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 pre-ovulatory 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.

Keywords Aging · Estrous cycle · Luteinizing hormone · Rodents

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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, for review). 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 (Legan and Karsch 1975; Kerdelhué et al. 2002). 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 (Nelson et al. 1982; McLean et al. 2012) 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 (Wise 1982; Akema et al. 1985; Cziesselsky et al. 2016) and illustrates the synchronization of the LH surge to the light/dark cycle by the master hypothalamic clock (Simonneaux and Bahougne 2015, for review). 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 LH β 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 $\pm$ standard error of the mean (SEM) of 4 to 8 mice of the same age. Further, for each age group, the mean ($\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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.

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 $4.6 \pm$

Fig. 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 μ L 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 $\pm$ 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.

Fig. 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 μ L 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 $\pm$ 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

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.

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.

Fig. 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 μ L 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

Changes in estrus cycle length and regularity in aging C57BL/6 J mice have already been studied (Nelson et al. 1982; Mobbs et al. 1984). 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 age-related 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 (Wise 1982; Nass et al. 1984; Akema et al. 1985; DePaolo and Chappel 1986; Matt et al. 1998) also reported a reduced LH peak amplitude in middle-age as compared to young female animals (Downs and Wise 2009, for review).

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 (Lederman et al. 2010; Ishii et al. 2013; Kunitura et al. 2017). 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 (Lloyd et al. 1994; Rubin et al. 1994, 1995; Downs and Wise 2009; Lederman et al. 2010; Ishii et al. 2013). 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 (Smith et al. 2006; Pinilla et al. 2012, for review). 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 (ER α) and responsiveness to E2 are significantly reduced (Lederman et al. 2010; Ishii et al. 2013; 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 (Lederman et al. 2010; Ishii et al. 2013; 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 middle-aged 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 Bahoune 2015, for review). 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 (Krajnak et al. 1998; Downs and Wise 2009) 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 (Vida et al. 2010; Piet et al. 2015), does not appear altered in middle-aged female rats (Krajnak et al. 1998). Finally, because the SCN clock resetting by transmitters like glutamate, N-methyl-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) (Perrott et al. 2017; Lawrence et al. 2018). 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, for reviews). It might be interesting to investigate whether SASP processes are involved in the age-induced alteration of the central regulation of the pre-ovulatory 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 individual 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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Author contributions TB and VS contributed to the study conception and design. Material preparation, data collection and analysis were performed by Bahougne. The first draft of the manuscript was written by Bahougne and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Declaration of interest All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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