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## Age-dependent change of RFRP-3 neuron numbers and innervation in female mice

Eleni Angelopoulou<sup>a, b, d</sup>, Andries Kalsbeek<sup>b, c, d</sup>, Valérie Simonneaux<sup>a, \*</sup>

<sup>a</sup> Institut des Neurosciences Cellulaire et Intégratives (UPR CNRS3212), Université de Strasbourg, 8, allée du Général Rouvillois, 67000 Strasbourg, France

<sup>b</sup> Netherlands Institute for Neuroscience (NIN), Amsterdam, the Netherlands

<sup>c</sup> Department of Endocrinology and Metabolism, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands

<sup>d</sup> Laboratory of Endocrinology, Amsterdam UMC, University of Amsterdam, Amsterdam Gastroenterology, Endocrinology & Metabolism, Amsterdam, the Netherlands

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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 age-dependent changes in the daily regulation of Arg-Phe amide-related peptide-3 (RFRP-3), a hypothalamic peptide involved in reproduction, in female C57BL/6 J mice of different age groups (4, 13, and 19 months old) sampled at their diestrus stage. We found an age-dependent decrease in the total number of RFRP-3 neurons and in the relative number of activated (i.e. c-Fos-positive) RFRP-3 neurons. RFRP-3 neuronal activation exhibited a daily variation in young and middle-aged mice, which was abolished in 19-month-old mice. We also found a daily variation in the number of RFRP-3 neurons receiving close vasopressin (AVP)- and vasoactive intestinal peptide (VIP)-ergic fiber appositions in mice aged 4 and 13 months, but not in 19-month-old mice. However, we found no daily or age-dependent changes in the AVP and VIP fiber density in the dorsomedial hypothalamus. Plasma LH levels were similar in mice aged 4 and 13 months, but were markedly increased in 19-month-old mice. The present findings indicate that the number of RFRP-3 positive neurons is 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.

### 1. 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 (Downs and Wise, 2009 for review). 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 for review). In female rodents, age-dependent suppression of hypothalamic GnRH neurons is associated with a delayed or attenuated preovulatory GnRH/LH surge (Scarborough and Wise, 1990; Rubin et al., 1994; Le et al., 2001; Bahoune et al., 2020). Many age-related alterations in the GnRH neu-

ronal system precede changes in the pituitary, the ovaries and the loss of estrous cyclicity (Kermath and Gore, 2012; Rubin, 2000; Yin and Gore, 2006). GnRH system dysfunction during aging may be intrinsic, but age-associated changes in its regulatory input pathways have not been thoroughly investigated yet.

GnRH neuronal activity is driven by coordinated inputs from upstream hypothalamic neuropeptide systems, notably 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 for review). 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 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).

\* Corresponding author at: Institut des Neurosciences Cellulaire et Intégratives, Université de Strasbourg, France.  
E-mail address: [simonneaux@inci-cnrs.unistra.fr](mailto:simonneaux@inci-cnrs.unistra.fr) (V. Simonneaux).

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In female rodents, both Kp and RFRP-3 neurons display coordinated daily rhythms, with Kp neuronal activity being increased and RFRP-3 neuronal activity being decreased at the time of sleep/wake transition (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). Indeed, experimental studies using SCN lesions or neuronal tracing have demonstrated that arginine vasopressin (AVP) fibers, which contact Kp neurons (Vida et al., 2010; Williams et al., 2011), and AVP and vasoactive intestinal peptide (VIP) fibers, which contact RFRP-3 neurons (Russo et al., 2015), all arise from the SCN.

Age-dependent changes in any of these regulatory networks, upstream of the GnRH neurons, may be involved in the induction of hypothalamic senescence and reproductive decline in female mammals. Therefore, 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 the number of RFRP-3 immunoreactive neurons, in the proportion of active (i.e. c-FOS expressing) RFRP-3 neurons and in the density of AVP-ergic and VIP-ergic fiber projections on RFRP-3 neurons in the hypothalamus of 4-, 13- and 19-month-old C57BL/6 J female mice sampled at their diestrus stage.

## 2. Materials and methods

### 2.1. Animals

Adult female C57BL/6 J mice up to the age of 19 months (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. 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).

### 2.2. Monitoring of estrous cycle and LH secretion

Female mice were studied at ages 4, 13 or 19 months during their diestrus stage as assessed by vaginal smears and circulating LH levels and as previously described (Bahougue et al., 2020; Steyn et al., 2013). Vaginal smears were performed at ZT2 during at least three 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 10× phosphate buffered saline, 0.25% of Tween-20 in milliQ H<sub>2</sub>O) 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 (Bahougue et al., 2020). Four concentrations of LH (1, 10, 20 and 30 ng/ml) were used as quality controls. The LH assay sensitivity was 0.5 ng/ml, the intra-assay variation was 11% and the inter-assay variation was 13%.

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

#### 2.3.1. Tissue processing

Adult female C57BL/6 J 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<sub>2</sub>. 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<sub>4</sub> 10 mM and lysine 75 mM in 100 mM phosphate buffer, pH 7.3). The brains were collected, post-fixed in periodate-lysine-paraformaldehyde for 12 h at room temperature, washed with PBS, dehydrated and embedded in polyethylene glycol as previously described (Klosen and Maessen, 1993).

Using a microtome, twelve series of 12 µm-thick coronal brain sections were cut throughout the DMH as described 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, yielding 6-7 DMH-containing brain sections) was rehydrated and mounted on a Super-Frost Plus (Menzel-Glaser, Braunschweig, Germany) slide. For each immunolabeling 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.

