


Figure 3: *LH secretion over successive preovulatory LH surges in 3 month- (A), 6 month- (B) and 12 month- (C) old C57BL/6 J female mice. At each age, LH value was measured by ELISA in 4 µL blood sampled hourly from 4 h before lights off (ZT 8) up to 4 h after lights off (ZT 16). Panel A shows LH secretion in 3 independent 3 month-old mice (mouse #1, mouse #2) at two consecutive proestrus and one mouse (mouse #3) with 3 consecutive superposed LH surges; Panel B shows LH secretion in 3 independent 6 month-old mice (mouse #8, mouse #9) at two consecutive proestrus and one mouse (mouse #10) with 3 consecutive superposed LH surges; Panel C shows LH secretion in 2 independent 12 month-old mice (mouse #20, mouse #21) at two consecutive proestrus and one mouse (mouse #21) with 2 consecutive superposed LH surges. ZT: zeitgeber time*

In the same line, the intra-individual variability in the estrus cycle length did not show significant difference from 3 to 12 months (0.9 ± 0.8, 0.4 ± 0.6, 1 ± 0, 2.9 ± 3 days, respectively).

Discussion

The recent development of a micro-assay for circulating LH in freely moving rodents has been an important advance in reproductive biology research, allowing LH secretion to be monitored every hour (or less) for several consecutive days in a single animal (Steyn et al., 2013). In this study, we took advantage of this method to investigate intra- and inter-variability in the preovulatory LH surge profile in individual C57BL/6 J mice of different ages, in addition to cytological estrus cycle analyses. From 3 up to 12 months, mice exhibited regular preovulatory LH surge at the late day/early night transition and normal estrous cycles. In 12 month-old mice however the timing of the LH surge was significantly delayed and its amplitude significantly reduced when compared to 3 month-old mice, and the estrous cycles tended to become longer and more irregular.

Changes in estrus cycle length and regularity in aging C57BL/6 J mice have already been studied (Mobbs et al., 1984; Nelson et al., 1982). Similar to our observation, it was found that from 4 to 11 months, mice exhibited no significant change in the estrus cycle duration (median length < 5 days) with 40 to 60% mice exhibiting regular cycles. Then, from 11 to 14 month, mice displayed a progressive lengthening of cycle duration (50% mice had a mean duration >5 days) and 40 to 90% mice became irregular and even acyclic (Nelson et al., 1982). Another study reported comparable agerelated changes in estrus cycle irregularity with female mice displaying no irregular cycle up to 8 months, and then 39% becoming irregular at 12–13 months and 79% at 16–20 months (Parkening et al., 1982).

From 3 to 9 months, the LH surge occurred with similar amplitude and at the same period around the light/dark transition, indicating that the pathway synchronizing the LH surge is functioning properly. By contrast, at 12 months, although there was a slight but not significant alteration in the estrous cycle length and regularity, the timing of the preovulatory LH surge was significantly delayed by about 2 h, and the amplitude reduced by about 50% as compared to earlier stages, suggesting that alteration in the central control of the LH surge is an early aging signal. Earlier studies on aging female mice (Parkening et al., 1982) and rats (Akema et al., 1985; DePaolo and Chappel, 1986; Matt et al., 1998; Nass

et al., 1984; Wise, 1982) also reported a reduced LH peak amplitude in middle-age as compared to young female animals (Downs and Wise, 2009).

In female rodents, reproductive senescence is a complex process involving progressive ovarian dysfunction (follicular and estrogen decrease) associated with an altered capacity of neuropeptides controlling the hypothalamic-pituitary axis, to respond to estradiol signal (Ishii et al., 2013; Kunimura et al., 2017; Lederman et al., 2010). The reduced secretion of LH at the surge in aging females does not result from a lower number of gonadotropin-releasing hormone (GnRH) neurons or GnRH release, nor a reduced pituitary responsiveness to GnRH, but rather is due to a marked decrease in GnRH neuronal activation at the time of the proestrus LH surge, indicating that mechanisms upstream of GnRH neurons are involved in this aging process (Downs and Wise, 2009; Ishii et al., 2013; Lederman et al., 2010; Lloyd et al., 1994; Rubin et al., 1995, 1994). The decline in the E2-dependent GnRH activation of LH at the surge may involve the anteroventral periventricular nucleus (AVPV) kisspeptin neurons, now recognized as the E2-dependant drivers of the preovulatory GnRH/LH surge (Pinilla et al., 2012; Smith et al., 2006). Indeed, a parallel decline in cFos positive kisspeptin neurons and cFos positive GnRH neurons at the time of the GnRH/LH surge has been reported in middle-aged female mice with irregular cycles (Zhang et al., 2014). Furthermore, in aging female, AVPV kisspeptin neurons expression of estradiol receptor (ERα) and responsiveness to E2 are significantly reduced (Ishii et al., 2013; Lederman et al., 2010; Zhang et al., 2014), an effect which may further be exacerbated by a natural age-dependant E2 decrease due to follicular depletion (Downs and Wise, 2009). By contrast, not significant changes in kisspeptin expression is reported in aging rodents (Ishii et al., 2013; Lederman et al., 2010; Zhang et al., 2014). Altogether these data suggest that AVPV kisspeptin neurons are likely among the earliest to undergo aging processes and thus participate in initiating the early reproductive decline. In agreement with this hypothesis, kisspeptin infusion has been shown to restore LH surge amplitude in middleaged female rats (Neal-Perry et al., 2009).

In addition to the reduced amplitude of the LH surge, our temporal resolution of LH monitoring reported that the LH surge timing exhibited a significant 2 h phase delay in the

12 month-old female mice. A similar phase delay, although of 1 h only, was previously reported in middle-aged female rats (Wise, 1982). It is now well established that the main circadian clock of the SCN is necessary for the proper timing of the preovulatory LH surge (Simonneaux and Bahougne, 2015). Notably, the daily rhythm in vasoactive intestinal peptide (VIP) in SCN neurons, which convey time of the day signal directly to GnRH neurons, is abolished in middle-aged female rats (Downs and Wise, 2009; Krajnak et al., 1998) and this may account for the LH surge phase delay. Contrastingly, the daily rhythm in SCN vasopressin neurons, known to project directly onto AVPV kisspeptin neurons (Piet et al., 2015; Vida et al., 2010), does not appear altered in middle-aged female rats (Krajnak et al., 1998). Finally, because the SCN clock resetting by transmitters like glutamate, Nmethyl-D-aspartate or serotonin is reduced in aging mice (Biello, 2009), it is possible that a less synchronized circadian clock is responsible for the LH surge delay in the 12 month-old mice. Whatever the mechanisms involved, it could be interesting to investigate whether the LH surge phase delay is associated with a similar shift in the rhythm of AVPV kisspeptin neuron's activity in middle-aged female rodents.

Cellular senescence is one of the hallmark of aging process and can be defined as an irreversible and stable arrest of the cell cycle causing inflammation through a complex senescence-associated secretory phenotype (SASP) (Lawrence et al., 2018; Perrott et al., 2017). Studies have reported that senescent cells and inflammation are implicated in general age-related dysfunction (Lawrence et al., 2018), including in female reproductive organs (Marquez et al., 2017; Shirasuna and Iwata, 2017). It might be interesting to investigate whether SASP processes are involved in the ageinduced alteration of the central regulation of the preovulatory LH surge.

The analysis of LH secretion in several individual mice over few successive proestrus has shown that all LH surges are gated at the light/dark transition although with some inter-individual variability in the ZT of the LH peak, as already reported in a previous study using similar LH analysis (Czieselsky et al., 2016). In this study, we followed individual mice over up to 3 consecutive proestrus in order to assess the intra-individual stability of the LH surge at different ages. We found low intra-individual variability in the ZT of the peak (< 2 h) over

successive proestrus LH surges, even in the oldest mice. Therefore, our data indicate that longitudinal analysis using micro LH assay in indivitual mice can be used over few weeks or months to study long term mechanisms regulating the LH surge timing.

Conclusions

This study has demonstrated that female mice in regular environmental conditions display stable LH surge timing and amplitude up to 9 months, implicating that the regulation or dysregulation of the preovulatory LH surge can be studied over extended longitudinal period of time. However, at 12 months the LH surge shows a 2 h delay, possibly due to an altered aging circadian clock, and a reduced amplitude, possibly resulting from a reduced sensitivity of AVPV kisspeptin neurons to E2 and hence a decreased release of AVPV kisspeptin onto GnRH neurons. Interestingly, the aged-related alteration in the preovulatory LH surge precedes overt modification in the estrous cycle which appears not significantly changed up to 12 month-old, thus supporting the current hypothesis that alteration in the central control of the LH surge is an early signal of reproductive aging in female. Understanding the underlying mechanisms of reproductive senescence is essential because the reproductive status affects health throughout life. Thus, menopause in women is reported to have a negative impact on inflammation, gynecologic cancer, and cardiovascular disease (Shi et al., 2016). Therefore, protocols aiming at maintaining/restoring reproductive activity (Habermehl et al., 2019) may help alleviating the negative impact of reproductive senescence on health.

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Chapter 6

Age-dependent modulation of RFRP-3 neurons in female mice

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Abstract

In female mammals, reproductive senescence is a complex process involving progressive ovarian dysfunction, associated with altered central control of the hypothalamic-pituitary-gonadal axis and desynchronization of the circadian system. The objective of this study was to investigate daily- and age-dependent changes in the regulation of Arg-Phe amide-related peptide-3 (RFRP-3), a hypothalamic peptide involved in reproduction, and correlate those changes to the luteinizing hormone (LH) secretion in female C57BL/6 J mice of different age groups (4-, 13-, and 19- months old). Plasma LH levels were similar in diestrus mice from 4 to 13 months, but were markedly increased in 19 month-old mice. We found an age-dependent decrease in the total number of RFRP-3 neurons and in the relative number of activated (c-Fos-positive) RFRP-3 neurons. In 19 month-old mice also the daily variation in RFRP-3 neuronal activation was abolished. We found no daily- or age-dependent changes in the arginine vasopressin (AVP)- and vasoactive intestinal peptide (VIP)-fiber density in the dorsomedial hypothalamus. However, we did record a daily variation in the number of RFRP-3 neurons receiving close AVP- and VIP-ergic fiber appositions in 4 and 13 month-old, but not 19 month-old mice. The present findings indicate that RFRP-3 neurons are downregulated during old age and that the daily changes in their innervation by the circadian peptides, AVP and VIP, are abolished. This age-associated reduced rhythmic activity of the inhibitory RFRP-3 system could be implicated in the elevated LH secretion observed during reproductive senescence.

Introduction

In female mammals, reproductive senescence is a complex process involving progressive ovarian dysfunction, associated with altered central control of the hypothalamic-pituitary-gonadal (HPG) axis and desynchronization of the circadian system. Female rodents undergo reproductive decline at middle-age during which they transition from regular estrous cycles to irregular cycles and eventually to acyclicity (estropause). Although age-associated changes occur at all levels of the HPG axis, several pieces of evidence suggest that the hypothalamus plays a primary role in the induction of reproductive senescence (Kermath and Gore, 2012). In rodents, age-dependent suppression of hypothalamic GnRH neurons is associated with a delayed or attenuated preovulatory GnRH/LH surge (Scarbrough and Wise, 1990; Rubin et al., 1994; Le et al., 2001; Bahougne et al., 2020). Many age-related alterations in the GnRH neuronal system precede changes in the pituitary, the ovaries and the loss of estrous cyclicity (Scarbrough and Wise, 1990). GnRH system dysfunction during aging may be intrinsic, however, age-associated changes in its regulatory input pathways have not been thoroughly investigated yet.

GnRH neuronal activity is driven by coordinated inputs from amongst others two hypothalamic neuropeptide systems, the kisspeptin (Kp) neurons, located in the preoptic area and the arcuate nucleus, and the (Arg)(Phe) amide-related peptide-3 (RFRP-3) neurons located in the dorsomedial hypothalamus (DMH) (Angelopoulou et al., 2019). In female mammals, Kp stimulates GnRH neuron activity (Pielecka-Fortuna et al., 2008; Piet et al., 2018) and LH release (Gottsch et al., 2004; Navarro et al., 2005; Smith et al., 2006), whereas RFRP-3 suppresses GnRH neuron activity and reduces the elevated preovulatory LH release (Ancel et al., 2017; Anderson et al., 2009; Ducret et al., 2009; Henningsen et al., 2017; Kriegsfeld et al., 2006; Pineda et al., 2010; Tsutsui et al., 2000). In female rodents, both Kp and RFRP-3 neurons display daily rhythms of activity that are thought to synchronize the LH surge with the beginning of the active period (Angelopoulou et al., 2019; Simonneaux, 2020). Notably, Kp neuronal activity increases while RFRP-3 neuronal activity decreases at the time of the LH surge (Chassard et al., 2015; Gibson et al., 2008; Henningsen et al., 2017; Robertson et al., 2009). Earlier studies have shown that these neuronal activities are

synchronized by daily signals derived from the main biological clock located in the suprachiasmatic nucleus (SCN), since monosynaptic connections exist between the SCN arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP) neurons on one side and Kp (Vida et al., 2010; Williams et al., 2011) and RFRP-3 neurons (Russo et al., 2015) on the other side. Age-dependent changes in any of these regulatory networks, upstream of the GnRH neurons, may play a key role in the induction of hypothalamic senescence and reproductive decline in female mammals.

In the present study, we investigated whether there are physiological correlates linking reproductive senescence with the RFRP-3 system and its innervation by the SCN-derived neuropeptides AVP and VIP. More specifically, we examined whether there are daily- and age-dependent changes in RFRP-3 expression, neuronal activation and frequency of AVP-ergic and VIP-ergic fiber projections on RFRP-3 neurons in the hypothalamus of 4, 13 and 19 months-old C57BL/6J female mice.

Materials and methods

Animals

Adult female C57BL/6J mice (Charles River, France) were housed two or three per cage under a 12-h light: 12-h dark cycle (lights on at 7:00 am given as zeitgeber 0 (ZT0)) with controlled temperature (22°C) and *ad libitum* access to food and water up to the age of 19 months. All protocols were reviewed by the Comite Regional d'Ethique en Matiere d'Experimentation Animale (CREMEAS) and approved by the French National Ministry of Education and Research (authorization #8452-2017010613574177 v2).

