REPRODUCTION
REVIEW

Ovarian function and reproductive senescence in the rat: role of ovarian sympathetic innervation

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Abstract

Successful reproduction is the result of a myriad interactions in which the ovary and the ovarian follicular reserve play a fundamental role. At present, women who delay maternity until after 30 years of age have a decreased fertility rate due to various causes, including damaged follicles and a reduction in the reserve pool of follicles. Therefore, the period just prior to menopause, also known as the subfertile period, is important. The possibility of modulating the follicular pool and the health of follicles during this period to improve fertility is worth exploring. We have developed an animal model to study the ovarian ageing process during this subfertile period to understand the mechanisms responsible for reproductive senescence. In the rat model, we have shown that the sympathetic nervous system participates in regulating the follicular development during ovarian ageing. This article reviews the existing evidence on the presence and functional role of sympathetic nerve activity in regulating the follicular development during ovarian ageing, with a focus on the subfertile period.

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Introduction

In recent times, women frequently decide to postpone motherhood until 30 years of age or later, thus representing a sociological change compared with the previous century (Fuentes *et al*. 2010, Mathews & Hamilton 2016). Pregnancy after 30 years of age is associated with a higher risk of miscarriage, hypertension and diabetes mellitus. It is also associated with an increased risk of genetic malformation of the foetus due to a greater probability of damaged follicles/oocytes (Wu *et al*. 2005, Schmidt *et al*. 2012, Waldenstrom *et al*. 2015). Therefore, it is important to know the sequence of events preceding menopause and the mechanisms mediating these events. Several conditions are associated with the onset of reproductive senescence in the female. However, in the human, one of the crucial factors is the loss of the pool of primordial follicles to the point of depletion (Gougeon 2003, Wilkosz *et al*. 2014). During the phase named the subfertile period (between 37.5 and 51 years of age), an accelerated loss of the follicular pool is observed. When the follicular pool reaches 1000 follicles, the ovary cannot maintain the hormonal feedback with the hypothalamus, and menopause is reached (close to 51 years of age) (te Velde 1998). Due to the constraints in using samples from human subjects, animal models are used to perform studies in this field. Their relatively short lifespan and the accessibility to samples from laboratory rats and mice make them a good model to study the mechanisms involved in reproductive senescence. Some authors hypothesised that in rats, the hypothalamus has a more important role than the ovary in achieving reproductive senescence (Aschheim 1965, Clemens *et al*. 1969, Peng & Huang 1972, Anderson *et al*. 2002, Finch 2014) because when rats become infertile, a vast number of primordial follicles are still in the ovary. However, it was demonstrated that ovarian ageing also contributes to anovulation, the condition characteristic of senescence in rats (Felicio *et al*. 1983). As rodents prove to be a very useful model to study follicular development and ovarian ageing, it is important to know the similarities and differences in reproductive senescence between them and humans. This would allow us to correctly extrapolate data from both mice and rats to humans. In Sprague–Dawley rats, the first signs of reproductive senescence occur at approximately 8–10 months of age

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and correspond both to a decrease in the number of developing follicles and to changes in oestrous cyclicity (Peng & Huang 1972, Acuna *et al*. 2009). This reduction in fertility continues gradually until 12 months of age. We characterised this period from 8 to 12 months old as the subfertile period in the rat because there is a gradual decrease in the number of developing follicles, corpora lutea and fertility (Acuna *et al*. 2009). From 12 months old and onward, almost no corpora lutea are observed in the ovaries of rats (Acuna *et al*. 2009), which is indicative of a virtual absence of ovulation. This coincides with the fact that no ova shed are found in the oviducts during oestrous from 12-months and onward (Chavez-Genaro *et al*. 2007). In fact, the percentage of successful pregnancies and the number of pups born per rat also decrease in the subfertile period (Fig. 1) (Jones & Krohn 1961, Acuna *et al*. 2009). In addition, foetal survival also influences fertility in this period. Although foetal survival is 92.5% in young 5-month-old rats, it gradually decreases as age increases, being only 33.8% in 11-month-old inbred rats (Mattheij & Swarts 1991). In this review article, we analyse the follicular dynamics in the ovaries of rats throughout the period of reproductive senescence and the role of sympathetic innervation in its control, with a particular focus on the subfertile period. Finally, we discuss a potential role for ovarian kisspeptin as a regulator of follicular dynamics during ovarian ageing.