#### 2.3.2. 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-52, RRID:AB\_2106783) 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 immunolabeling, antibodies from the first immunolabeling were eluted with 2 × 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 GA197, a rabbit polyclonal antiserum raised against the C-terminal sequence of the RFRP precursor peptide, which is similar for RFRP-1 and RFRP-3, i.e. this antibody does not discriminate between RFRP-1 and RFRP-3 (1:15000; Greg M. Anderson Lab; University of Otago; New Zealand Cat# GA197, RRID: AB\_2877670). GA197 was 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). Specificity

of the antibody for RFRP-3 (Zizwan et al., 2009), c-Fos (Parker et al., 2017), Neurophysin II (Gibson et al., 2015) and VIP (Dedeene et al., 2019) primary antibodies has been demonstrated in previous studies.

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

Quantification was done by the first author while blind to the animal's identity. 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 of the total number of RFRP-3-ir neurons and the number of RFRP-3 neurons co-labeled with c-Fos was done on these five sections. Counting results were then averaged to give a mean number of RFRP-3 neurons per DMH brain section. Thus, for each mouse, data shown are the mean number of RFRP-3 neurons, the mean number of c-Fos expressing RFRP-3 neurons, and the percentage of RFRP-3 neurons expressing c-Fos per DMH section.

Regarding the quantification of AVP and VIP-ir fibers, we performed two kinds of counting. First, the overall density of AVP and VIP-ir fibers was quantified bilaterally in the selected regions of interest (ROI), i.e. in the DMH. DMH boundaries were defined by neuroanatomical landmarks such as the median eminence, the tuberoinfundibular sulcus and the pituitary stalk, and the anatomical position of the RFRP-3 neurons. Fiber density was quantified by manually counting 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 was given as the total number of crossing points/total grid number of the ROIs. Secondly, the number of RFRP-3 neurons receiving direct AVP- and VIP-fiber projections was counted manually on both sides of the DMH. AVP-ergic or VIP-ergic appositions were defined as terminal fibers directly contacting RFRP-3 cell somas. For each mouse, data shown are the mean number of RFRP-3 neurons with close AVP or VIP fiber appositions per DMH section, and the percentage of RFRP-3 neurons with close AVP or VIP fiber appositions per DMH section.

### 2.3.4. Data analysis

Statistical analysis was performed with GraphPad Prism 6. All values are given as mean  $\pm$  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* (i.e. *Interaction*), followed by Tukey's multiple comparisons post-hoc test. Differences were considered significant for  $P < 0.05$ .

## 3. Results

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

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

Young mice displayed regular 4–5 day cycles, middle-aged mice displayed longer cycles of 7–8 days and old mice displayed irregular cycles lasting up to 10–12 day (pseudopregnancy stage) (Fig. 1A, B, C). 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. 1D). No significant time-of-day differences were found (Two Way ANOVA, *Daytime*:  $F(1, 24) = 0.4491, p = 0.5091$ ; Fig. 1D).