Monitoring of estrous cycle and LH secretion

Female mice were studied at 4, 13 or 19 month-old during their diestrus stage as assessed by vaginal smears and circulating LH levels and as previously described (Bahougne et al., 2020). Vaginal smears were performed at ZT2 during at least two consecutive cycles and all mice, whatever their age, were sampled at the diestrus stage (appearance of leucocytes).

On the day of diestrus, before sacrifice, 4 µl of blood was sampled from the tip of the tail and immediately diluted in 116 µl of PBST (10% of 10x phosphate buffered saline, 0.25% of Tween-20 in milliQ H2O) and stored at −80 °C until the LH assay. LH concentration was determined by an ELISA using anti-bovine LHβ as capture antibody (monoclonal antibody, 518B7, NHPP, Torrance, California), rabbit anti-mouse LH as first antibody (polyclonal antibody, rabbit LH antiserum, AFP240580Rb, NHPP, Torrance, California), goat anti-rabbit IgG as secondary antibody (D048701–2, Dako Cytomation, Polyclonal Goat Anti-Rabbit, Denmark) and mouse LH as standard (mLH, AFP-5306A, NHPP, Torrance, California) as previously described (Bahougne et al., 2020). Four concentrations of LH (1, 10, 20 and 30 ng/ml) were used as quality controls.

Neuroanatomical investigation of age-dependent changes in the daily regulation of RFRP3 neurons

Tissue Processing

Adult female C57BL/6J mice at diestrus stage were sacrificed at 2 different time points (ZT0 and ZT12; n = 5 per experimental point) by exposure to an increasing concentration of CO_2. Animals were intracardially perfused with 10 ml phosphate buffer saline 0.1 M (PBS, pH 7.4) followed by 20 ml of periodate-lysine-paraformaldehyde fixative (formaldehyde 4%, $NaIO_4$ 10 mM and lysine 75 mM in 100 mM phosphate buffer, pH 7.3). The brains were collected, post-fixated in periodate-lysine-paraformaldehyde for 12 h at room temperature, washed with PBS, dehydrated and embedded in polyethylene glycol as previously described (Klosen et al., 1993).

Twelve series of 12 µm-thick coronal brain sections were cut using a microtome throughout the DMH as presented in the Paxinos mouse brain atlas and using neuroanatomical landmarks, such as the SCN, the third ventricle and the mammillary bodies. For each mouse, one section in every twelve (i.e. 1 section every 144 µm, giving 6-7 DMH-containing brain sections) was rehydrated and mounted on a SuperFrost Plus (Menzel-Glaser, Braunschweig, Germany) slide. For each immunolabelling experiment, DMH-containing slides of all mice of

different age and time points were processed at the same time in order to limit variations in the labeling background.

Double c-Fos/RFRP-3, AVP/RFRP-3 and VIP/RFRP-3 immunohistochemistry

The number of c-Fos expressing RFRP-3 neurons and the number of RFRP-3 neurons with close AVP- and VIP-fiber appositions was assessed by dual immunohistochemistry. Brain sections were first incubated either with a rabbit polyclonal antiserum raised against c-Fos (1:2000; Santa Cruz Biotechnology Cat# sc-413, RRID:AB_627251) or a rabbit polyclonal antiserum raised against neurophysin II, a cleavage product of prepro-vasopressin (1:15000; Sigma-Aldrich Cat# N0774, RRID:AB_260747) or a rabbit polyclonal antiserum raised against VIP (1:2000; Sigma-Aldrich Cat# HPA017324, RRID:AB_1858754) diluted in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20, for 24 h at room temperature. Brain sections were washed with PBS, incubated with biotinylated donkey antirabbit (1:2000; Jackson Labs; in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20) for 1 h at room temperature and then washed again with PBS. Immunoreactive signal was amplified by a treatment with the avidin biotin complex coupled to peroxidase (1:250; Vector Laboratories) for 1 h at room temperature, then revealed using a solution of 0.5 mg/mL 3,3-diaminobenzidine (DAB; Sigma-Aldrich) diluted in water and 0.001% hydrogen peroxide urea (Sigma-Aldrich) for 30 min at room temperature. Before performing the second immunolabelling, antibodies from the first immunolabelling were eluted with 2 x 15 min washes in a solution of 100 mM glycine containing 0.3% Triton X-100 (pH 2.2) at room temperature. Brain sections were then incubated with a rabbit polyclonal antiserum raised against RFRP-3 (1:15000; Greg M. Anderson Lab; University of Otago; New Zealand Cat# GA197, RRID: AB_2877670) diluted in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20 overnight at room temperature. The sections were then washed with PBS, incubated with biotinylated donkey anti-rabbit (1:2000; Jackson Labs; in 154 mM PBS buffer containing 10% donkey serum and 0.3% Tween 20) for 1 h at room temperature, and then washed again with PBS. The RFRP-3 signal was detected using streptavidin-peroxidase at 1/3000 (Roche) for 1 h at room temperature and revealed with Fast blue-BB (Sigma-

Aldrich) for 10 min at room temperature. Finally, sections were mounted with CC/mount (Sigma-Aldrich), dehydrated in toluene twice for 10 min, and mounted with Eukitt resin (Sigma-Aldrich).

Quantification of immunolabeled RFRP-3 cells and AVP- and VIP-ergic fibers

Five sections located at a comparable rostro-caudal level in the middle of the DMH were taken into account for the analysis in order to limit differences stemming from rostro-caudal variations. These five sections were selected based on neuroanatomical markers such as the median eminence, the tuberoinfundibular sulcus and the pituitary stalk. Counting was done by the first author when unaware of the animal's identity. For each mouse, the total number of RFRP-3-immunoreactive (ir) neurons, the number of RFRP-3 neurons containing nuclear c-Fos, and the number of RFRP-3 neurons receiving direct AVP- and VIP-fiber projections were counted manually on both sides of the brain. For each mouse, the number of RFRP-3 neurons is given as the total number in 5 sections, the relative number of c-Fos expressing RFRP-3 neurons is given as a percentage of the total number of RFRP-3 neurons, and the relative numbers of RFRP-3 neurons with close AVP- or VIP-fiber appositions are given as a percentage of the total number of RFRP-3 neurons.

The overall density of AVP- and VIP-ir fibers was quantified in selected regions of interest (ROI) bilaterally in the DMH defined by neuroanatomical landmarks (such as the median eminence, the tuberoinfundibular sulcus and the pituitary stalk), the DMH boundaries and the anatomical position of the RFRP-3 neurons by counting manually the number of points at which a labeled fiber crossed the intersections of a grid applied on the ROIs. For each mouse AVP- and VIP-ir fiber density is given as total number of crossing points/total grid number of the ROIs.

Data analysis

Statistical analysis was performed with GraphPad Prism 6. All values are given as mean ± SEM (n =5 per group). Two-way ANOVA was used to assess significant effects of *Daytime* and *Age*, as well as possible interaction effects of *Daytime* and *Age,* followed by Tukeys multiple comparisons post-hoc test. Differences were considered significant for $P < 0.05$.

Results

Age-related changes in the daily pattern of RFRP-3 expression and neuronal activation in female C57BL/6J mice

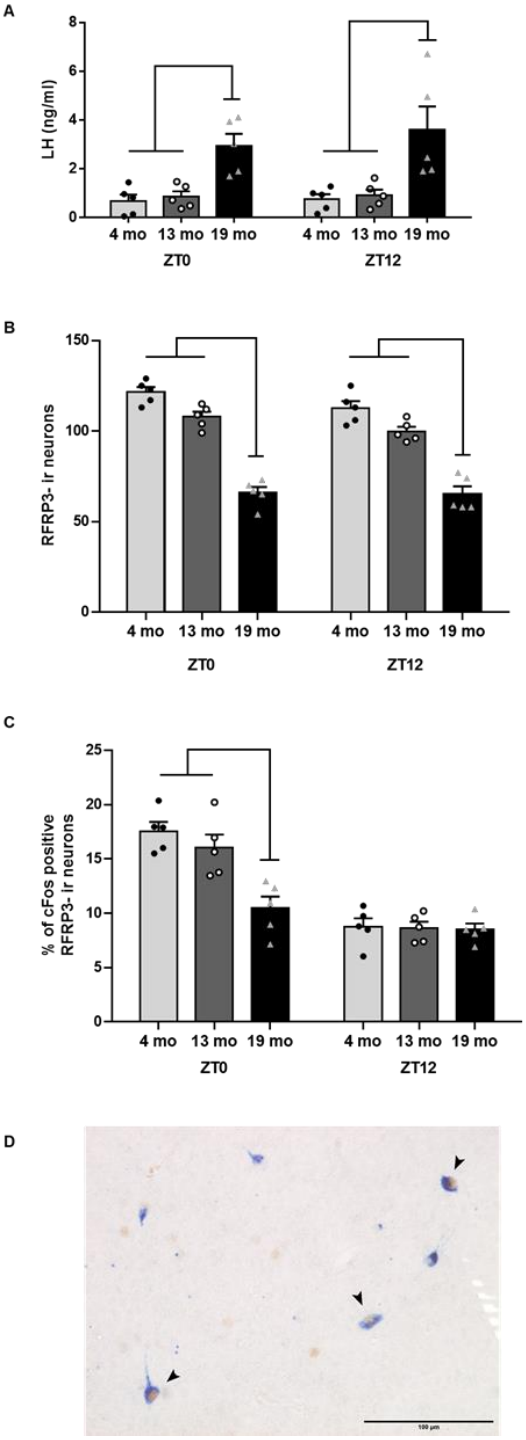
First, we investigated whether RFRP-3 neurons display differential regulation according to time-of-day using two time points (ZT0 and ZT12) in young (4 month-old), middle-aged (13 month-old) and old (19 month-old) female mice sampled at the diestrus stage.

Young (4 month-old) mice displayed regular 4-5 day cycles, middle-aged (13 month-old) mice displayed regular 7-8 day cycles and old (19 month-old) mice displayed irregular 10-12 day cycles (pseudopregnancy stage). Young and middle-aged mice exhibited low circulating LH levels, contrary to old mice that exhibited a significant increase in the LH levels (Two Way ANOVA, *Age*: $F(2, 24) = 17.63$, $p < 0.0001$; Fig. 1A). No significant time-of-day differences were found as expected (Two Way ANOVA, *Daytime*: $F(1, 24) = 0.4491$, $p = 0.5091$; Fig. 1A).

The number of RFRP-3-immunostained neurons did show a significant daily variation (Two Way ANOVA, *Daytime*: $F(1, 24) = 4.568$, $p = 0.0430$; Fig. 1B). In addition, we found a clear effect of age (Two Way ANOVA, *Age*: $F(2, 24) = 129.6$, $p < 0.0001$). A moderate, non-significant decrease in the number of RFRP3-ir neurons was recorded in the middle-aged group ($p=0.076$ (ZT0); $p=0.107$ (ZT12)) and a further prominent decrease of approximately 50% in the number of RFRP3-ir neurons in the old group (Fig. 1B).

Then, we examined whether there are daily- and age-related changes in RFRP-3 neuronal activation, as assessed by the number of c-Fos-positive RFRP-3 neurons (Fig. 1C, D). We

found a significant effect of time of day (Two Way ANOVA, *Daytime*: $F(1, 24) = 70.49$, $p < 0.0001$; Fig. 1C), with both young and middle-aged mice displaying a marked decrease of approximately 50% in the number of c-Fos-positive RFRP-3 neurons at the beginning of the dark (active) period (ZT12) as compared to the beginning of the light (resting) period (ZT0). Notably, 19 month-old mice displayed a significant decrease in the number of c-Fos-positive RFRP-3 neurons at the beginning of the day (ZT0) compared to young and middle-aged mice (Two Way ANOVA, *Age*: $F(2, 24) = 9.513$, $p = 0.0009$; Fig. 1C), but they did not exhibit daily variation in the number of c-Fos-positive RFRP-3 neurons (Tukeys multiple comparisons test, $p = 0.6194$; Fig. 1C). This differential effect of time-of-day within the different age groups was also reflected in the significant effect of *Interaction* ($F(2, 24) = 8.340$, $p = 0.0018$).


A
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B
RFRP3- Ir neurons
150
100
50
0
4 mo 13 mo 19 mo
ZT0
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ZT12
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% of cFos positive
RFRP3- Ir neurons
25
20
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Figure 1. *RFRP-3 immunoreactivity and neuronal activity in the dorsomedial hypothalamus of female C57BL/6J mice of three different ages at two daily time points. (A) LH blood concentrations; (B) number of RFRP-3-ir neurons; (C) percentage of c-Fos-expressing RFRP-3-ir neurons; (D) photograph showing c-Fos-positive (brown) and c-Fos-negative RFRP3-ir neurons (blue) in a 4 month-old (mo) female mouse sampled at ZT0. Zeitgeber (ZT) 0 is light onset and ZT12 is light offset; bars in A-C represent mean ± SEM (n = 5) values of 4 mo (light grey), 13 mo (dark grey) and 19 mo (black) groups of mice. Statistics in A-C show the post-hoc results for the effects of Age (p<0.05). Scale bar in D = 100 µm.*

Age-related changes in the daily pattern of RFRP-3 innervation by AVP and VIP projections in female C57BL/6J mice

In this experiment we evaluated whether the density of AVP- and VIP-containing fibers in the DMH and the number of RFRP-3 neurons with close AVP-ir or VIP-ir fiber appositions exhibited changes according to the two time points examined (ZT0 and ZT12).

We recorded no daily- (Two Way ANOVA, *Daytime*: F(1, 24) = 1.570, p = 0.2222; Fig. 2A) or age-dependent (Two Way ANOVA, *Age*: F(2, 24) = 0.3332, p = 0.7199; Fig. 2A) variations in DMH AVP-ir fiber density. Similarly, we found no daily- (Two Way ANOVA, *Daytime*: F(1, 24) = 1.440, p = 0.2419; Fig. 3A) or age-dependent (Two Way ANOVA, *Age*: F(2, 24) = 0.5989, p = 0.5574; Fig. 3A) changes in VIP-ir fiber density in the DMH.