Oestrous cycle of ageing rats and mice

The daily examination of vaginal smears is commonly used to estimate the stages of the oestrous cycle in rats and mice. The differences in the proportion of different types of cells observed in the vaginal lavages reflect the changes occurring in the vaginal epithelium due to hormonal variation during the oestrous cycle

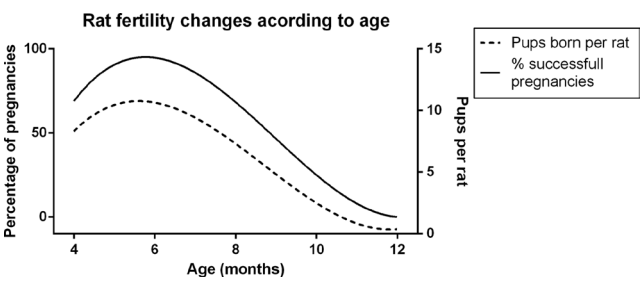

Figure 1 Representation of fertility indexes between 4 and 12 months in rats. The figure shows the average number of pups born per rat and the percentage of successful pregnancies occurring between 4 and 12 months. The information was obtained from Jones & Krohn (1961) and Acuna and coworkers (2009). The percentage of successful pregnancies was assessed as the number of pregnancies over the number of times in which females presented positive for sperm in vaginal smears. The number of pups per rat was evaluated as the number of living pups born per mother in the first 12 h from delivery.

(Westwood 2008, Cora *et al*. 2015). In young cyclic rats and mice, the oestrous cycle is 4–5 days in duration and comprises 4 stages: proestrus, oestrous, metestrus and diestrus. Ovulation occurs in the afternoon of proestrus. One of the first changes occurring during ageing is a lengthening of the oestrous cycle from 4 to 5 days to more than 5 days. This lengthening of the cycle is often considered irregular cyclicity and typically occurs by 8–12 months old, or even earlier, in both mice (Nelson *et al*. 1985, Finch 2014) and rats (Nelson *et al*. 1985, Sone *et al*. 2007). In addition, along with the changes in the cycle length, some animals do not display the logical sequence of proestrus–oestrus–metestrus–diestrus regularly; rather, they stay in the same phase for 3–5 days between regular cycles (Marcondes *et al*. 2002). After exhibiting irregular cycles, both rats and mice become acyclic. A representation of this cycling behaviour is schematised in Fig. 2. In both rats and mice, the end of normal ovarian cycles varies among the cohorts of animals and can be reached between 10 and 16 months old (Aschheim 1974, Merry & Holehan 1979, Felicio *et al*. 1984). In one study, nearly 75% (43/59) of the mouse cohort showed persistent epithelial cornification in their vaginal smears after ceasing to cycle (Felicio *et al*. 1984). This period is termed constant oestrous (CE). A CE is characterised by more than 15 days of continuous cornification cytology in the vaginal smear (Felicio *et al*. 1984, Westwood 2008). The CE in rats results in low and constant levels of oestradiol, estrone, testosterone, androstenedione and progesterone (with minimal levels of 20-α-OH-progesterone) compared with younger rats in the classic oestrous stage during regular cycles. Additionally, the oestradiol/progesterone ratio is increased (Huang *et al*. 1978, Lu *et al*. 1979, Westwood 2008, Fernandois *et al*. 2012). This low steroidogenesis is due to a low follicular development and a near absence of corpora lutea, reflecting anovulation (Acuna *et al*. 2009). The CE stage can be found from 10 months old and onward and is commonly followed by a period of irregular length, known as pseudopregnancy (PP) (by 19 months and onward). PP is characterised by vaginal leukocyte cytology for more than 10 days, but this can be interspersed with 1–2 days of cornified cytology or oestrous (Felicio *et al*. 1984). In the PP stage, both rats and mice can have a scarce number of corpora lutea, indicating that the animals may ovulate. The presence of corpora lutea is accompanied by high levels of progesterone and 20-α-OH-progesterone, which leads to a decreased oestradiol/progesterone ratio (Lu *et al*. 1979, Sone *et al*. 2007, Westwood 2008). Finally, both rats and mice reach an anoestrous state (AS) by 22–25 months old (Aschheim 1961, Huang & Meites 1975, Nelson *et al*. 1985, Sone *et al*. 2007). In AS, the rats have no cyclic activity and only vaginal leukocyte cytology accompanied by low levels of steroid hormones due to the lack of follicles (Ingram 1959,