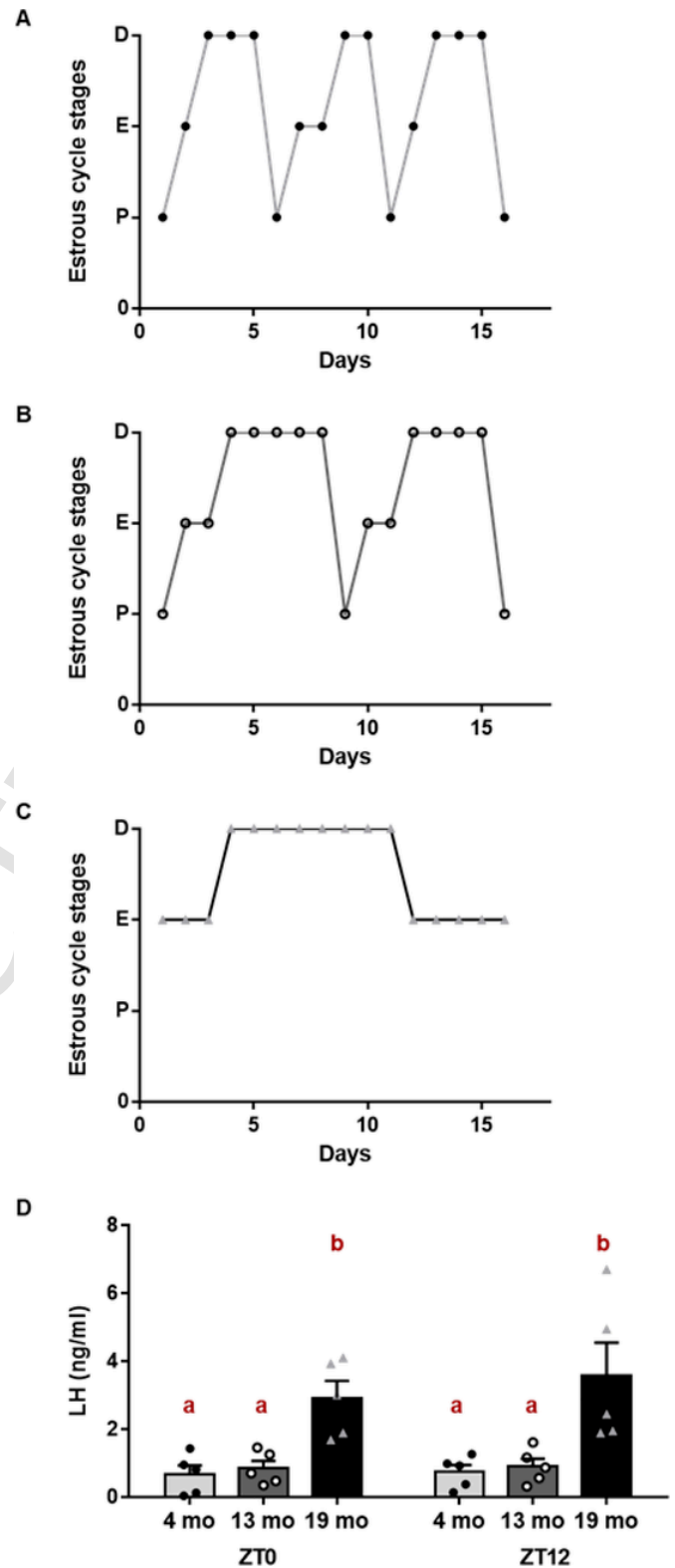


Fig. 1. Estrous cyclicity and LH secretion in female C57BL/6 J mice of three different ages. Representative estrous cycles tracked for 16 consecutive days in a 4-month-old (mo) (A) a 13 mo (B) and a 19 mo (C) female mouse; letters on the y axis refer to stages of the estrous cycle; P: proestrus, E: estrus, and D: diestrus; (D) LH blood concentrations in 4, 13 and 19 mo female mice sampled at ZT0 and ZT12 on the day of diestrus. Zeitgeber (ZT) 0 is light onset and ZT12 is light offset; bars in D represent mean  $\pm$  SEM ( $n = 5$ ) values of 4 mo (light grey), 13 mo (dark grey) and 19 mo (black) groups of mice. Different lowercase

◀ letters in D indicate that groups are statistically different according to the post-hoc results for the effects of Age or Daytime ( $p < 0.05$ ).

The number of RFRP-3-immunostained neurons showed a significant, albeit moderate, daily variation (Two Way ANOVA, *Daytime*:  $F(1, 24) = 4.568$ ,  $p = 0.0430$ ; Fig. 2A), with mean values being lower at ZT12 in all 3 age groups, although these differences did not reach significance in the individual age groups. In addition, we found a significant effect of age (Two Way ANOVA, *Age*:  $F(2, 24) = 129.6$ ,  $p < 0.0001$ ) in the number of RFRP-3-ir neurons with a moderate decrease (about 15%) in the middle age group, and prominent decrease (approximately 50%) in the old group as compared to the young group (Fig. 2A).

Next, we examined whether there are daily or age-related changes in RFRP-3 neuronal activation, as assessed by the number of c-Fos-positive RFRP-3 neurons (Fig. 2B - D). We found a significant effect of time of day (Two Way ANOVA, *Daytime*:  $F(1, 24) = 70.49$ ,  $p < 0.0001$ ; Fig. 2B, C), 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). At ZT0, the number of c-Fos-positive RFRP-3 neurons shows a moderate decrease in middle-aged mice (approximately 15%) but a marked decrease in aged mice (approximately 65%) as compared to young mice (Two Way ANOVA, *Age*:  $F(2, 24) = 9.513$ ,  $p = 0.0009$ ; Fig. 2B), so that old mice exhibit no more daily variation in the number of c-Fos-positive RFRP-3 neurons (Tukey's multiple comparisons test,  $p = 0.6194$ ; Fig. 2B, C).

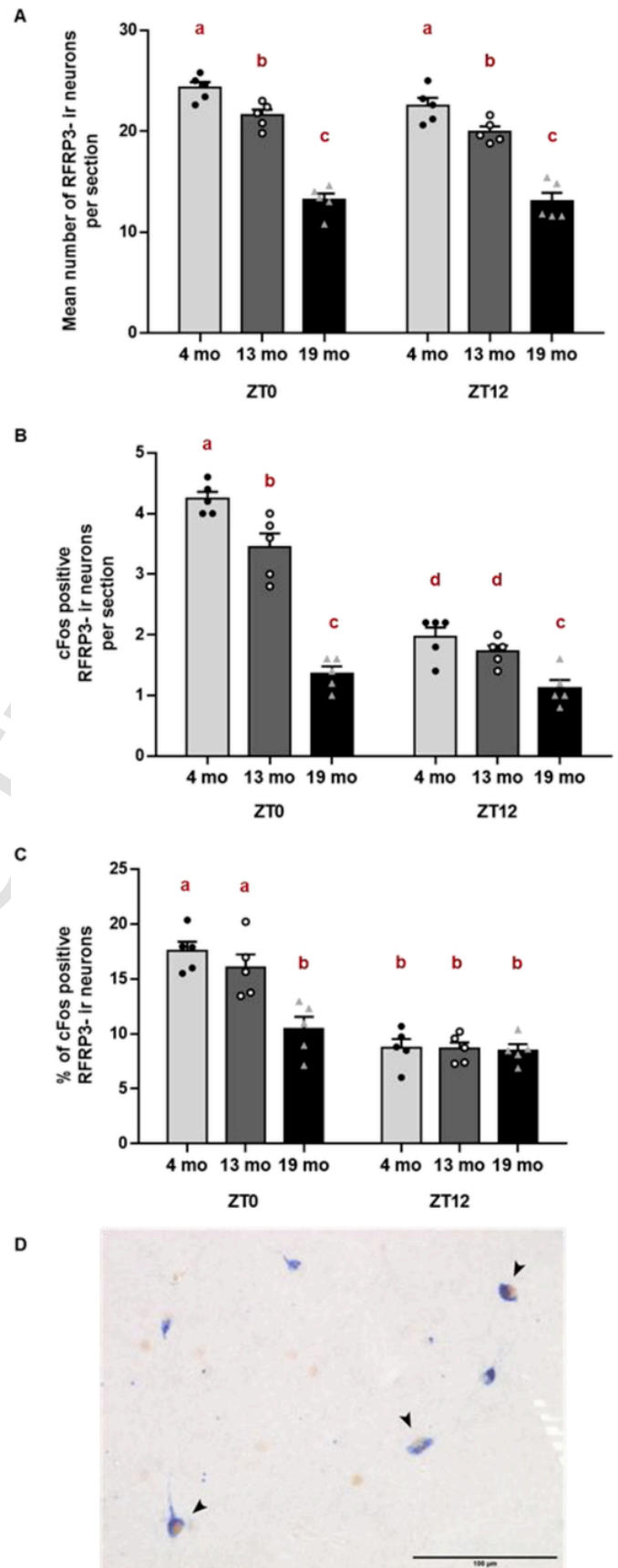
Comparable results were obtained when considering the mean number of c-Fos-positive RFRP-3 neurons per section (Fig. 2B) or the percentage of c-Fos-expressing RFRP-3-ir neurons (Fig. 2C).