When measuring daily- and age-related changes in the percentage of RFRP-3 neurons with close AVP-ir fiber appositions (Fig. 2B, C), we recorded a significant effect of time of day (Two Way ANOVA, *Daytime*: F(1, 24) = 8.616, p = 0.0072), as well as a significant effect of age (Two Way ANOVA, *Age*: F(2, 24) = 13.56, p = 0.0001) and a significant *Interaction* effect (F(2, 24) = 3.927 p = 0.0335). Overall, the percentage of RFRP-3 neurons with close AVP-ir fiber appositions was increased at ZT12 due to an increase especially in young and middle-aged mice. Old mice did not exhibit such a daily variation in the percentage of RFRP-3 neurons with close AVP-ir fiber appositions, but at ZT0 their number of RFRP-3 neurons with close AVP-ir fiber appositions was higher as compared to young and middle-aged mice (p = 0.0004 and p = 0.0018, respectively).

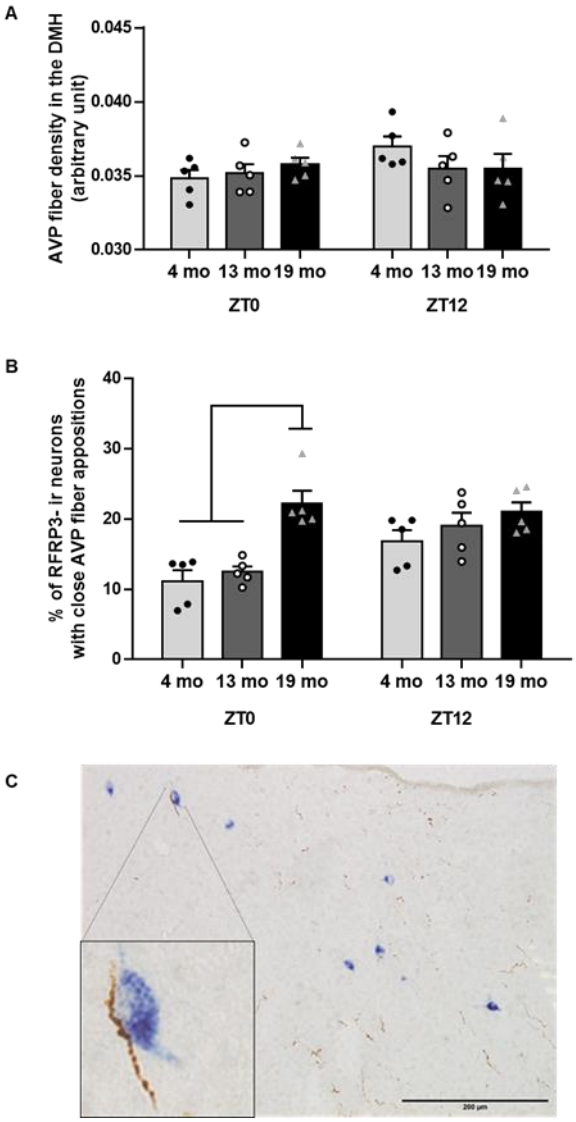

Figure 2. *Density of arginine vasopressin (AVP)-immunostained fibers in the dorsomedial hypothalamus and AVP contacts with RFRP-3 neurons in female C57BL/6J mice of three different ages at two daily time points. (A) AVP-ir fiber density (arbitrary units) in the dorsomedial hypothalamus; (B) percentage of RFRP3-ir neurons with close AVP-ir fiber appositions; (C) photograph showing RFRP3-ir neurons (blue) and AVP-containing fibers (brown) in a 4 month-old (mo) female mouse sampled at ZT12. Zeitgeber (ZT) 0 is light onset and ZT12 is light offset; bars in A &*

B represent mean ± SEM (n = 5) values of 4 mo (light grey), 13 mo (dark grey) and 19 mo (black) groups of mice. Statistics in B show the post-hoc results for the effects of Age (p<0.05). Scale bar in C = 200 µm.

When analyzing daily- and age- dependent changes in the percentage of RFRP-3 neurons with close VIP-ir fiber appositions, we recorded a significant effect of time of day (Two Way ANOVA, *Daytime*: $F(1, 24) = 31.50$, $p < 0.0001$), due to a significant decrease in the number of RFRP-3 neurons with close VIP-ir fiber appositions at the beginning of the dark period (ZT12) as compared to the early light period (ZT0) in both young (Tukeys multiple comparisons test, $p = 0.0019$; Fig. 3B) and middle-aged (Tukey's multiple comparisons test, $p = 0.0003$; Fig. 3B) mice. However, no daily variation in the percentage of RFRP-3 neurons with close VIP-ir fiber appositions was found in the old mice (Tukeys multiple comparisons test, $p > 0.9999$; Fig. 3B). Furthermore, old mice exhibited the highest number of RFRP-3 neurons with close VIP-ir fiber appositions (Two Way ANOVA, *Age*, $F(2, 24) = 26.74$, $p < 0.0001$), and this effect was most prominent at ZT12 as indicated by the significant effect of *Interaction* ($F(2, 24) = 7.945$, $p = 0.0022$; Fig. 3B).

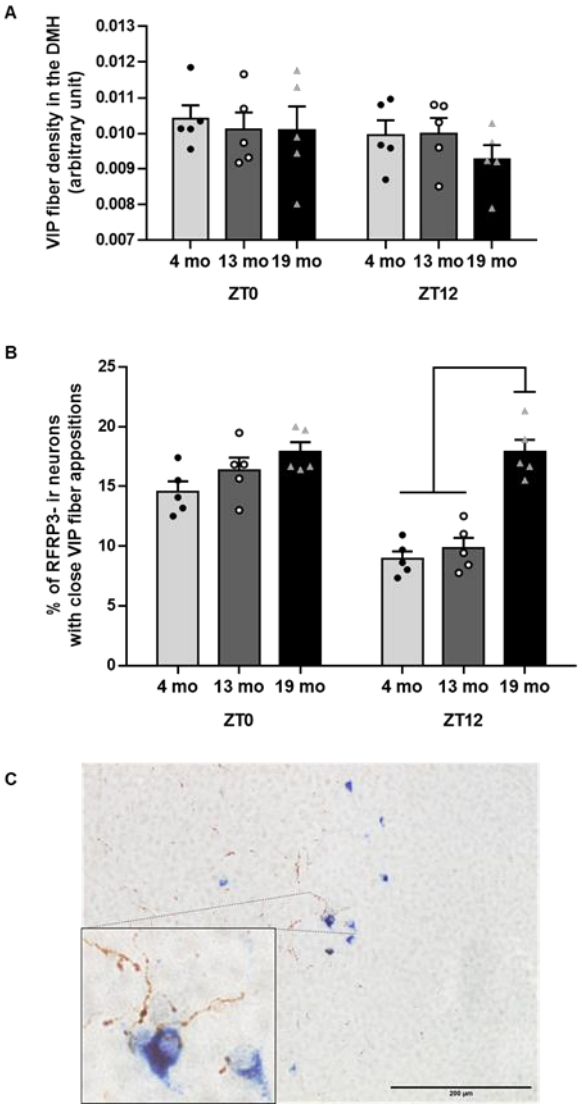

Figure 3. *Density of vasoactive intestinal peptide (VIP)-immunostained fibers in the dorsomedial hypothalamus and VIP contacts with RFRP-3 neurons in female C57BL/6J mice of three different ages at two daily time points. (A) VIP-ir fiber density (arbitrary units) in the dorsomedial hypothalamus; (B) percentage of RFRP3-ir neurons with close VIP-ir fiber appositions; (C) photograph showing RFRP3-ir neurons (blue) and VIP-containing fibers (brown) in a 4 month-old (mo) female mouse sampled at ZT0. Zeitgeber (ZT) 0 is light onset and ZT12 is light offset; bars represent mean ± SEM (n = 5) values of 4 mo (light grey), 13 mo (dark grey) and 19 mo (black) groups of mice. Statistics in B show the post-hoc results for the effects of Age (p<0.05). Scale bar in C = 200 µm.*

Discussion

Female mammals display regular daily and ovarian cycles driven by estradiol-sensitive neural circuits in the hypothalamus (Angelopoulou et al., 2019). Age-related hypothalamic decline leads to senescent changes in the reproductive function (Yin and Gore, 2006). In this study, we investigated whether there are daily- and age-dependent changes in the number and regulation of hypothalamic RFRP-3 neurons, using a neuroanatomical approach. We found an age-dependent down-regulation of RFRP-3 neurons and a loss of daily variation in the innervation of RFRP-3 neurons by the circadian peptides AVP and VIP, notably in the 19 month-old mice.

In female rodents, reproductive aging is marked by the onset of longer irregular cycles (> 4-6 days) at the age of 8-12 months, followed by a period of constant estrous (CE) or persistent vaginal cornification at the age of 10-16 months. The CE period is followed by a prolonged diestrus phase with intermittent ovulation known as repetitive pseudo-pregnancy (RPP), before reaching the anoestrous stage at the age of 22-25 months (Cruz et al., 2017). In this study we evaluated daily changes in the LH production in mice of different ages in addition to the cytological analysis of the estrous cycle. Young (4 months-old) and middle-aged (13 months-old) mice in diestrus displayed constant low plasma LH levels and regular estrous cycles, even though the cycles of middle-aged mice were longer (6-7 days). However, old (19 months-old) mice in diestrus (RPP) displayed a marked increase in the plasma LH levels on both experimental time points, while estrous cyclicity was abolished (PP stage).

Although age-related changes occur at the level of the pituitary and the ovary, evidence suggests that the aging hypothalamus is the driving force in the induction of reproductive senescence in rodents. While menopause in women is characterized by follicular depletion, a study showed that rat ovaries maintain a stock of follicles throughout life (Mandl and Shelton, 1959). Ovarian transplantation studies showed that old anestrous rats cannot reinitialize cycling activity when receiving ovaries from young animals. On the other hand, young rats maintain their regular estrous cyclicity when transplanted with old rat ovaries (Peng and Huang, 1972). Moreover, electrical stimulation of the hypothalamus induces

ovulation in old acyclic rats (Clemens et al., 1969). Altogether, these findings support the idea that altered hypothalamic function accounts for reproductive decline and point towards changes in the GnRH neuronal system as the central regulator of the reproductive axis. Earlier studies in female rodents showed that both the number of GnRH cells and GnRH neuronal activation during the preovulatory GnRH/LH surge decreased in middle-age (Funabashi and Kimura, 1995; Lloyd et al., 1994; Miller et al., 1990; Yin et al., 2009). This age-related suppression of the GnRH system may be partly due to changes in the upstream neuronal circuitry that regulates the activity of these neurons.

Kisspeptin (Kp), one of the main hypothalamic modulators of the GnRH system that potently stimulates GnRH/LH release, also undergoes age-dependent changes. A previous study in female mice demonstrated that both Kp neuronal activity during the GnRH/LH surge and the expression of ERα in Kp neurons decrease at advanced ages. Furthermore, a subset of Kp neurons displays characteristics of cellular senescence (atypical morphology and increased expression of markers of cellular aging) in middle aged mice (Zhang et al., 2014). Other studies showed that both the number of Kp cells and the levels of Kp mRNA expression in the anteroventral periventricular nucleus decrease during the preovulatory LH surge in middle-aged rats (Lederman et al., 2010; Neal-Perry et al., 2009). Moreover, central administration of Kp in the preoptic area (POA) restored the attenuated amplitude of the LH surge in middle-aged rats (Neal-Perry et al., 2009). Therefore, age-related alterations in the GnRH system may be driven in part by altered Kp signaling, along with changes in other regulatory systems, extrinsic to GnRH neurons.

Our current study demonstrates a significant decrease in the number of RFRP3-ir neurons in old mice. This result is in contrast to a previous study in male mice, which reported a decrease in RFRP-3 cell number in adulthood followed by an increase during old age (Sethi et al., 2010). Of note, developmental alterations in RFRP-3 expression have been reported in another study showing that the total number of RFRP-3 neurons decreases during puberty in both male and female mice (Poling et al., 2012). Altogether, the previous and current findings suggest a strong effect of aging on the RFRP-3 expression and provide evidence for sex-specific regulation of the RFRP-3 system during old age. Moreover, our

findings further underscore the importance of down-regulation of the inhibitory RFRP-3 system for the facilitation of LH secretion in females. Notably, the decreased number of RFRP-3 neurons in old mice might be responsible in part for the uplift of the inhibition of the reproductive axis and account for the increased LH release at advanced ages.

We confirmed that the RFRP-3 neuronal activation is specifically decreased at the beginning of the dark period in young and middle-aged mice. Here, we show that old mice display a lower percentage of c-Fos–positive RFRP-3 neurons at the beginning of the light period compared to the other age groups, but not at the beginning of the dark period, therefore leading to a loss of the daily variation in the RFRP-3 neuronal activity in these old mice. Notably, another study also reported an age-dependent alteration in the daily rhythm of both kisspeptin and GnRH neuronal activity, showing a decreased activity during the late afternoon in middle-aged mice (Zhang et al., 2014).