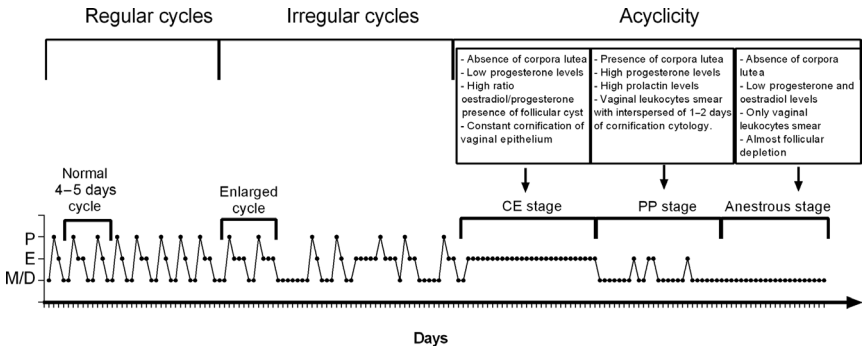

Figure 2 Schematic representation of the changes occurring in the oestrous cycle in rats or mice during reproductive ageing. The figure shows the stage of the oestrous cycle in the *Y*-axis; proestrus (P), oestrous (E), metestrus and diestrus (M/D). The *X*-axis indicates the time course in days. A normal cycle is represented by a 4- or 5-day cycle length with the sequence P–E–M–D. The prolonged/irregular cycles are represented by cycles of more than 5 days in length or cycles interspaced by several days of acyclicity. The acyclic stage is represented by the loss of cycles and includes constant oestrous (CE), pseudopregnancy (PP) and anoestrous. Ovarian and hormonal profiles listed above the scheme for each stage were obtained from Westwood (2008).

Aschheim 1961, Huang & Meites 1975, Finch 2014). Not all animals change from irregular cycles to CE and then PP (Finch *et al*. 1984, Finch 2014). Instead, they show different sequences of events, including going directly from irregular cycling to the anoestrous stage. In one study, 25% of the mice went directly to the AS stage after the cessation of cyclicity (Felicio *et al*. 1984).

Although our focus in this review is analysing the ovarian changes during the subfertile period and their relationship with the sympathetic control of the ovary, it is important to mention that the hypothalamus plays a central role in the onset of reproductive senescence in mice and rats. The cessation of cyclical activity is related to the exhaustion of the follicular pool, whereas the transition from regular to irregular cycles that occurs during the subfertile period is critically influenced by the hypothalamus (Brann & Mahesh 2005). In relation to this, GnRH pulses and hence, LH secretion, are attenuated in middle-aged rats (8–12-month-old) (Rubin 2000). In addition, the central response to the steroid-induced LH peak in middle-aged rats is also attenuated in comparison to young rats (Gee *et al*. 1984, Wise 1984, Rubin 2000, Temel *et al*. 2002). Interestingly, oestrogens cause epigenetic modifications, switching the *Kiss1* promoter to an active form, resulting in an increase in AVPV-specific *Kiss1* gene expression (Tomikawa *et al*. 2012). Thus, the lower oestradiol levels and the low sensitivity to oestradiol may be the cause of the reduction in kisspeptin expression (Kermath *et al*. 2014) and, consequently, irregular and lower GnRH/LH surges in aged rats. This suggestion is supported by the fact that kisspeptin infusion directly into the medial preoptic area restores the attenuated LH surge in middle-aged rats (Neal-Perry *et al*. 2009). In addition to the failure in the kisspeptin system, other mechanisms could explain the alteration in GnRH/LH secretion. For further reading on this topic, we suggest the following works: (Brann & Mahesh 2005, Yin & Gore 2006, Kermath & Gore 2012).