### 3.2. Age-related changes in the daily pattern of RFRP-3 innervation by AVP and VIP projections in female C57BL/6 J 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 age-dependent changes at two time points (ZT0 and ZT12).

We recorded no daily (Two Way ANOVA, *Daytime*:  $F(1, 24) = 1.570$ ,  $p = 0.2222$ ; Fig. 3A) or age-dependent (Two Way ANOVA, *Age*:  $F(2, 24) = 0.3332$ ,  $p = 0.7199$ ; Fig. 3A) 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. 5A) or age-dependent (Two Way ANOVA, *Age*:  $F(2, 24) = 0.5989$ ,  $p = 0.5574$ ; Fig. 5A) changes in VIP-ir fiber density in the DMH.

On average, we found that each RFRP-3 neuron receives  $1.02 \pm 0.04$  AVP-ir fiber appositions. When measuring daily and age-related changes in the mean number of RFRP-3 neurons with close AVP-ir fiber appositions per section (Fig. 3B), we did not record an effect of time of day (Two Way ANOVA, *Daytime*:  $F(1, 24) = 4.200$ ,  $p = 0.0515$ ), nor an effect of age (Two Way ANOVA, *Age*:  $F(2, 24) = 0.8310$ ,  $p = 0.4478$ ). However, when measuring daily and age-related changes in the percentage of RFRP-3 neurons with close AVP-ir fiber appositions (Fig. 3C), 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 ( $F(2, 24) = 3.927$ ,  $p = 0.0335$ ). Notably, at ZT0 the number of RFRP-3 neurons with close AVP-ir fiber appositions was higher in old as compared to young and middle-aged mice ( $p = 0.0004$  and  $p = 0.0018$ , respectively). Contrary to the young and middle-aged mice, old mice did not exhibit a daily variation in the percentage of RFRP-3 neurons with close AVP-ir fiber appositions. Fig. 4 shows representative RFRP-3 neurons



◀ **Fig. 2.** RFRP-3 immunoreactivity and neuronal activity in the dorsomedial hypothalamus of female C57BL/6 J mice of three different ages sampled on diestrus at two daily time points. (A) mean number of RFRP-3-ir neurons per section; (B) mean number of c-Fos-expressing RFRP-3-ir neurons per section (C) percentage of c-Fos-expressing RFRP-3-ir neurons; (D) photograph showing c-Fos-positive (brown) and c-Fos-negative (blue) RFRP-3-ir neurons 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  $\pm$  SEM ( $n = 5$ ) values of 4 mo (light grey), 13 mo (dark grey) and 19 mo (black) groups of mice. Different lowercase letters in A-C indicate that groups are statistically different according to the post-hoc results for the effects of Daytime or Age ( $p < 0.05$ ). Scale bar in D = 100  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