The above described changes in the daily rhythms of neuronal activity in the hypothalamus during old age may be associated with aging-dependent alterations in the circadian system. With aging, the circadian regulation of many physiological and behavioral processes deteriorates. Age-related changes in circadian rhythms include decreased amplitude and period length, increased fragmentation and desynchronization (Carskadon et al., 1982; Martin et al., 1986; Pittendrigh and Daan, 1974; Rosenberg et al., 1991; Shibata et al., 1994; van Gool et al., 1987). Notably, we recently reported that the daily surge of LH is delayed by 2 h and has a reduced amplitude in middle-aged mice (Bahougne et al., 2020). Such alterations in circadian rhythmicity are associated with age-dependent modifications in the SCN, including impairment in the SCN output pathways that regulate the GnRH/LH surge. Numerous studies have shown that the SCN molecular core clock mechanism is sustained during aging, but the SCN output signal is compromised (Asai et al., 2001; Nakamura et al., 2011; Polidarová et al., 2017; Yamazaki et al., 2002). Electrophysiological studies in rodents demonstrated aging-associated changes in the SCN electrical activity, including a decreased amplitude during daytime, desynchronization of SCN neurons and aberrant SCN firing patterns (Watanabe et al., 1995; Satinoff et al., 1993; Farajnia et al., 2012; Nakamura et al., 2011). Aging may also affect the synthesis of neuropeptides that act as synchronizers within

the SCN and/or as output signals of the SCN. In humans, the total SCN AVP cell number decreases at advanced ages (Swaab et al., 1985). In addition the circadian rhythm of AVP synthesis in the human SCN is disrupted during senescence, showing loss of diurnal oscillations, reduced amplitude and reversed diurnal pattern (Hofman and Swaab, 1994). Senescent rodents exhibit no changes in the SCN volume and in the total SCN cell number (Roozendaal et al., 1987). However, the number of AVP (- 31%) and VIP (- 36%) neurons in the rodent SCN decrease during aging (Roozendaal et al., 1987; Chee et al., 1988). Middle-aged rodents maintain robust daily rhythms of SCN AVP mRNA, but the rhythmicity of VIP mRNA levels is attenuated during senescence (Krajnak et al., 1998). Altogether, these findings show that age-related impairments in the SCN electrical activity and circadian expression of neuropeptides probably result in a compromised SCN output signal and may be responsible for alterations in the LH secretion during senescence.

As previously reported in female Syrian hamsters (Russo et al., 2015) hypothalamic RFRP-3 neurons are contacted by AVP- and VIP-ir fibers. In this study, we found no daily variation in the AVP- and VIP-ir fiber density in the DMH, but we did record a daily variation in the relative number of RFRP-3 neurons that receive close AVP- and VIP-fiber appositions in young and middle age mice. In old mice, no changes in the overall density of AVP and VIP fibers in the DMH was observed, but the daily variation in the number of RFRP-3 neurons with close AVP- and VIP- fiber appositions was lost. More precisely, at the beginning of the light period young and middle-aged mice display a decreased percentage of RFRP-3 neurons with close AVP fiber appositions and at the beginning of the dark period a decreased percentage of RFRP-3 neurons with close VIP fiber appositions. In old mice, RFRP-3 neurons exhibit no more morning decrease in AVP-ir fibers and evening decrease in VIP-ir fibers. This loss of daily variation in the peptidergic input on RFRP-3 neurons may be partly responsible for the observed loss of the daily regulation of RFRP-3 neuronal activity. Indeed, both peptides may be involved in the daily regulation of RFRP-3 neurons, since previous studies have reported that VIP inhibits (Russo et al., 2015) and AVP activates (Angelopoulou et al., 2021) RFRP-3 neurons at specific time points.

Conclusions

This study shows that RFRP-3 expression decreases and daily rhythms in RFRP-3 neuronal activity are abolished in old female mice. Furthermore, old female mice exhibit a loss of daily variation in the AVP-ergic and VIP-ergic input on RFRP-3 neurons. Our study suggests that during aging, the down-regulation of RFRP-3 neurons may account for the subsequent increase of LH release, and that age-dependent alterations in the function of the SCN may lead to changes in the SCN output pathways towards the RFRP-3 neurons that regulate LH production. However, it is essential to investigate whether there are also age-dependent changes in the daily expression of ERα, VPAC1/VPAC2 and V1a receptors in RFRP3 neurons, in order to identify the full mechanism of RFRP-3 neuron regulation during old age.

CRediT statement

Eleni Angelopoulou: Methodology, Investigation, Formal analysis, Writing- Original draft, Visualization. **Valérie Simonneaux**: Supervision, Conceptualization, Reviewing and Editing, Funding acquisition. **Andries Kalsbeek**: Supervision, Validation**,** Reviewing and Editing, Funding acquisition.

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The authors have declared that no conflicts of interest exist.

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PART V

DISCUSSION

Chapter 7

General discussion and future perspectives

General Discussion

In this thesis a number of experiments are described in which we investigated whether there are daily-, estral- and age- dependent alterations in hypothalamic RFRP-3 neurons and how these alterations correlate with factors that alter pituitary LH secretion using the C57BL/6J mice (*Mus Musculus*) as a model organism as well as a diverse toolbox of anatomical, electrophysiological and biochemical techniques. In a first set of experiments, we analyzed in female mice whether there are daytime- and estrous cycle stage-dependent changes in RFRP-3 expression, neuronal activity, and/or the density of AVP- and VIP-ergic fiber projections onto these RFRP-3 neurons. Subsequently, the changes found were compared to the profiles of LH secretion. In a next experiment we investigated whether AVP and VIP regulate the electrical activity of RFRP-3/ EYFP-labelled neurons in hypothalamic sections of transgenic RFRP/Cre female mice. Subsequently, we tested 3 hypotheses: 1) disturbing the biological clock, through a single phase shift or exposure to chronic shifts, impairs estrous cyclicity and LH surge generation, leading to reduced fertility; 2) aging changes the central control of the HPG axis resulting in impaired preovulatory LH surge characteristics and 3) age-dependent alterations in the SCN output pathways lead to changes in the hypothalamic circuits that regulate LH production, notably in the RFRP-3 system.

The data presented in this thesis show that indeed a clear daily rhythm is present in the RFRP-3 neuronal activity and in the neuropeptidergic input on the RFRP-3 neurons. We reported that RFRP-3 firing rates change according to the time-of-day and estrous stage and that the circadian peptide, AVP, has a functional role in the control of RFRP-3 firing activity (chapters 2 & 3). In chapter 4, we confirmed our hypothesis that disruptions in the biological clock through shifted light/dark cycles induce alterations in the generation of the LH surge and lead to decreased fecundity. In chapter 5, we demonstrated a prominent effect of aging on the preovulatory LH surge characteristics and in chapter 6, we reported the altered SCN control of the RFRP-3 system in the aging hypothalamus.

1. Daily and estral regulation of RFRP-3 neurons

This part of the thesis **(Chapters 2 and 3)** focused on the question whether RFRP-3 neurons act as an interface for SCN-derived output pathways and E2 feedback in order to convey daily and ovarian signals to the HPG axis. We took two approaches to investigate the effects of the time of day and estrous stage on RFRP-3 neuronal activity. Using a neuroanatomical approach, we found that also in mice c-Fos expression in the RFRP-3 neurons decreases during the day-to-night transition, independently of the estrous cycle stage, in agreement with previous findings in Syrian hamsters (Gibson et al., 2008; Henningsen et al., 2017). On proestrus during the day-to-night transition, when the LH surge is generated, we found a decreased RFRP-3 neuronal activity along with a concurrent increase in Kp neuronal activity (Chassard et al., 2015; Henningsen et al., 2017; Robertson et al., 2009). In contrast, on diestrus, when circulating LH levels are constantly low, the RFRP-3 neuronal activity was similarly decreased, but Kp neuronal activity remained stable at low levels (Chassard et al., 2015; Henningsen et al., 2017; Robertson et al., 2009). Therefore, we concluded that RFRP-3 neuronal activation (as attested by c-Fos expression) is tightly regulated by the time of day, but not by the estrous cycle status even though previous studies reported that a subset of RFRP-3 neurons express ERα and E2 administration suppresses RFRP expression (Kriegsfeld et al., 2006; Poling et al., 2012). Next, using an electrophysiological approach, we found that RFRP-3 neurons display different firing rates according to the time of day and estrous cycle status. RFRP-3 neurons fired action potentials at an about twice times higher frequency in proestrus as compared to diestrus, regardless of the time of the day. However, RFRP-3 firing activity was more regular during the day-to-night transition compared to an earlier time of the day in both diestrus and proestrus.

Altogether, these neuro-anatomical and electrophysiological data strengthen the importance of RFRP-3 neurons, along with Kp neurons, in the daily and estral control of the reproductive axis. However, our data acquired by means of immunohistochemistry and electrophysiology show some discrepancies. While we recorded a clear effect of estrous cycle stage on the RFRP-3 electrical activity, we found no effect of estrous cycle stage on c-Fos expression in RFRP-3 neurons. Expression of the immediate early gene (IEG) c-*fos*, has

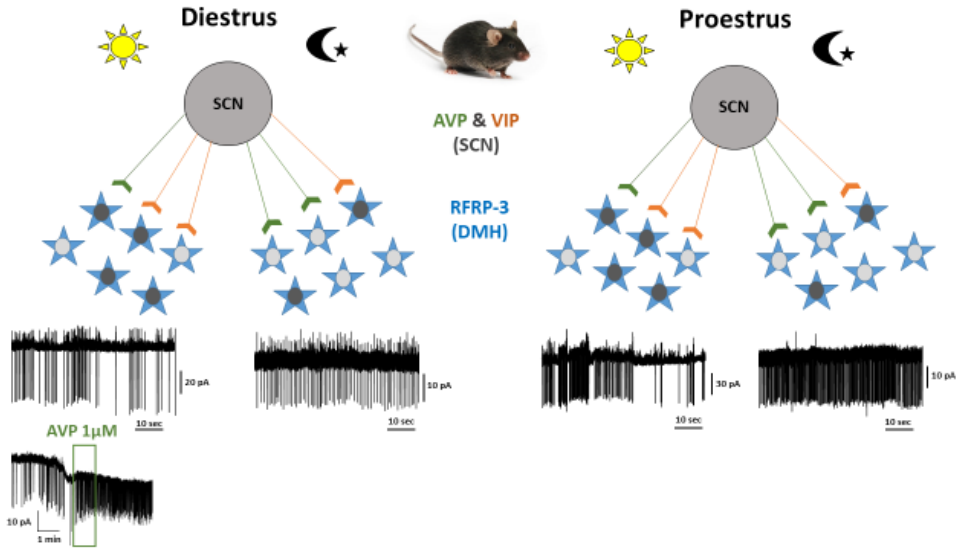
been extensively used as a marker of neuronal activity, despite numerous studies demonstrating that it is not always a reliable marker of functional activity. Originally, c-Fos induction was attributed to numerous challenges, including neurotropic factors, neurotransmitters, depolarization and increased calcium influx (Kovács, 1998). However, calcium influx during spike activity alone cannot induce c-Fos expression. Antidromic activation experiments failed to induce c-Fos, thus demonstrating that c-Fos induction requires receptor activation rather than spike activity (Luckman et al., 1994). Moreover, another study showed that c-Fos expression is associated with the neuronal firing pattern and not the firing rate (Guo et al., 2007). These findings clearly indicate that there is not a one-to-one relation between electrical activity and c-Fos expression. On the other hand, it is important to highlight that c-Fos expression reflects *in vivo* neuronal activation, while our electrophysiological studies were performed *ex vivo*, thus the above-mentioned differences could also stem from peripheral signaling that was lost in the isolated hypothalamic slices.

Next, we investigated whether the circadian neuropeptides, AVP and VIP, regulate RFRP-3 neurons in a time- and estrous cycle stage-dependent way. First, using a neuro-anatomical approach, we demonstrated that mouse RFRP-3 neurons receive close appositions from AVP-ergic and VIP-ergic fibers, as was previously shown also in Syrian hamsters (Russo et al., 2015). In addition, we revealed that regardless the estrous cycle stage, during late afternoon the number of RFRP-3 neurons receiving close AVP- and VIP-ergic fiber appositions increases, concomitantly with an increase in the AVP-ergic fiber density in the DMH. Thus, both AVP and VIP signaling on RFRP-3 neurons increases prior to the light-dark transition. Notably, the overall AVP-ergic fiber input on RFRP-3 neurons is similar in both diestrus and proestrus, which reflects the pattern of AVP gene expression in the SCN which is not sexually differentiated or affected by E2 (Krajnak et al., 1998). In contrast, although VIP mRNA was shown to be sexually differentiated in the SCN, we did not record any E2-dependent changes in the VIP-ergic fiber input on RFRP-3 neurons (Krajnak et al., 1998; Mahoney et al., 2009).

Secondly, we used an electrophysiological approach to investigate the functional role of AVP and VIP in the control of activity of RFRP-3 neurons and demonstrated that during

diestrus AVP increases the RFRP-3 neuron firing frequency at midday and not at the day-to-night transition, while it has no effect at both time points in proestrus. Our findings suggest that AVP may coordinate RFRP-3 neurons in a specific time window under low E2 levels (diestrus) and that high E2 levels (proestrus) may act in a suppressive manner by disabling the excitatory effect of AVP on RFRP-3 neurons. Moreover, we demonstrated that VIP has no effect on the RFRP-3 neuron firing frequency at the day-to-night transition during either diestrus or proestrus. Our findings are opposing an earlier study in Syrian hamsters in which it was shown that central injections of VIP decreased the RFRP-3 neuronal activation in a time-dependent manner, being effective in the afternoon, but not in the morning, while central administration of AVP had no effect (Russo et al., 2015). Although it is possible that these discrepancies are due to the limitations of c-Fos as a functional marker of electrical activity or due to species-dependent differences, it is important to highlight the different methodologies used. Our electrophysiological study was performed *ex vivo*, in isolated hypothalamic slices, while the Russo et al. study involved c-Fos ICC and *in vivo* administration of the peptides.

In conclusion, this part of our study provided further evidence that RFRP-3 neurons act as a locus of relay, where daily and ovarian cues are conveyed to the HPG axis. We reported temporal changes in the RFRP-3 neuronal activity and in the frequency of AVP- and VIP-ergic fiber projections on RFRP-3 neurons. We demonstrated that RFRP-3 electrical activity exhibits estrous stage-dependent changes with higher spike frequency in proestrus, and that AVP application activates RFRP-3 firing activity only at midday of the diestrus stage, while VIP had no effect at any experimental point (graphical abstract 1). Our current hypothesis is that the increased AVP input during late afternoon induces an excitatory effect on the slow firing RFRP-3 neurons at diestrus, but is inefficient in further exciting the highly firing RFRP-3 neurons in proestrus.