Follicular development during the subfertile period in rats

The ovarian reserve of primordial follicles declines with increasing age in different mammals, including the rat (Mandl & Shelton 1959, Jones & Krohn 1961, Almeida *et al*. 2012, Atkins *et al*. 2014). In humans, it has been estimated, using mathematical models that the rate of follicular loss during life occurs as a biphasic exponential rate of decay. This loss of follicles accelerates when the subfertile period that precedes menopause is reached (Richardson *et al*. 1987, Faddy *et al*. 1992, Hale *et al*. 2014). However, other authors state that the follicular decay is constantly accelerating and that the increase in follicular loss during the subfertile period may be an experimental issue (Hansen *et al*. 2008). In mice, it has been proposed that the decline in the number of primordial follicles occurs in a constant proportion to the existing number of follicles (Jones & Krohn 1961). In addition to the loss of primordial follicles, a feature of ovarian ageing is the change in the expression profile of some key genes between aged and young primordial follicles. In summary, some altered genes include *Brac1*, *Rad51*, *Ercc2 H2ax*, GRP78, FIGNL1, Calreticulin, BOK and Peroxiredoxin 2 and 3 (Govindaraj & Rao 2015, Govindaraj *et al*. 2015). Altogether, the change in the expression of these factors could explain the decrease in DNA repair, protein folding and anti-apoptotic properties of aged primordial follicles. Therefore, the fate of aged primordial follicles could be altered in comparison with a primordial follicle in a younger animal. Then, the function and development of the follicle in subsequent stages could be affected. Independent of this, the recruitment of primordial follicles into the growing pool of follicles does not depend on the cyclic activity of gonadotropins. Instead, it appears to be regulated by a coordinated machinery of paracrine factors, which exert an inhibitory control of primordial follicle activation (Adhikari & Liu 2009). In the review by McGee and Hsueh (2000), it is suggested

to denominate 'initial recruitment' as the transition of a primordial follicle into a primary follicle to differentiate this process from the cyclic recruitment of antral follicles performed by FSH.

If the distribution of developing follicles is analysed, the number of small developing follicles (preantral stage) are observed to decrease by more than half by 8 months of age, and this number of follicles is maintained relatively constantly until 14 months of age (Acuna *et al*. 2009). Meanwhile, the number of antral follicles also decreases from 8 months old and onward (Lerner *et al*. 1990, Acuna *et al*. 2009). However, it has been demonstrated that the number of antral follicles >400 µm and preovulatory follicles remains unchanged (Jones & Krohn 1961, Lerner *et al*. 1990, Fernandois *et al*. 2016). This increase in the proportion of larger-sized follicles indicates that ovaries of aged rats within the subfertile period utilise primordial follicles more efficiently. They have low initial recruitment and low growth of small follicles but have a proportionally higher number of follicles reaching the preovulatory stage at the proestrus phase (Jones & Krohn 1961, Peluso *et al*. 1979). However, this higher proportion of preovulatory follicles is not proportionally reflected in more ovulation as the number of corpora lutea decreases with age and is virtually absent from 12 months of age, despite antral follicles continuing to grow (Chavez-Genaro *et al*. 2007, Acuna *et al*. 2009), indicating that preovulatory follicles take a pathway alternative to ovulation. This alternative pathway could be the formation of precystic and cystic structures (as discussed below). However, together with the decrease in healthy preantral and antral follicles, a decrease in the atretic follicle count in ageing rats from 12 months old has been characterised (Peluso *et al*. 1979, Acuna *et al*. 2009). In addition, Nishijima and coworkers (2013) showed that atretic follicles begin to increase from 18 months old and onward in rats. As the decrease in healthy follicles in the subfertile period is not explained by the process of atresia, an alternative explanation is required. A lower recruitment of follicles and the deviation of follicles to abnormal structures should explain this low follicular development. The presence of luteinized follicles has been observed in ageing rats, structures that are abnormal in young rats (Acuna *et al*. 2009). Luteinized follicles are characterised by the presence of luteinized granulosa cells (they contain a big cytoplasm similar to luteal cells), and an antral cavity (although there is no oocyte) (Smirnova 1964, Moon *et al*. 1993). These alternative structures, which appear in the ovary of rats during the subfertile period, may produce hormones and paracrine factors, which potentially affect the development of other follicles.

The lower number of developing follicles is probably responsible for the low serum levels of steroid hormones observed in middle-aged rats, particularly oestradiol (Lu *et al*. 1979, Anzalone *et al*. 2001, Acuna *et al*. 2009, Fernandois *et al*. 2012). This could account for the prolonged or irregular cycles observed during the subfertile period as low numbers of antral follicles lead to an extended follicular phase in which the levels of oestradiol sufficient to induce LH secretion are reached later or not at all. This may manifest in additional days in the diestrus or oestrous stage in the vaginal smear.