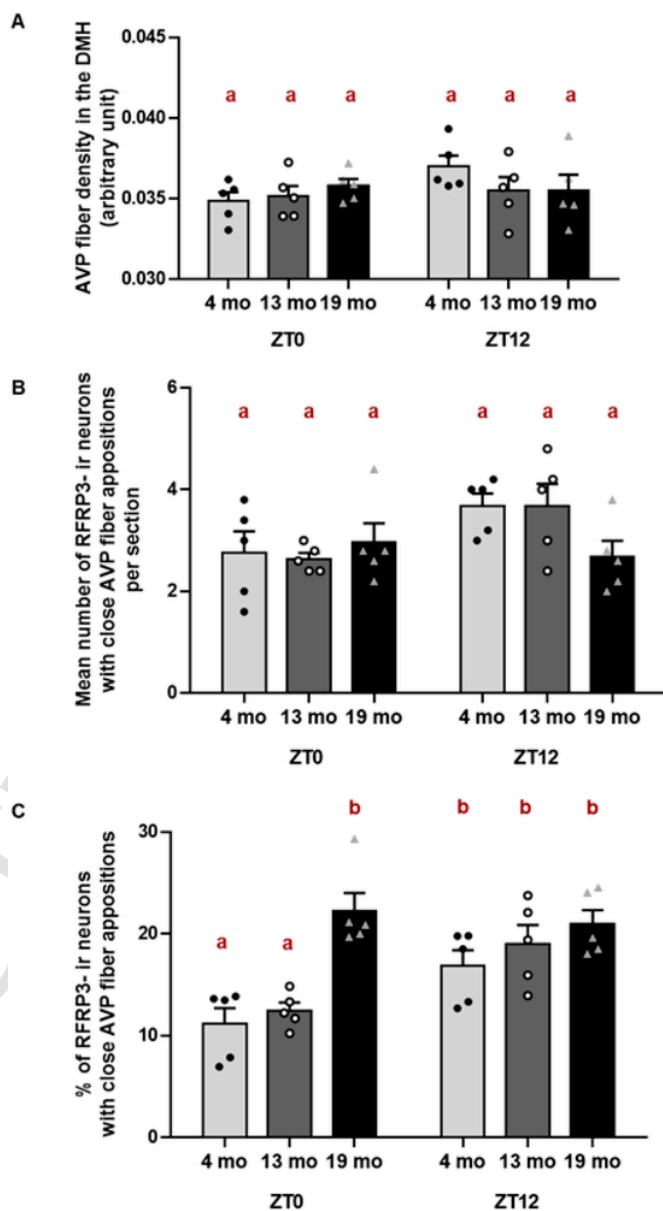
contacted by AVP-ir fibers at the two time points in young, middle-aged and old mice.

On average, we found that each RFRP-3 neuron receives  $1.02 \pm 1.01$  VIP-ir fiber appositions. When analyzing daily and age-related changes in the mean number of RFRP-3 neurons with close VIP-ir fiber appositions per section (Fig. 5B), we recorded a significant effect of time of day (Two Way ANOVA, *Daytime*:  $F(1, 24) = 56.39$ ,  $p < 0.0001$ ), with 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 (Tukey's multiple comparisons test,  $p < 0.0001$ ; Fig. 5B) and middle-aged (Tukey's multiple comparisons test,  $p < 0.0001$ ; Fig. 5B) mice. However, no daily variation in the number of RFRP-3 neurons with close VIP-ir fiber appositions was found in the old mice (Tukey's multiple comparisons test,  $p > 0.9999$ ; Fig. 5B). When analyzing the daily and age-dependent changes in the percentage of RFRP-3 neurons with close VIP-ir fiber appositions, we recorded a similar significant effect of time of day (Two Way ANOVA, *Daytime*:  $F(1, 24) = 31.50$ ,  $p < 0.0001$ ), with a significant decrease in the percentage 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 (Tukey's multiple comparisons test,  $p = 0.0019$ ; Fig. 5C) and middle-aged (Tukey's multiple comparisons test,  $p = 0.0003$ ; Fig. 5C) mice. No daily variation in the percentage of RFRP-3 neurons with close VIP-ir fiber appositions was found in the old mice (Tukey's multiple comparisons test,  $p > 0.9999$ ; Fig. 5C). Furthermore, old mice exhibited the highest relative number of RFRP-3 neurons with close VIP-ir fiber appositions (Two Way ANOVA, *Age*,  $F(2, 24) = 26.74$ ,  $p < 0.0001$ ; Fig. 5C). Fig. 6 shows representative RFRP-3 neurons contacted by VIP-ir fibers at the two time points in young, middle-aged and old mice.