Graphical abstract 1: *Working model illustrating the regulation of RFRP-3 neurons by daily and ovarian cues in female mice. Neurons of the suprachiasmatic nuclei (SCN) synthesizing vasopressin (AVP) and vasoactive intestinal peptide (VIP) exhibit daily variation controlled by an intrinsic circadian clock and the daily change in light input. The SCN AVP and VIP fiber outputs on RFRP-3 neurons located in the dorsomedial hypothalamus (DMH), also exhibit daily variation (AVP innervation peaks at ZT8-ZT12 and drops at ZT0, VIP innervation peaks at ZT8 and drops at ZT12). Furthermore, the activity of RFRP-3 neurons (attested by c-Fos induction) decreases at the light/dark transition. Daily variation in AVP and VIP outputs and RFRP-3 neuron activity are similar in both diestrus and proestrus. However, RFRP neuron firing rate, as illustrated at the representative traces recorded at two time points (ZT6-10 and ZT11-14), is higher in proestrus as compared to diestrus, independently of the time of the day. AVP(1 µM) application increases RFRP neuron firing in the afternoon of diestrus, but not at the other stages of the estrous cycle indicating that AVP signalling on RFRP neurons may depend on circulating ovarian steroids. VIP (1µM) application exhibits no effect at the light/dark transition. Thus, based on the ICC data we hypothesize that the SCN AVP and VIP outputs coordinate the activity of GnRH neurons indirectly via the RFRP-3 neurons. The electrophysiological data show that the SCN AVP output activates low-firing RFRP-3 neurons in the afternoon under low estradiol levels (diestrus), thereafter inhibiting GnRH neurons, while the SCN AVP output cannot activate high-firing RFRP-3 neurons under high estradiol levels (proestrus), therefore facilitating GnRH release. Altogether, this coordinated pathway, acting synergistically with the kisspeptin system (not in the graph), is proposed to time the preovulatory GnRH/LH surge at the light/dark transition of the proestrus stage.*

Future directions

Future studies should address our hypothesis further, mainly regarding the estrous stage-dependent regulation of RFRP-3 neurons. Recording the electrical activity in ovariectomized and E2-treated ovariectomized RFRP-Cre mice would allow us to confirm the effect of E2 on RFRP-3 neurons. In order to complement our data and confirm that the fluctuating E2 levels control the effect of AVP on RFRP neurons, it is necessary also to examine the effect of AVP in ovariectomized and E2-treated ovariectomized mice. Interestingly, an earlier study reported a role of E2 in the activation of AVPV Kp neurons by AVP (Piet et al., 2015), suggesting that this could also be the case with RFRP-3 neurons. Further experiments should also explore the effect of endogenous AVP release using AVP receptor antagonists. The use of an antagonist cocktail for AMPA, NMDA and GABA receptors will demonstrate whether AVP activates RFRP neurons directly or via an indirect mechanism. Future studies should also address whether RFRP-3 neurons express AVP receptors (V1aR) and whether there are time of day- and E2-dependent changes in the expression of V1aR and ERα that could mediate the daily changes in the RFRP-3 neuronal activity that we demonstrated. The issue of VIP action on RFRP-3 neurons should also be further examined. It should be assessed whether mouse RFRP-3 neurons express VIP receptors (VPAC1 and VPAC2) and if so whether their expression is time of day- and E2-dependent. Interestingly, in Syrian hamsters less than 10% of RFRP-3 neurons express VPAC1 and VPAC2, thus indicating a possible indirect effect of the peptide (Russo et al., 2015). Although in our study, we found that VIP did not have any effect on the RFRP-3 neuron activity during the day-to-night transition, further studies should examine the effect of VIP at midday in both diestrus and proestrus, since that is the time point at which we recorded the stimulatory effect of AVP in diestrus. Furthermore, the availability of RFRP-Cre mice should help to better understand the role of RFRP-3 in female reproduction, notably in the LH surge onset. Thus, using Cre-dependent expression of Channelrhodopsin-2 in RFRP-3 neurons *in vivo* and optogenetically tuning the frequency, duration, and circadian time of the stimulation, would allow us to dissect the specific role of RFRP-3 in controlling the GnRH activity and LH release.

2. Effect of circadian disruption on reproductive activity

In this part of the thesis **(Chapter 4)**, we investigated the effect of shifted light/dark cycles on the robustness of estrous cyclicity and timing of the preovulatory LH surge in female mice. We demonstrated that a single 10-hour phase advance or delay of the light/dark cycle leads to temporary changes in the function of the reproductive system. Notably, during the first cycle after a phase shift, only half of the mice exhibited an LH surge, which occurred 2 hours in advance. During the second cycle, mice exhibited prolonged estrous cycle duration, while in the third cycle, proper estrous cyclicity and timing of the LH surge were recovered. In agreement with our findings, a study in Syrian hamsters showed that the rhythms of E2, progesterone and FSH re-entrained during the third estrous cycle after photoperiod reversal (a single 12-hour shift) (Finkelstein et al., 1978).

Next, we demonstrated that chronic exposure to disrupted light/dark cycles markedly impairs the occurrence of the preovulatory LH surge, leading to reduced fertility. Chronic rotation of 3 days 10-hour phase advance/4 days 10-hour phase delay had a minor effect on estrous cyclicity, however, after 9 months of chronic shifts mice tended to have longer, irregular estrous cycles, while a part of them became acyclic. By contrast, chronic exposure to shifts in the light/dark cycle strongly impaired the occurrence of the preovulatory LH surge. Only a few mice exhibited a preovulatory LH surge, but with a reduced amplitude and at an out of phase timing. Given our observation that female mice kept under a stable 12L/12D cycle maintain estrous cyclicity and preovulatory LH surge characteristics until 9 months (Chapter5), we conclude that the dramatic alteration in the LH surge occurrence and timing observed under chronic shifts is not attributable to aging.

Moreover, we found that chronic exposure to disrupted light/dark cycles reduces gestational success, with shifted mice delivering half the number of pups than mice kept under a stable 12L/12D cycle do. Shifted mice possibly had a delayed ovulation since most of them gave birth 5 days later than the majority of the control mice. A previous study investigating the impact of chronodisruptive conditions on gestation (chronic 6-hour phase advance or delay every 5 days during pregnancy) showed decreased full-term gestational

success in the shifted mice with a larger impact in the chronic phase advance group (Summa et al., 2012).

Altogether, our results suggest that single phase shifts have a moderate effect on female reproduction, whereas chronic phase shifts lead to a severely impaired preovulatory LH surge, which in turn may reduce fertility and gestational success. These findings confirm the requirement of a functional and well-synchronized circadian system for proper female reproduction.

Future directions

Studies showed that the SCN clock forwards daily cues to the reproductive system either directly via a VIP-ergic input on GnRH neurons, or indirectly via an AVP-ergic input on Kp neurons and both an AVP- and VIP-ergic input on RFRP-3 neurons, which in turn drive the GnRH-induced LH surge. Therefore, future studies should investigate how shifts in the light/dark cycle (single or chronic) alter the SCN output pathways towards the reproductive system. Assessing the density of AVP- and VIP-ergic fiber projections on Kp, RFRP-3 and GnRH neurons, as well as the Kp, RFRP-3 and GnRH neuronal activity following the phase shifts, would allow us to identify the exact mechanism through which chronodisruptive conditions affect the neural networks that control female fertility. It is also possible that in addition to the SCN clock, shift work conditions impair peripheral reproductive clocks. To test this hypothesis, we could compare the endogenous circadian oscillations of bioluminescence from cultured AVPV explant, pituitary, uterus and ovary sampled from PER2:luciferase mice exposed to a normal light/dark cycle or to a chronic shift. Finally, future experiments should also examine whether properly timed central administration of AVP and VIP could overcome reproductive deficits observed in shifted mice, such as the lost LH surge in mice after chronic exposure to phase shifts.

3. Age-dependent regulation of RFRP-3 neurons and LH secretion

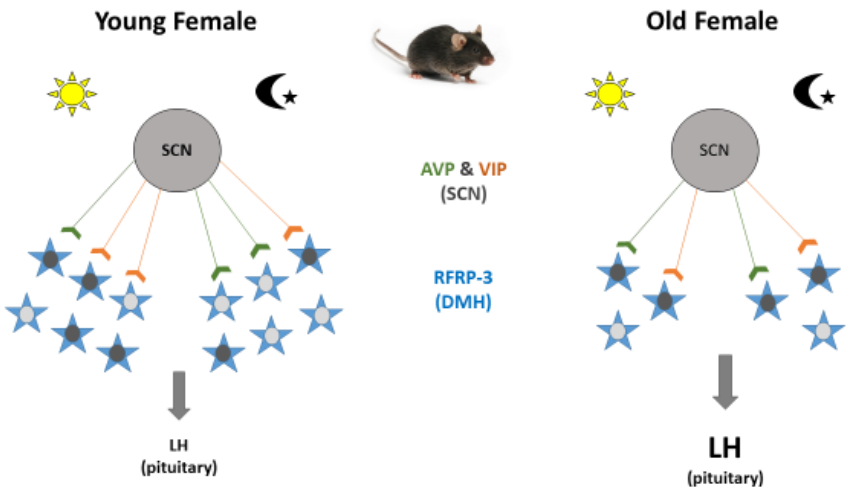
In this part of the thesis **(Chapters 5 and 6)**, we investigated the effect of aging on the RFRP-3 system and its daily peptidergic innervation and on LH secretion in female mice. We demonstrated that the characteristics of the LH surge in proestrus are sustained until middle-age, while old mice display a delayed LH surge with half the amplitude. We also found that the level of LH secretion in diestrus is maintained until middle-age, even when the estrous cycles become irregular, while at old age, when estrous cyclicity is lost, the circulating levels of LH increase prominently.

Using a neuroanatomical approach, we found that the number of RFRP-3-ir neurons decreases during middle and old age. Our findings are opposing an earlier study in male mice which reported that the number of RFRP-3 neurons increases during old age (Sethi et al., 2010). However, together these findings point towards a strong effect of aging on the RFRP-3 expression and provide evidence for a sex-dependent regulation of the RFRP-3 system during reproductive senescence. Notably, the decreased number of RFRP-3 neurons observed at advanced ages was associated with a marked increase in LH secretion at diestrus. This might be partly due to the uplift of inhibitory RFRP-3 signal towards the HPG axis. Regardless the age, we recorded a moderate daily variation in the number of RFRP-3 neurons, but during senescence the daily variation in the RFRP-3 neuronal activity was abolished. Indeed, during old age, we recorded a prominent decrease in the RFRP-3 neuronal activity (as attested by c-Fos expression) at the beginning of the light period, but not at the beginning of the dark period. Of note, another study reported decreased Kp and GnRH neuronal activity during the late afternoon at middle-age (Zhang et al., 2014).

Regardless of the age, we found no daily variation in the AVP- and VIP-ergic fiber density in the DMH. Next, we recorded a daily variation in the relative number of RFRP-3 neurons with close AVP- and VIP-fiber appositions during youth and middle-age. However, during old age the daily variation in the number of RFRP-3 neurons with close AVP- and VIP- fiber appositions was abolished. Notably, until middle-age the percentage of RFRP-3 neurons with close AVP fiber appositions decreased at the beginning of the light period, while the

percentage of RFRP-3 neurons with close VIP fiber appositions decreased at the beginning of the dark period. In contrast, during old age the morning decrease in the number of RFRP-3 neurons with close AVP fiber appositions was not observed nor the evening decrease in the number of RFRP-3 neurons with close VIP fiber appositions. This loss of daily variation in the peptidergic input on RFRP-3 neurons may account in part for the observed loss of the daily regulation of RFRP-3 neuronal activity. Indeed, both peptides may be involved in the daily regulation of RFRP-3 neurons, since we previously showed that AVP activates the RFRP-3 electrical activity at midday and central administration of VIP was shown to decrease the RFRP-3 neuronal activity in the afternoon (Russo et al., 2015).

In conclusion, in the last part of our study we showed that during old age the circulating LH levels increase independently of the time of day, possibly due to altered control in the hypothalamic and circadian circuits that control the GnRH/LH secretion. We demonstrated that during aging the RFRP-3 expression decreased, while daily variations in both the RFRP-3 neuronal activity and the SCN input on RFRP-3 neurons were lost (graphical abstract 2). Our current hypothesis is that age-dependent alterations in the SCN output pathways affect the regulation of the hypothalamic circuits that control GnRH/LH secretion. Importantly, the down-regulation of the RFRP-3 system and its altered control by the circadian peptides, may be partly responsible for the increased LH release at advanced ages and account for the reproductive decline.



Graphical abstract 2: *Working model illustrating the effect of age on the regulation of RFRP-3 neurons by daily cues in female mice at the diestrus stage. Neurons of the suprachiasmatic nuclei (SCN) synthesizing vasopressin (AVP) and vasoactive intestinal peptide (VIP) make close appositions with RFRP-3 neurons, located in the dorsomedial hypothalamus (DMH). Young females exhibit daily variation in the SCN AVP and VIP fiber outputs on RFRP-3 neurons (AVP innervation peaks at ZT12 and drops at ZT0, VIP innervation peaks at ZT0 and drops at ZT12). In contrast, old females exhibit decreased RFRP-3 expression, and no daily variation in both the SCN AVP and VIP fiber outputs on RFRP-3 neurons, and the RFRP-3 activity. Thus, it is hypothesized that reduced inhibitory RFRP-3 signal to GnRH neurons at old age may lead to constantly elevated levels of LH.*

Future directions

Our study indicated RFRP-3 as a possible driving co-factor for impaired female reproductive activity during senescence. Therefore, interventions that target restoration of the RFRP-3 signaling could provide more insight into the mechanisms behind hypothalamic decline, reproductive aging and gonadotropin secretion. Future studies should examine whether exogenous administration of RFRP-3 could overcome reproductive patterns observed in old females, such as the elevated LH levels and the delayed LH surge. We demonstrated that daily variation in RFRP-3 activity is abolished at advanced ages, therefore it would be interesting to test the effect of central administration of RFRP-3, especially during the light period, on LH secretion. Furthermore, due to our observation that the daily variation in the number of RFRP-3 neurons with close AVP- and VIP-fiber appositions is lost during senescence, it is necessary to examine the effect of exogenous administration of AVP and VIP on the RFRP-3 activity in old females. In our studies, we reported an elevated number of RFRP-3 neurons with close AVP- and VIP-fiber appositions during midday in young and middle-aged females, thus it would be interesting to investigate whether central administration of AVP and VIP before the day-to-night transition can restore the daily variation in the RFRP-3 neuronal activity in old females. Finally, although cumulating evidence shows that reproductive senescence is initiated by the aging hypothalamus and not by ovarian decline, it would still be interesting to investigate the RFRP-3 system and its innervation by circadian peptides in ovariectomized and E2-treated ovariectomized mice, in order to confirm that the down-regulation of the RFRP-3 system is due to age-associated changes in the hypothalamic and circadian systems and not due to changes in the circulating levels of E2.

4- Concluding clinical relevance

Age-related female infertility is becoming more common because, for a variety of socio-economic reasons, many women choose to delay child bearing. In women, reproductive decline starts gradually, approximately at age 32 and progresses more rapidly after age 37, reflecting primarily a decrease in oocyte quality and quantity (Broekmans et al., 2006; Faddy et al., 1992). In addition, the flexible working time arrangements, such as night work, increasingly found in Western societies, have a great impact on the reproductive health of women. Circadian disruptions impair many physiological systems, including female reproduction. Night shift work is associated with increased risk of menstrual irregularity, endometriosis and prolonged time to conception (Fernandez et al., 2020; Simonneaux and Bahougne, 2015). For decades, research has been centered on the mechanism underlying ovarian decline while the contribution of the aging brain has not been fully considered. Although extrapolating data from rodents to women is always challenging, it is imperative to explore the contribution of the aging hypothalamus. Previous experimental work revealed that both the GnRH and the Kp system are down-regulated during old age. Our study has been the first to describe the age-related changes in the RFRP-3 system that could contribute to the suppression of female fecundity at advanced ages. On the other hand, the neural circuits mediating reproductive failure due to circadian asynchrony have not been studied at all yet. Increasing our knowledge on how aging and circadian disruptions, such as shifts in the sleep/wake cycle, impact female reproduction can help us develop strategies that counteract reproductive decline. Targeting the neural circuits that are negatively affected could contribute to the delay of reproductive failure and prolong the time window of fertility for women in the near future. However, due to the decreased oocyte quality during old age, an extended fertility window could induce other deficiencies (miscarriages, chromosomal abnormalities in embryos, etc). Therefore, additional treatment strategies should be developed contemporaneously, as for example the recently developed technique of transfer of autologous mitochondria to compromised oocytes (Kristensen et al., 2017).

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APPENDICES

Summary

Female reproduction is regulated by neuroendocrine mechanisms operating along the hypothalamic-pituitary-gonadal (HPG) axis. In female mammals, the reproductive system displays regular cycles that depend both on the biological clock (SCN) synchronized to the light/dark cycle and on estradiol, whose concentration varies according to oocyte maturation. Exploring the neural pathways by which daily and ovarian cues are integrated into the HPG axis and notably GnRH neurons, represents an exciting opportunity to get a further understanding of the role of certain neuropeptides in female reproduction. Notably, two hypothalamic neuropeptides RFRP-3 and kisspeptin, reported to regulate GnRH neuron activity and GnRH/LH release in female mammals, have emerged as critical for the integration of such cues within the reproductive axis. The goal of this PhD project was to investigate the daily-, estral- and age-dependent regulation of RFRP-3 neurons in order to decipher its role in the temporal control of reproduction.

In **Chapters 2 & 3**, we investigated how RFRP-3 neurons are regulated by daily and estrogenic cues in female C57BL/6J mice. First, we examined whether there are daytime- and estrous stage-dependent changes in RFRP-3 expression, neuronal activity as measured by c-Fos expression, and in the number of RFRP-3 neurons that receive close AVP- and VIP-ergic fiber projections, knowing that most AVP- and VIP fibers are derived from the biological clock in the SCN. Next, we investigated the effect of time of day and estrous cycle status on the RFRP-3 firing activity and whether AVP and VIP regulate the electrical activity of RFRP-3/EYFP fluorescent protein labelled neurons in hypothalamic sections of transgenic RFRP/Cre female mice. We demonstrated that c-Fos expression in the RFRP-3 neurons decreased during the day-to-night transition in both proestrus and diestrus. Therefore, on proestrus, the decrease in RFRP-3 neuronal activity coincides with an increase in LH secretion during the day-to-night transition. These data also show that the daily pattern in RFRP-3 c-Fos activation is independent of the stage of the estrous cycle. On the other hand, the electrophysiological experiments showed that the RFRP-3 neurons do exhibit different firing rates according to the estrous stage. We observed that RFRP-3 neurons fire action potentials at an about twice higher frequency in proestrus compared to diestrus. We found

no time of the day difference in firing rate, but we did find that RFRP-3 electrical activity is more regular during the day-to-night transition compared to an earlier time period in both diestrus and proestrus. Altogether, our results suggest that RFRP-3 neurons may be involved in the daily and estral regulation of the reproductive axis. Next, we investigated whether mouse RFRP-3 neurons receive direct AVP- and VIP-ergic fiber projections and whether there are daily- or estrous cycle stage-dependent variations in the fiber innervation. Indeed, we found that RFRP-3 neurons receive close AVP- and VIP-ergic fiber appositions. Furthermore, we reported a coordinated increase in the number of RFRP-3 neurons with close AVP- and VIP-ergic fiber appositions during late afternoon, concurrently with an increase in the AVP-ergic fiber density in the DMH. Thus, prior to the light-dark transition, there was an increased input of AVP and VIP signaling on RFRP-3 neurons. The overall AVP- and VIP-ergic fiber input on RFRP-3 neurons was similar in both diestrus and proestrus, which might reflect the E2 independent daily rhythms in SCN. Since the presence of peptidergic fiber appositions on neuronal cell bodies does not prove that they make synapses or that there is a direct post-synaptic effect of the peptide, we also examined the effect of AVP and VIP on the RFRP-3 neuronal firing activity either at ZT6-10 or at ZT11-14 on diestrus and proestrus. We demonstrated that AVP increases RFRP-3 neuron firing frequency at midday and not during the day-to-night transition of diestrus, while it has no effect at either time point in proestrus. These findings indicate that AVP may coordinate the RFRP-3 system in a specific time window under low E2 milieu (diestrus) and that E2 (at proestrus) may act in a suppressive manner by disabling the excitatory effect of AVP on RFRP-3 neurons. Finally, we observed that VIP had no effect on the RFRP-3 neuron firing frequency during the day-to-night transition in either diestrus or proestrus.

In **Chapter 4**, we investigated the effect of shifted light/dark cycles on the robustness of estrous cyclicity and timing of the preovulatory LH surge in female C57BL/6J mice. We demonstrated that mice exposed to a single 10-hour phase advance or delay of the light/dark cycle exhibit temporary changes in the occurrence and timing of the LH surge. During the first cycle after a phase shift, only half of the mice exhibited an LH surge, which also occurred 2 hours in advance. During the second cycle, the duration of the estrous cycle

was prolonged and only during the third cycle, proper estrous cyclicity and timing of the LH surge were attained again. Next, we showed that chronic exposure to disrupted light/dark cycles markedly impaired the occurrence of the preovulatory LH surge, leading to reduced fertility. Chronic rotation of 3 days with a 10-hour phase advance and subsequently 4 days with a 10-hour phase delay had a minor effect on estrous cyclicity, although after 9 months of chronic shifts mice tended to have longer, irregular estrous cycles, while a part of them became acyclic. By contrast, chronic exposure to such a shift protocol strongly impaired the occurrence of the preovulatory LH surge. Only a few mice exhibited a preovulatory LH surge, but with a reduced amplitude and its timing was out of phase. Moreover, we found that chronic exposure to continuously changing light/dark cycles reduced gestational success, with shifted mice delivering half the number of pups compared to mice kept under a stable 12L/12D cycle. Shifted mice possibly had a delayed ovulation since most of them gave birth 5 days later than the majority of the control mice. Altogether, our results suggest that single phase shifts have a moderate effect on female reproduction, whereas chronic phase shifts lead to a severely impaired preovulatory LH surge, which in turn may reduce fertility and gestational success.

In **Chapter 5**, we investigated whether there are age-dependent alterations in the characteristics of the preovulatory LH surge in female C57BL/6 J mice. We demonstrated that the amplitude and timing of the LH surge were similar in 3-, 6- and 9-month-old mice. However, 12-month-old mice exhibited a prominent decrease (more than 50%) of peak LH secretion and a 2-hour delay in the timing of the LH surge.

In **Chapter 6**, we examined whether reproductive senescence may be associated with changes in the RFRP-3 system and its peptidergic innervation by AVP and VIP fibers in female C57BL/6 J mice. We showed that during diestrus basal, non-surge levels of LH secretion were similar in young (4-month-old) and middle-aged (13-month-old) mice, while these levels were prominently increased at an advanced age (19-month-old). Next, we demonstrated that the number of RFRP-3-ir neurons decreases during middle and old age, while the daily variation in the RFRP-3 neuronal activity, observed in young and middle age mice, is lost during senescence. Of note, we recorded a prominent decrease in RFRP-3

neuronal activity (as attested by c-Fos expression) at the beginning of the light period, but not at the beginning of the dark period in senescent mice. Regardless of age, we recorded no daily variation in the AVP- and VIP-ergic fiber density in the DMH, but we did find a daily variation in the percentage of RFRP-3 neurons with close AVP- and VIP-fiber appositions at young and middle-age. During old age, this daily variation in the number of RFRP-3 neurons with close AVP- and VIP- fiber appositions was lost. Notably, until middle-age the percentage of RFRP-3 neurons with close AVP fiber appositions at the beginning of the light period decreased, as well as the percentage of RFRP-3 neurons with close VIP fiber appositions at the beginning of the dark period. Consequently, during senescence the morning decrease in the percentage of RFRP-3 neurons with close AVP fiber appositions was not observed anymore nor the evening decrease in the percentage of RFRP-3 neurons with close VIP fiber appositions. This loss of daily variation in the peptidergic input on RFRP-3 neurons may be responsible in part for the observed loss of the daily regulation of RFRP-3 neuronal activity.

In conclusion, in this thesis we provided further evidence that RFRP-3 neurons act as a locus of relay for daily and ovarian cues that are conveyed to the HPG axis. We reported temporal changes in RFRP-3 neuronal activity and in the frequency of AVP- and VIP-ergic fiber projections on RFRP-3 neurons. We demonstrated that RFRP-3 electrical activity exhibits estrous stage-dependent alterations with a higher spike frequency in proestrus, and that AVP application activates RFRP-3 firing activity only at midday of the diestrus stage, while VIP had no effect during the day-to-night transition of either diestrus or proestrus. We also showed that single phase shifts have a moderate effect on female reproduction, whereas chronic phase shifts lead to a severely impaired preovulatory LH surge, which in turn may reduce fertility and gestational success. Finally, we demonstrated that during senescence RFRP-3 expression decreases, while daily variations in both RFRP-3 neuronal activity and SCN input on RFRP-3 neurons were lost. Altogether, our findings highlight the requirement of a functional and well-synchronized circadian system for proper female reproduction and showcase the impact of RFRP-3 on the daily control of reproduction.

Samenvatting

De vrouwelijke voortplanting wordt gereguleerd via neuro-endocriene mechanismen die aangestuurd worden door de hypothalamus-hypofyse-gonadale (HPG) as. Bij vrouwelijke zoogdieren vertoont het voortplantingssysteem regelmatige cycli die zowel afhangen van de biologische klok (SCN), gesynchroniseerd met de licht-donkercyclus, als van oestradiol, waarvan de concentratie varieert naargelang de rijping van de eicel. Het onderzoeken van de neurale projecties waarlangs de dagelijkse en ovarium signalen geïntegreerd worden in de HPG-as en in het bijzonder in de GnRH neuronen, biedt gelijk ook een mooie mogelijkheid om de rol van bepaalde neuropeptiden in de vrouwelijke voortplanting beter te begrijpen. Twee hypothalame neuropeptiden, RFRP-3 en kisspeptide, die de activiteit van GnRH neuronen en de afgifte van GnRH en LH bij vrouwelijke zoogdieren reguleren, blijken cruciaal te zijn voor de integratie van deze signalen in de voortplantings-as. Het doel van dit promotieproject was de dagelijkse, oestral- en leeftijdsafhankelijke regulatie van RFRP-3 neuronen te onderzoeken om zo de rol van deze neuronen in de temporele controle van de voortplanting te ontcijferen.