#### 4. Discussion

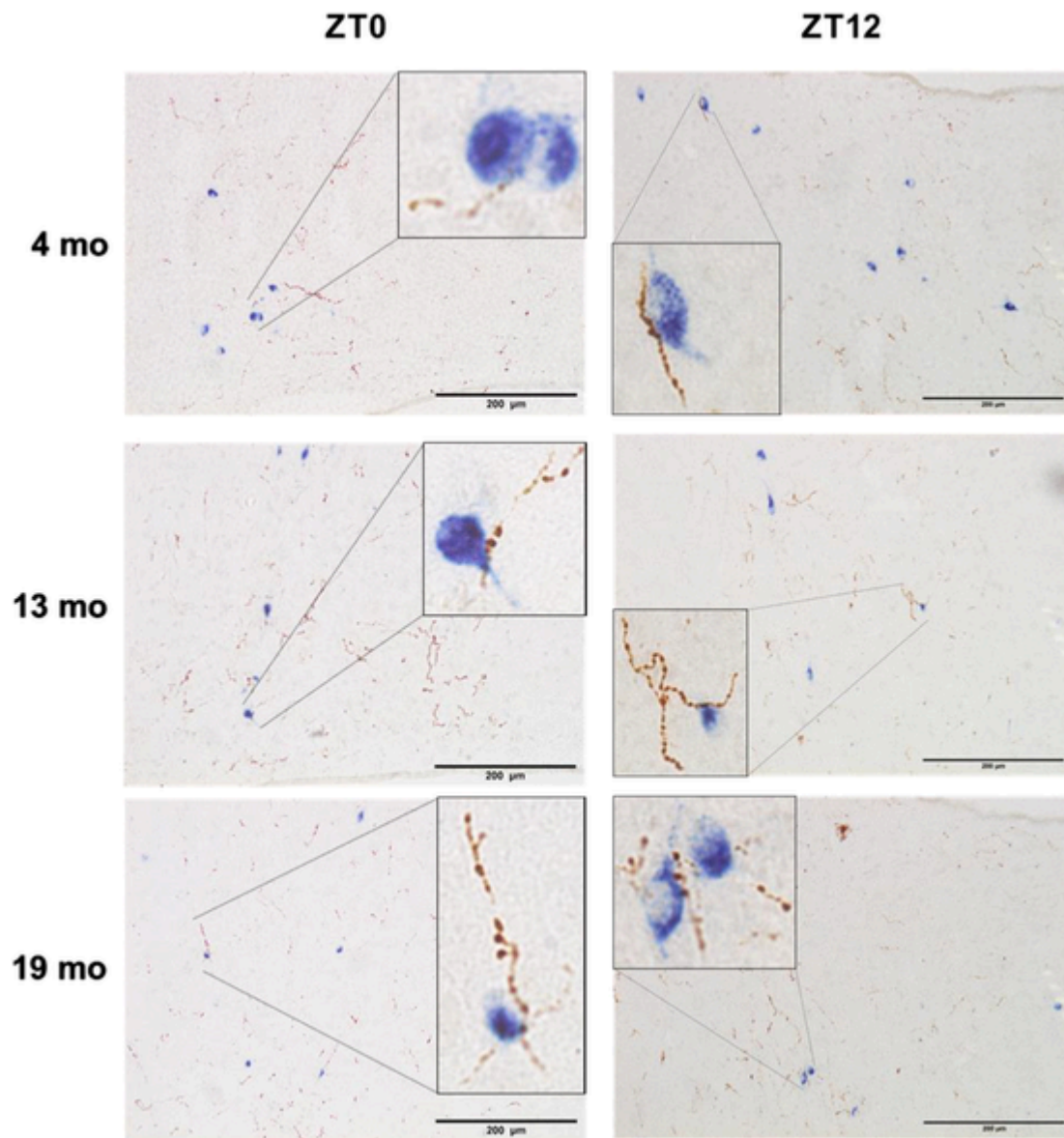
Female mammals display regular daily and ovarian cycles driven by estradiol-sensitive neural circuits in the hypothalamus (Angelopoulou et al., 2019 for review). Age-related hypothalamic decline leads to senescent changes in reproductive function (Yin and Gore, 2006). In this study, we used a neuroanatomical approach to investigate whether there are age-dependent changes in the number, activation and innervation of hypothalamic RFRP-3 neurons in female mice sampled at light onset and light offset of their day of diestrus. We found an age-dependent down-regulation of the number of RFRP-3 positive neurons and the number of c-Fos activated RFRP-3 neurons. We also reported a loss of daily variation in the number of RFRP-3 neurons showing c-Fos activation and innervation by the circadian peptides AVP and VIP, most 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 or persistent vaginal cornification at the age of 10–16 months. The constant estrous period is followed by a prolonged diestrus phase with intermittent ovulation known as repeti-



**Fig. 3.** Density of arginine vasopressin (AVP)-immunostained fibers in the dorsomedial hypothalamus and AVP contacts with RFRP-3 neurons in female C57BL/6 J mice of three different ages sampled on diestrus at two daily time points. (A) AVP-ir fiber density (arbitrary units) in the dorsomedial hypothalamus; (B) mean number of RFRP-3 neurons with close AVP-ir fiber appositions per section; (C) percentage of RFRP-3-ir neurons with close AVP-ir fiber appositions. Zeitgeber (ZT) 0 is light onset and ZT12 is light offset; bars represent mean  $\pm$  SEM ( $n = 5$ ) values of 4 mo (light grey), 13 mo (dark grey) and 19 mo (black) groups of mice. Different lowercase letters in A-C indicate that groups are statistically different in terms of the post-hoc results for the effects of Daytime or Age ( $p < 0.05$ ).

tive pseudo-pregnancy, before reaching the anoestrous stage at the age of 22–25 months (Cruz et al., 2017 for review). In this study we evaluated basal LH production at diestrus in mice of different ages in addition to the cytological analysis of the estrous cycle. Young (4-month-old) and middle-aged (13-month-old) mice in diestrus displayed constant low plasma LH levels and estrous cyclicity, yet with cycle duration being longer in middle-aged (6–7 days) as compared to young (4–5 days) mice. Contrastingly, old (19-month-old) mice displayed a marked increase in the plasma LH levels on both experimental time points, associated with a loss of estrous cyclicity.