In **Hoofdstuk 2 & 3** onderzochten we hoe de activiteit van RFRP-3 neuronen gereguleerd wordt door de dagelijkse en oestrogene signalen in vrouwelijke C57BL/6J muizen. Eerst onderzochten we of er dagelijkse- en oestruscyclus afhankelijke veranderingen zijn in RFRP-3 expressie, neuronale activiteit zoals gemeten door c-Fos expressie, en in het aantal RFRP-3 neuronen die AVP- en VIP-erge vezelprojecties ontvangen, wetende dat de meeste van deze AVP- en VIP-vezels afkomstig zijn van de biologische klok in de SCN. Vervolgens onderzochten we het effect van het tijdstip en de oestruscyclus op de RFRP-3 vuurfrequentie en of AVP en VIP de elektrische activiteit reguleren van RFRP-3/EYFP gelabelde fluorescente neuronen in hypothalame coupes van transgene RFRP/Cre vrouwelijke muizen. We toonden aan dat de c-Fos expressie in de RFRP-3 neuronen afnam tijdens de dag-nacht overgang, zowel in pro-oestrus als in di-oestrus. Tijdens proestrus valt de afname van de neuronale RFRP-3 activiteit tijdens de dag-nacht overgang dus samen met een toename van de LH secretie. Deze gegevens tonen ook aan dat het dagelijkse patroon in RFRP-3 c-Fos activatie onafhankelijk is van het stadium van de oestruscyclus. Anderzijds

toonden de elektrofysiologische experimenten aan dat de RFRP-3 neuronen wel verschillende vuurfrequentie' patronen vertonen afhankelijk van de fase van de oestruscyclus. We zagen dat RFRP-3 neuronen actiepotentialen afvuren met een ongeveer twee keer hogere frequentie in pro-oestrus vergeleken met di-oestrus. We vonden geen verschillen afhankelijk van de tijd van de dag, maar wel dat zowel in di-oestrus als in pro-oestrus de RFRP-3 vuurfrequentie regelmatiger is tijdens de overgang van dag naar nacht in vergelijking met een eerdere periode. Al met al suggereren onze resultaten dat RFRP-3 neuronen betrokken zijn bij zowel de dagelijkse als de oestrogene regulatie van de voortplantings-as. Vervolgens onderzochten we of RFRP-3 neuronen in de muis directe AVP- en VIP-erge vezelprojecties ontvangen en of er dagelijkse of oestruscyclus afhankelijke variaties zijn in de vezelinnervatie. Inderdaad vonden we dat RFRP-3 neuronen nauwe AVP- en VIP-erge vezel-apposities ontvangen. Bovendien zagen we een toename van het aantal RFRP-3 neuronen met nauwe AVP- en VIP-erge vezel apposities tijdens de late namiddag, samenvallend met een toename van de AVP-erge vezeldichtheid in de DMH. Vóór de licht-donker overgang was er dus een verhoogde input van AVP- en VIP-signalering op RFRP-3 neuronen. De totale AVP- en VIP-erge vezelinput op RFRP-3 neuronen was vergelijkbaar in di-oestrus als pro-oestrus, wat de E2 onafhankelijke dagelijkse ritmes in de SCN zou kunnen weerspiegelen. Omdat de aanwezigheid van peptiderge vezelcontacten op de cellichamen niet bewijst dat ze synapsen maken of dat er een direct post-synaptisch effect van het peptide is, onderzochten we ook het effect van AVP en VIP op de vuurfrequentie van RFRP-3 neuronen op ZT6-10 of ZT11-14 tijdens di-oestrus en pro-oestrus. We toonden aan dat AVP de RFRP-3 vuurfrequentie verhoogt in de middag, maar niet tijdens de dag-naar-nacht overgang tijdens di-oestrus, terwijl het op beide tijdstippen geen effect had tijdens pro-oestrus. Deze bevindingen wijzen erop dat in een laag E2 milieu (di-oestrus) AVP het RFRP-3 systeem kan moduleren in een specifiek tijdsinterval en dat in een milieu met hoge E2 (in pro-oestrus) E2 door een remmend effect het exciterende effect van AVP op RFRP-3 neuronen uit kan schakelen. Tenslotte stelden we vast dat zowel tijdens di-oestrus als pro-oestrus VIP geen effect had op de vuurfrequentie van RFRP-3 neuronen tijdens de overgang van dag naar nacht.

In **Hoofdstuk 4** onderzochten we het effect van een verschoven licht-donker cyclus op de robuustheid van de oestruscyclus en de timing van de pre-ovulatoire LH-surge bij vrouwelijke C57BL/6J muizen. We toonden aan dat muizen die worden blootgesteld aan èèn enkele fase-versnelling of -vertraging van de licht-donkercyclus van 10 uur, tijdelijke veranderingen vertonen in het optreden en de timing van de LH-surge. Tijdens de eerste cyclus na een faseverschuiving vertoonde slechts de helft van de muizen een LH-surge, die ook 2 uur eerder optrad. Tijdens de tweede cyclus werd de duur van de oestruscyclus verlengd en pas tijdens de derde cyclus werd de juiste timing van de oestruscyclus en LH-surge weer bereikt. Vervolgens toonden we aan dat chronische blootstelling aan verstoorde licht-donker cycli het optreden van de pre-ovulatoire LH-surge duidelijk verminderde, wat resulteerde in verminderde vruchtbaarheid. Chronische rotatie van 3 dagen met een fase-versnelling van 10 uur en vervolgens 4 dagen met een fase-vertraging van 10 uur had in eerste instantie een gering effect op de oestruscyclus. Na 9 maanden van chronische verschuivingen hadden muizen echter de neiging langere en onregelmatige oestrus cycli te hebben, terwijl een deel van de muizen acyclisch werd. Bovendien verminderde de chronische blootstelling aan een dergelijk verschuivingsprotocol sterk het optreden van de preovulatoire LH-surge. Slechts een paar muizen vertoonden een preovulatoire LH-surge, en dan ook nog met een verminderde amplitude en de timing was uit fase. Bovendien vonden we dat chronische blootstelling aan continu veranderende licht-donker cycli het zwangerschapssucces verminderde, waarbij “verschoven muizen” de helft minder pups ter wereld brachten dan muizen die onder een stabiele 12L/12D cyclus werden gehouden. De “verschoven muizen” hadden mogelijk ook een vertraagde ovulatie, aangezien de meeste van hen 5 dagen later bevielen dan de meerderheid van de controlemuizen. Al met al suggereren onze resultaten dat eenmalige faseverschuivingen een matig effect hebben op de vrouwelijke voortplanting, maar dat chronische faseverschuivingen leiden tot een sterk verminderde preovulatoire LH-surge, die op zijn beurt de vruchtbaarheid en het zwangerschapssucces kan verminderen.

In **Hoofdstuk 5** hebben we onderzocht of er leeftijdsafhankelijke veranderingen zijn in de kenmerken van de preovulatoire LH-surge bij vrouwelijke C57BL/6 J muizen. We toonden

aan dat de amplitude en timing van de LH-surge vergelijkbaar waren in 3-, 6- en 9-maanden oude muizen. Echter, 12-maanden oude muizen vertoonden een duidelijke afname van de piek LH secretie (met meer dan 50%) en een vertraging van 2 uur in de timing van de LH-surge.

In **Hoofdstuk 6** onderzochten we in vrouwelijke C57BL/6 J muizen of reproductieve veroudering geassocieerd kan worden met veranderingen in het RFRP-3 systeem en de peptiderge innervatie door AVP en VIP vezels. We toonden aan dat tijdens de di-oestrus de basale, niet-piek niveaus van LH secretie vergelijkbaar waren in jonge muizen (4 maanden oude) en muizen van middelbare leeftijd (13 maanden oude), terwijl deze niveaus duidelijk verhoogd waren in oude muizen (19 maanden oud). Vervolgens toonden we aan dat het aantal RFRP-3-ir neuronen afneemt tijdens middelbare en hoge leeftijd, terwijl de dagelijkse variatie in de RFRP-3 neuronale activiteit, zoals gemeten met c-Fos expressie en waargenomen in jonge en middelbare leeftijd muizen, verloren gaat in oude muizen. Opmerkelijk is dat we in oude muizen een prominente afname in RFRP-3 neuronale activiteit registreerden (zoals blijkt uit de c-Fos expressie) aan het begin van de lichtperiode, maar niet aan het begin van de donkerperiode. Ongeacht de leeftijd, registreerden we geen dagelijkse variatie in de AVP- en VIP-erge vezeldichtheid in de DMH, maar we vonden wel een dagelijkse variatie in het percentage RFRP-3 neuronen met nauwe AVP- en VIP-vezel apposities op jonge en middelbare leeftijd. Op oudere leeftijd ging deze dagelijkse variatie in het aantal RFRP-3 neuronen met nauwe AVP- en VIP-vezel apposities verloren. Opmerkelijk was ook dat tot middelbare leeftijd het percentage RFRP-3 neuronen met nauwe AVP-vezel apposities aan het begin van de lichtperiode afnam, evenals het percentage RFRP-3 neuronen met nauwe VIP-vezel apposities aan het begin van de donkerperiode. Bijgevolg werd in de oude muizen de ochtenddaling van het percentage RFRP-3 neuronen met nauwe AVP-vezel apposities niet meer waargenomen, noch de avonddaling van het percentage RFRP-3 neuronen met nauwe VIP-vezel apposities. Dit verlies van dagelijkse variatie in de peptiderge input op RFRP-3 neuronen zou gedeeltelijk verantwoordelijk kunnen zijn voor het waargenomen verlies in de dagelijkse variatie van RFRP-3 neuronale activiteit.

Concluderend, in dit proefschrift hebben we verder bewijs geleverd dat RFRP-3 neuronen fungeren als een relais voor dagelijkse en ovarium signalen die worden doorgegeven aan de HPG-as. We rapporteerden temporele veranderingen in RFRP-3 neuronale activiteit en in de frequentie van AVP- en VIP-erge vezelprojecties op RFRP-3 neuronen. We toonden aan dat de elektrische activiteit van RFRP-3 neuronen afhankelijk is van de fase van de oestruscyclus, met een hogere vuurfrequentie in pro-oestrus, en dat AVP de RFRP-3 vuurfrequentie enkel activeert op de middag van de di-oestrus fase, terwijl VIP geen effect had tijdens de dag-nacht overgang tijdens di-oestrus noch pro-oestrus. We toonden ook aan dat een eenmalige faseverschuiving van de licht/donker cylcus een matig effect heeft op de vrouwelijke voortplanting, maar dat chronische faseverschuivingen resulteren in een ernstig verminderde pre-ovulatoire LH-surge, die op zijn beurt de vruchtbaarheid en het zwangerschapssucces kan verminderen. Tenslotte toonden we aan dat tijdens veroudering de RFRP-3 expressie afneemt, terwijl de dagelijkse variaties in zowel RFRP-3 neuronale c-Fos activiteit als in SCN input op RFRP-3 neuronen verloren gingen. Al met al benadrukken onze bevindingen dat een functioneel en goed gesynchroniseerd circadiaan systeem een vereiste is voor een goede vrouwelijke voortplanting en dat RFRP-3 neuronen waarschijnlijk een belangrijke spelen in de regulatie van het (dagelijkse) ritme van de voortplanting.

Résumé

La reproduction féminine est régulée par des mécanismes neuroendocriniens opérant le long de l'axe hypothalamo-hypophyso-gonadique (HPG). Chez les mammifères femelles, le système reproducteur présente des cycles réguliers qui dépendent à la fois de l'horloge biologique des noyaux suprachiasmatiques (SCN) synchronisée au cycle lumière/obscurité et de l'estradiol, dont la concentration varie en fonction de la maturation des ovocytes. L'exploration des voies neuronales par lesquelles les signaux journaliers et ovariens sont intégrés dans l'axe HPG et notamment les neurones à GnRH, représente une opportunité intéressante pour mieux comprendre le rôle de certains neuropeptides dans la reproduction féminine. Deux neuropeptides hypothalamiques, l' (Arg)(Phe) related peptide-3 (RFRP-3) et la kisspeptine, qui régulent l'activité des neurones GnRH et la libération de GnRH/LH chez les mammifères femelles, sont notamment apparus comme essentiels pour l'intégration de ces signaux dans l'axe reproducteur. L'objectif de ce projet de thèse était d'étudier la régulation des neurones RFRP-3 en fonction de la journée, du cycle estrien et de l'âge, afin de déchiffrer son rôle dans le contrôle temporel de la reproduction.

Dans les **chapitres 2 et 3**, nous avons étudié comment les neurones à RFRP-3 sont régulés par des signaux journaliers et œstrogéniques chez les souris femelles C57BL/6J. Tout d'abord, nous avons examiné s'il existe des changements dépendants du jour et du stade estral dans l'expression de RFRP-3, l'activité neuronale mesurée par l'expression de c-Fos, et dans le nombre de neurones à RFRP-3 contactés par des projections de fibres contenant de l'arginine vasopressine (AVP) et du peptide intestinal vasoactif (VIP) , sachant que la plupart de ces fibres proviennent de l'horloge biologique des SCN. Ensuite, nous avons étudié l'effet de l'heure de la journée et du stade estrien, et l'effet d'application d'AVP et de VIP sur l'activité électrique des neurones à RFRP-3 marqués à la protéine jaune fluorescente (EYFP) dans des sections hypothalamiques de souris femelles transgéniques RFRP/Cre. Nous avons démontré que l'expression de c-Fos dans les neurones à RFRP-3 diminuait pendant la transition jour-nuit, tant en proestrus qu'en diestrus. Par conséquent, en proestrus, la diminution de l'activité des neurones à RFRP-3 coïncide avec une augmentation de la sécrétion de LH pendant la transition jour-nuit. Ces données montrent

également que l'activation journalière des neurones à RFRP-3 est indépendant du stade du cycle estral. D'autre part, les expériences électrophysiologiques ont montré que les neurones à RFRP-3 présentent des taux d'excitation différents selon le stade estral. Nous avons observé que les neurones à RFRP-3 émettent des potentiels d'action à une fréquence environ deux fois plus élevée en proestrus qu'en diestrus. Nous n'avons pas trouvé de différence dans la fréquence des potentiels d'action en fonction du moment de la journée, mais nous avons constaté que l'activité électrique du RFRP-3 est plus régulière pendant la transition jour-nuit par rapport à une période plus précoce à la fois en diestrus et en proestrus. Dans l'ensemble, nos résultats suggèrent que les neurones à RFRP-3 peuvent être impliqués dans la régulation journalière et estrale de l'axe reproducteur. Ensuite, nous avons cherché à savoir si les neurones RFRP-3 de la souris reçoivent des projections de fibres AVP- et VIP-ergiques et s'il existe des variations journalières ou liées au stade du cycle estral dans l'innervation des fibres. Nous avons constaté que les neurones à RFRP-3 reçoivent des appositions de fibres AVP- et VIP-ergiques. De plus, nous avons observé une augmentation coordonnée du nombre de neurones à RFRP-3 avec des appositions de fibres AVP- et VIP-ergiques en fin d'après-midi, en même temps qu'une augmentation de la densité de fibres AVP-ergiques dans le DMH. Ainsi, avant la transition lumière-obscurité, il y avait une augmentation de la signalisation AVP- et VIP-ergique sur les neurones à RFRP-3, sans différence entre le diestrus et le proestrus, ce qui pourrait refléter des rythmes journaliers indépendants de l'E2 dans les SCN. La présence d'appositions de fibres peptidergiques sur les corps cellulaires neuronaux ne prouve pas qu'elles créent des synapses ou qu'il existe un effet post-synaptique direct du peptide. C’est pourquoi, nous avons également examiné l'effet de l'AVP et du VIP sur l'activité électrique des neurones à RFRP-3 à ZT6-10 ou à ZT11-14 en diestrus et en proestrus. Nous avons démontré que l'AVP augmente la fréquence des potentiels d’action des neurones à RFRP-3 à midi et non pendant la transition jour-nuit du diestrus, alors qu'il n'a pas effet à aucun des deux moments du proestrus. Ces résultats indiquent que l'AVP peut coordonner le système à RFRP-3 dans une fenêtre temporelle spécifique dans un milieu à faible taux d'E2 (diestrus) et que l'E2 (en proestrus) peut agir de manière suppressive en désactivant l'effet excitateur de l'AVP sur les neurones à RFRP-3. Enfin, nous avons observé que le VIP n'avait aucun effet sur la fréquence des potentiels

d’action des neurones à RFRP-3 pendant la transition jour-nuit, que ce soit en diœstrus ou en proœstrus.