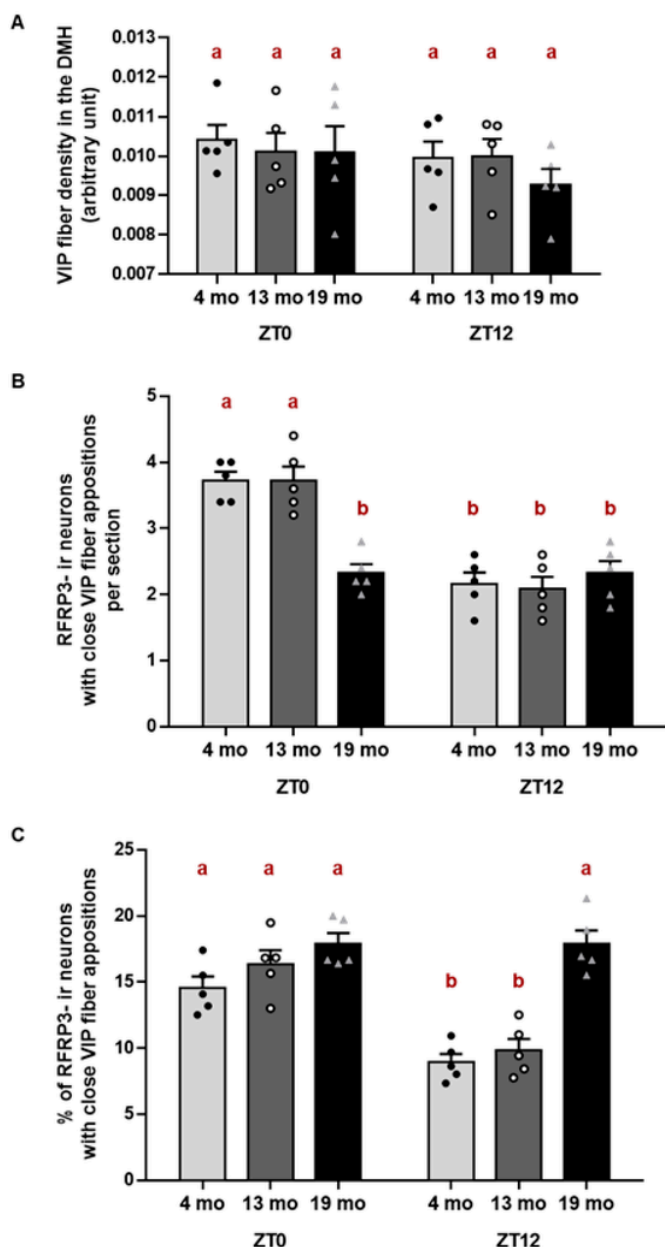


**Fig. 4.** Photographs showing RFRP3-ir neurons (blue) and AVP-containing fibers (brown) in 4, 13, and 19-month-old (mo) female mice sampled on diestrus at ZT0 and ZT12. In the old mice the RFRP neuron was contacted by > 1 AVP fiber. Zeitgeber (ZT) 0 is light onset and ZT12 is light offset. Scale bars are = 200  $\mu$ m. Zoomed-in panels display neurons and fiber appositions in high clarity. Different magnifications were used here to better portray the fiber projections onto each neuron. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Although age-related changes occur at the level of the pituitary and the ovary, evidence suggests that the aging hypothalamus is also involved in the induction of reproductive senescence in rodents. Indeed, ovarian transplantation studies showed that old anoestrus rats cannot reinitialize estrous cyclicity when receiving ovaries from young animals, while on the other hand, young rats maintain their regular estrous cyclicity after transplantation with old rat ovaries (Peng and Huang, 1972 for review). 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. Earlier studies in female rodents showed that both the number of GnRH cells and the preovulatory GnRH neuronal activation decrease 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.

Kp, one of the main hypothalamic activators of the GnRH system, also undergoes age-dependent changes. Previous studies in female mice and rats demonstrated that the number of Kp cells, Kp neuronal activity and the expression of estrogen receptor  $\alpha$  in Kp neurons decrease at advanced ages, with Kp neurons displaying characteristics of cellular senescence in middle-aged mice (Lederman et al., 2010; Neal-Perry et al., 2009; Zhang et al., 2014). 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 RFRP-3-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 neu-



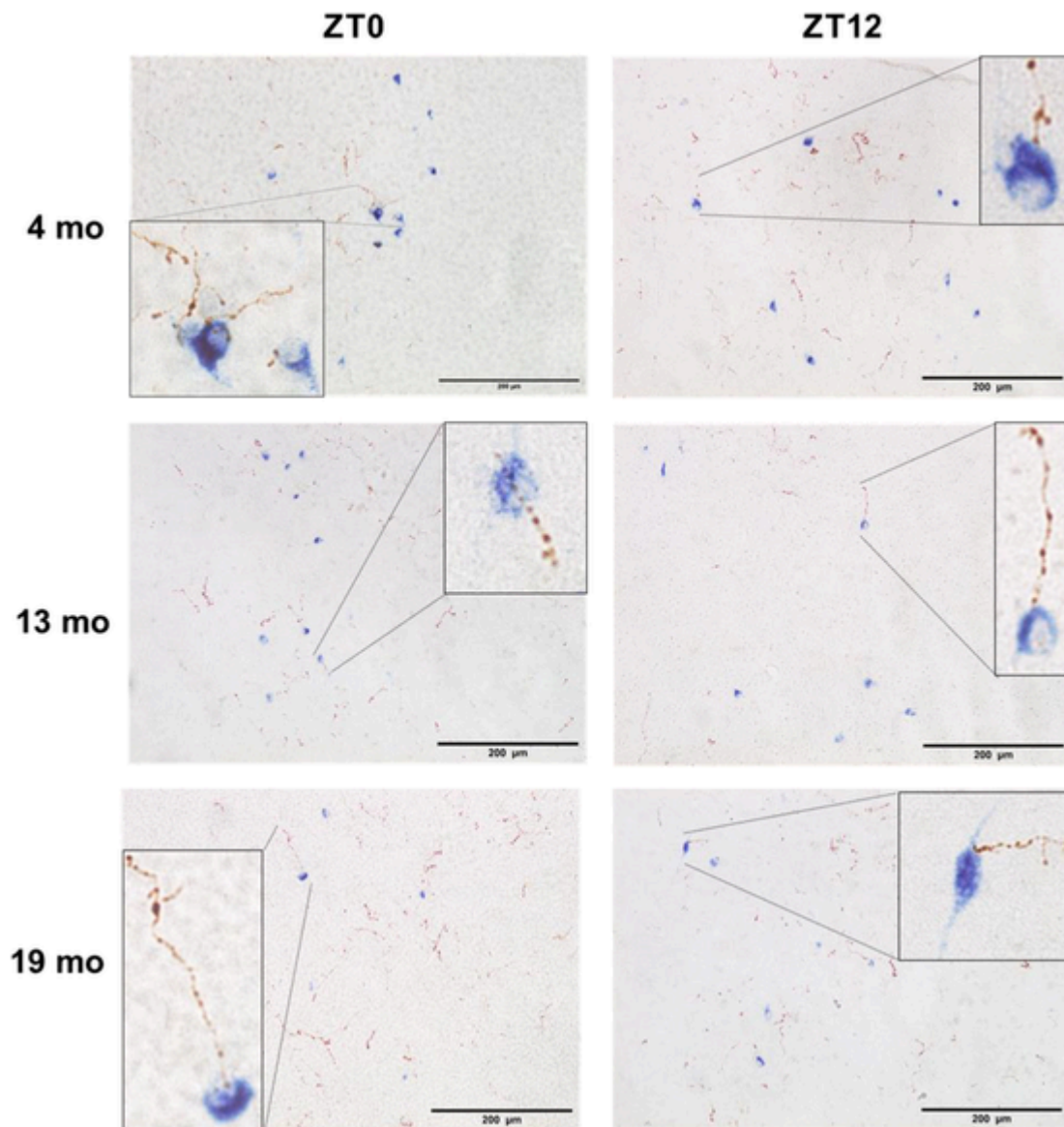
**Fig. 5.** Density of vasoactive intestinal peptide (VIP)-immunostained fibers in the dorsomedial hypothalamus and VIP contacts with RFRP-3 neurons in female C57BL/6 J mice of three different ages sampled on diestrus at two daily time points. (A) VIP-ir fiber density (arbitrary units) in the dorsomedial hypothalamus; (B) mean number of RFRP-3 neurons with close VIP-ir fiber appositions per section; (C) percentage of RFRP-3 neurons with close VIP-ir fiber appositions. Zeitgeber (ZT) 0 is light onset and ZT12 is light offset; bars represent mean  $\pm$  SEM ( $n = 5$ ) values of 4 mo (light grey), 13 mo (dark grey) and 19 mo (black) groups of mice. Different lowercase letters in A-C indicate that groups are statistically different in terms of the post-hoc results for the effects of Daytime or Age ( $p < 0.05$ ).

rons 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. Remarkably, the decreased number of RFRP-3 neurons in old mice is associated with an increased basal LH release at diestrus. Given the reported inhibitory effect of RFRP-3 on LH secretion in females (Angelopoulou et al., 2019 for review), this finding suggest a possible involvement of RFRP-3 neurons in the aging reproductive axis.

We found a small but significant ( $p = 0.0430$ ) daily variation in RFRP-3-ir cell numbers in all age groups, and confirmed that RFRP-3 neuronal activation is specifically decreased at the beginning of the dark/active 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 Kp 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. Numerous studies have shown that the SCN molecular core clock mechanism is sustained during aging, but that 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 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 with advancing age (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 SCN volume or total SCN cell number (Rooyendaal et al., 1987). However, the number of AVP (-31%) and VIP (-36%) neurons in the rodent SCN decreased during aging (Rooyendaal 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 could result in a compromised SCN output signal towards downstream hypothalamic targets.

As previously reported in female Syrian hamsters (Russo et al., 2015) hypothalamic RFRP-3 neurons are contacted by AVP- and VIP-ir fibers arising from the SCN. In young and middle-aged mice, 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. Regarding AVP, the increased appositions may be due to the small decrease in the number of RFRP-3 positive neurons at ZT12. However, it is not clear what could cause the decreased VIP appositions at ZT12, since the density of the VIP-ir fibers in the DMH shows no daily variation. In old mice, no changes in the overall density of AVP and VIP fibers in the DMH were observed, and in contrast to younger mice, there is no more daily variation in the number of RFRP-3 neurons with close AVP and VIP-fiber appositions. In summary, we observed in young and middle-aged mice that at the beginning of the light period, there is a decreased percentage of RFRP-3 neurons with close AVP fiber appositions while at the begin-



**Fig. 6.** Photographs showing RFRP3-ir neurons (blue) and VIP-containing fibers (brown) in 4, 13, and 19-month-old (mo) female mice sampled at ZT0 and ZT12 at diestrus. In the young mouse at ZT0 an example can be seen of an RFRP neuron contacted by > 1 VIP fiber. Zeitgeber (ZT) 0 is light onset and ZT12 is light offset. Scale bars are = 200 µm. Zoomed-in panels display neurons and fiber appositions in high clarity and different magnifications were used to better portray the fiber projections onto each neuron shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ning of the dark period there is a decreased percentage of RFRP-3 neurons with close VIP fiber appositions. In old mice, RFRP-3 neurons cease to exhibit a morning decrease in AVP-ir fibers or 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.

## 5. Conclusions

This study shows that RFRP-3 expression decreases and that the daily rhythm in RFRP-3 neuronal activity is 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 age-dependent alterations in the function of the SCN may

lead to changes in the SCN output pathways towards the RFRP-3 neurons, which in turn may impact their daily activity. Furthermore, as RFRP-3 is reported to inhibit GnRH neurons, the down-regulation of the number of RFRP-3 neurons may account for the subsequent increase of basal LH release in aged mice. However, it is essential to investigate whether there are also age-dependent changes in the daily expression of estrogen, VIP and AVP receptors in RFRP3 neurons, in order to identify the full mechanism of RFRP-3 neuron regulation during old age.

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## 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.

## Declaration of Competing Interest

The authors have declared that no conflicts of interest exist.

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