Dans le **chapitre 4**, nous avons étudié l'effet des cycles lumière/obscurité décalés sur la robustesse de la cyclicité estrale et le moment du pic préovulatoire de LH chez les souris C57BL/6J femelles. Nous avons démontré que les souris exposées à une avance ou un retard unique de 10 heures du cycle lumière/obscurité présentent des changements temporaires dans l'apparition du pic préovulatoire de LH. Au cours du premier cycle suivant un décalage de phase, seule la moitié des souris a présenté un pic de LH, qui s'est également produit 2 heures à l'avance. Au cours du deuxième cycle, la durée du cycle estral a été prolongée et ce n'est qu'au cours du troisième cycle que la cyclicité estrale et le moment du pic préovulatoireet de LH ont été restaurés. Ensuite, nous avons montré que l'exposition chronique à des cycles lumière/obscurité perturbés diminuait considérablement l'apparition du pic préovulatoire de LH, entraînant une réduction de la fertilité. Une rotation chronique de 3 jours avec une avance de phase de 10 heures, puis de 4 jours avec un retard de phase de 10 heures, a eu un effet mineur sur la cyclicité estrale, bien qu'après 9 mois de rotations chroniques, les souris avaient tendance à avoir des cycles estriens plus longs et irréguliers, tandis qu'une partie d'entre elles devenaient acycliques. En revanche, l'exposition chronique à un tel protocole de décalage a fortement perturbé l'apparition du pic préovulatoire de LH. Seules quelques souris ont présenté un pic de LH, mais avec une amplitude réduite et un décalage dans le temps. De plus, nous avons constaté que l'exposition chronique à des cycles lumière/obscurité altérés réduisait le succès de la gestation. En effet les souris désynchronisées donnaient naissance moitié moins de petits par rapport aux souris maintenues dans un cycle lumière/obscurité stable. Les souris décalées ont probablement eu une ovulation retardée puisque la plupart d'entre elles ont donné naissance à leur petits 5 jours plus tard que la majorité des souris témoins. Dans l'ensemble, nos résultats suggèrent que les déphasages uniques ont un effet modéré sur la reproduction des femelles, tandis que les déphasages chroniques conduisent à une altération sévère du pic préovulatoire de LH, qui peut à son tour réduire la fertilité et le succès gestationnel.

Dans le **chapitre 5**, nous avons cherché à savoir si les caractéristiques du pic préovulatoire de LH chez les souris femelles C57BL/6 J étaient modifiées en fonction de l'âge. Nous avons démontré que l'amplitude et le moment du pic de LH étaient similaires chez les souris âgées de 3, 6 et 9 mois. Cependant, les souris de 12 mois présentaient une diminution importante (plus de 50 %) du pic de sécrétion de LH et un retard de 2 heures dans le moment de la montée de LH.

Dans le **chapitre 6**, nous avons examiné si la sénescence reproductive pouvait être associée à des modifications du système à RFRP-3 et de son innervation peptidergique par les fibres AVP et VIP chez les souris femelles C57BL/6 J. Nous avons montré que pendant le diestrus, les niveaux basaux de la sécrétion de LH étaient similaires chez les souris jeunes (4 mois) et d'âge moyen (13 mois), tandis que ces niveaux augmentaient de manière significative à un âge avancé (19 mois). Ensuite, nous avons démontré que le nombre de neurones à RFRP-3 diminue au cours de l'âge moyen et de la vieillesse, tandis que la variation journalière de l'activité des neurones à RFRP-3, observée chez les souris jeunes et d'âge moyen, disparaît au cours de la sénescence. Il est à noter que nous avons enregistré une diminution importante de l'activité des neurones à RFRP-3 (attestée par l'expression de c-Fos) au début de la période de lumière, mais pas au début de la période d'obscurité chez les souris sénescentes. Indépendamment de l'âge, nous n'avons enregistré aucune variation journalière de la densité des fibres à AVP et VIP dans le DMH, mais nous avons constaté une variation journalière du pourcentage de neurones à RFRP-3 avec des appositions de fibres à AVP et à VIP à un âge jeune et moyen. Pendant la vieillesse, cette variation journalière du nombre de neurones RFRP-3 ayant des appositions de fibres à AVP et VIP a disparu. Notamment, jusqu'à l'âge moyen, le pourcentage de neurones à RFRP-3 avec des appositions de fibres AVP au début de la période diurne a diminué, ainsi que le pourcentage de neurones à RFRP-3 avec des appositions de fibres VIP au début de la période nocturne. Par conséquent, au cours de la sénescence, la diminution du pourcentage de neurones à RFRP-3 avec des appositions de fibres AVP n'a plus été observée le matin, ni la diminution du pourcentage de neurones à RFRP-3 avec des appositions de fibres VIPle soir. Cette perte de variation journalière de l'entrée peptidergique sur les neurones à RFRP-3 pourrait être

responsable en partie de la perte observée du rythme journalier de l'activité des neuronale à RFRP-3.

En conclusion, dans cette thèse, nous avons fourni des preuves supplémentaires que les neurones à RFRP-3 agissent comme un lieu de relais pour les signaux journaliers et ovariens qui sont transmis à l'axe HPG. Nous avons rapporté des changements temporels dans l'activité des neurones à RFRP-3 et dans la densité des fibres AVP- et VIP-ergiques sur les neurones du RFRP-3. Nous avons démontré que l'activité électrique des neurones à RFRP-3 présente des modifications dépendantes du stade estral avec une fréquence plus élevée en proestrus, et que l'application d'AVP augmente la fréquence des potentiels d'action des neurones à RFRP-3 uniquement à midi du stade diestrus, tandis que le VIP n'a aucun effet pendant la transition jour-nuit du diestrus ou du proestrus. Nous avons également montré que les déphasages simples ont un effet modéré sur la reproduction des femelles, tandis que les déphasages chroniques conduisent à une altération sévère du pic préovulatoire de LH, qui peut à son tour réduire la fertilité et le succès gestationnel. Enfin, nous avons démontré qu'au cours de la sénescence, l'expression du RFRP-3 diminue, tandis que les variations journalières de l'activité des neurones à RFRP-3 et de la signalisation peptidergique des SCN sur les neurones à RFRP-3 ont disparu. Dans l'ensemble, nos résultats soulignent la nécessité d'un système circadien fonctionnel et bien synchronisé pour une bonne reproduction féminine et mettent en évidence l'impact du RFRP-3 sur le contrôle journalier de la reproduction.

PhD Portfolio

Name PhD candidate: Eleni Angelopoulou

PhD period: October 2016 – September 2021

Name PhD supervisors: V. Simonneaux and A. Kalsbeek

1. PhD Training:

a. Socio-professional

1st Colloquium of Young Physicists - Chemists of EUCOR- the European Campus. Energy Transition.	2017	Strasbourg, France.
MOOC Intégrité scientifique dans les métiers de la recherche (Bordeaux)	2019	Strasbourg, France.
First steps with Zotero	2020	Strasbourg, France.
Programme Valorisation des compétences, NCT.	2021	Strasbourg, France.

b. Scientific

Les outils de gestion des élevages de rongeurs et intérêt de la cryopréservation	2017	Strasbourg, France.
Formation relative à l'expérimentation animale.	2018	Strasbourg, France.
Initiation à la Chirurgie Expérimentale (rongeurs).	2018	Strasbourg, France.

c. Workshops, meetings and schools

Weekly research meetings and Journal Clubs	2016-2019	Strasbourg, France.
Does optogenetics still shine after 11 years?	2016	Strasbourg, France.
Circadian clocks and metabolic health: from basic science to clinical implications.	2016	Strasbourg, France.

Electrophysiology School of Strasbourg	2017	Strasbourg, France.
Basic and Clinical aspects of neurobiology of rhythms Summer School	2017	Strasbourg, France.
Weekly research meetings Endocrinology and Metabolism, Amsterdam UMC.	2019-2020	Amsterdam, the Netherlands.

2. Conferences

a. Oral presentations

NeuroTime Anual meeting: "Shining light on RFRP-3 neurons in female mice."	2017	Amsterdam, the Netherlands.
42ème colloque de la Société de Neuroendocrinologie: "The role of RFRP-3 neurons in the daily control of female reproduction."	2017	Dijon, France.

b. Poster presentations

9th International Congress of Neuroendocrinology: "Electrical firing characteristics of RFRP-3 neurons in female mice."	2018	Toronto, Canada.
46éme Congrès de la Société Francophone de Chronobiologie:"Electrical firing characteristics of RFRP-3 neurons in female mice."	2018	Rabat, Maroc.
XVI European Biological Rhythms Society Congress: "The role of RFRP-3 neurons in the daily control of reproduction in female mice."	2019	Lyon, France.

3. Prizes

Travel Grant from the Société de Neuroendocrinologie to attend the 42ème colloque	2017	Dijon, France.
Travel Grant from the Société Francophone de Chronobiologie to attend the 46éme Congrès.	2018	Rabat, Maroc.

Best Poster prize during the 46éme Congrès de la Société Francophone de Chronobiologie.	2018	Rabat, Maroc.

4. Publications

a. Peer reviewed

Angelopoulou, E., Inquimbert, P., Klosen, P., Anderson, G., Kalsbeek, A., Simonneaux, V., 2021a. Daily and Estral Regulation of RFRP-3 Neurons in the Female Mice. Journal of Circadian Rhythms 19, 4. https://doi.org/10.5334/jcr.212

Angelopoulou, E., Kalsbeek, A., Simonneaux, V., 2021b. Age-dependent modulation of RFRP-3 neurons in female mice. Neuropeptides 102146. https://doi.org/10.1016/j.npep.2021.102146

Bahougne, T., **Angelopoulou, E.**, Jeandidier, N., Simonneaux, V., 2020a. Individual evaluation of luteinizing hormone in aged C57BL/6 J female mice. Geroscience 42, 323–331. https://doi.org/10.1007/s11357-019-00104-z

Bahougne, T., Kretz, M., **Angelopoulou, E.**, Jeandidier, N., Simonneaux, V., 2020b. Impact of Circadian Disruption on Female Mice Reproductive Function. Endocrinology 161. https://doi.org/10.1210/endocr/bqaa028

b. Reviews

Angelopoulou, E., Quignon, C., Kriegsfeld, L.J., Simonneaux, V., 2019. Functional Implications of RFRP-3 in the Central Control of Daily and Seasonal Rhythms in Reproduction. Front Endocrinol (Lausanne) 10. https://doi.org/10.3389/fendo.2019.00183

Simonneaux, V., Bahougne, T., **Angelopoulou, E.**, 2017. Daily rhythms count for female fertility. Best Pract. Res. Clin. Endocrinol. Metab. 31, 505–519. https://doi.org/10.1016/j.beem.2017.10.012

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Eleni ANGELOPOULOU

Régulation des neurones à RFRP-3 en fonction de la journée, du cycle estral et de l'âge et leur rôle dans la sécrétion de l'hormone lutéinisante chez les souris femelles

Résumé

Dans cette thèse, nous avons démontré que les neurones RFRP-3 agissent comme un lieu de relais pour les signaux journaliers et ovariens qui sont transmis à l'axe HPG. Nous avons noté des changements temporels dans l'activité neuronale du RFRP-3 et dans la densité des projections des fibres AVP et VIP sur les neurones à RFRP-3. Nous avons démontré que l'activité électrique du RFRP-3 présente des altérations dépendantes du stade œstral avec une fréquence de spikes plus élevée en proestrus. L'application d'AVP augmente l'activité électrique du RFRP-3 uniquement en milieu de journée au stade du diestrus, tandis que le VIP n'a aucun effet pendant la transition jour-nuit. Enfin, nous avons démontré qu'au cours de la sénescence, l'expression du RFRP-3 diminue, tandis que les variations journalières de l'activité neuronale du RFRP-3 et de la sortie du SCN sur les neurones du RFRP-3 ont disparues. Nos résultats soulignent la nécessité d'un système circadien fonctionnel pour une bonne reproduction féminine et montrent l'impact du RFRP-3 sur le contrôle quotidien de la reproduction.

Arg-Phe amide-related peptide, arginine vasopressine, vasoactive intestinal peptide

Résumé en anglais

In this thesis, we provided evidence that RFRP-3 neurons act as a locus of relay for daily and ovarian cues that are conveyed to the HPG axis. We reported temporal changes in the RFRP-3 neuronal activity and in the density of AVP and VIP fiber projections on RFRP-3 neurons. We demonstrated that the RFRP-3 electrical activity exhibits estrous-stage dependent alterations with a higher spike frequency in proestrus. AVP application increases the RFRP-3 firing activity only at midday of the diestrus stage, while VIP had no effect during the day-to-night transition of either diestrus or proestrus. Finally, we demonstrated that during senescence the RFRP-3 expression decreases, while daily variations in both the RFRP-3 neuronal activity and the SCN output on RFRP-3 neurons were lost. Altogether, our findings highlight the requirement of a functional and well-synchronized circadian system for proper female reproduction and display the impact of RFRP-3 on the daily control of reproduction.

Arg-Phe amide-related peptide, arginine vasopressin, vasoactive intestinal peptide