Ovulation and spontaneous follicular cyst formation during the subfertile period in rats

It is well known that in the rat, ovulatory capacity and fertility are decreased from 8 to 10 months old and onward (Jones & Krohn 1961, Mattheij & Swarts 1991, Niggeschulze & Kast 1994, Chavez-Genaro *et al*. 2007, Acuna *et al*. 2009). This can be evaluated by counting the number of corpora lutea present in the ovary, the number of ovulated oocytes and the number of pups born per litter at different ages. This period has been characterised by a dramatic decrease in the number of corpora lutea with increasing age from 10 to 12 months old (Acuna *et al*. 2009), even though the number of preovulatory follicles is maintained (Peluso *et al*. 1979). We demonstrated that 12-month-old Sprague–Dawley rats have a very low number of corpora lutea, along with an increase in type III follicles and follicular cysts (Acuna *et al*. 2009). Type III follicles are structures with very similar morphology to preovulatory follicles but can be found in the oestrous stage, indicating that these follicles did not ovulate after the LH preovulatory peak. Type III follicles were first described by Brawer and coworkers in the rat model of polycystic ovary syndrome induced by oestradiol valerate. Histologically, they are formed by approximately 5 layers of granulosa cells, an invagination of the theca layer, loss or discontinuation of the basal membrane and usually a size bigger than 750 µm in diameter. Regarding the oocyte nucleus, an apparently healthy germinal vesicle can be observed (Brawer *et al*. 1989, Lara *et al*. 2000, Fernandois *et al*. 2012). These types of follicles lose their oocytes and change into follicular cysts, which do not contain oocytes and have only one layer of granulosa cells. Although both type III follicles and follicular cysts are almost absent in young control rats, they are typically observed in rat models of polycystic ovary syndrome induced by oestrogens and in aged rats (Brawer *et al*. 1989, Acuna *et al*. 2009, Fernandois *et al*. 2012, 2016). Comparing the rat model of oestradiol valerate-induced polycystic ovaries with ageing rats could give us insights into the mechanisms that control ovulation and cystic structure generation during ageing. It is possible that preovulatory follicles are deviating from ovulation into the formation of cysts (Convery *et al*. 1990, Lara *et al*. 2000, Fernandois *et al*. 2012). In the model of polycystic ovaries induced by oestradiol exposure, there is an increase in the content and release of norepinephrine

(NE) in the ovary (see below). This led us to investigate the role of sympathetic activity in ovarian ageing.

Sympathetic innervation and ovarian ageing

The sympathetic innervation of the ovary has been described using different techniques in humans, monkeys and rodents (mice and rats) (Stefenson *et al*. 1981, Burden *et al*. 1983, 1985, Gerendai *et al*. 1995, 1998, 2002). Using histofluorescence, nerve fibres, mainly noradrenergic, were shown to be present in the ovary (Burden *et al*. 1983, 1985). These were associated with not only the vasculature but also the ovarian follicles, which were densely marked around the thecal zone (Stefenson *et al*. 1981). The mapping of the nerve projection from the sympathetic pathway was studied in rats with retrograde viral tracers by Gerendai and coworkers. In these experiments, a hypothalamic–spinal medulla–ganglion–ovary pathway was elucidated (Gerendai *et al*. 1995, 1998, 2002). In rats, the sympathetic nerve fibres projecting to the ovary come from the coeliac ganglion via two different routes: the nerve plexus of the ovary, whose nerve fibres project mainly to the ovarian blood vessels, and the superior ovarian nerve (SON), whose fibres project into the follicles (Baljet & Drukker 1979, Lawrence & Burden 1980). It was demonstrated that cholinergic ganglionic stimulation during oestrous increases the NE in the ovarian compartment compared to that in control (Hanada *et al*. 2011, Daneri *et al*. 2013). Hanada and coworkers (2011) have shown that the nerve fibres associated with blood vessels regulating ovarian blood flow are mainly unmyelinated C fibres, which are maintained in number, size, conduction ability and vasoconstrictor response in aged PP rats compared to that in young rats. This means that the sympathetic adrenergic vasoconstrictor response in the ovary is well preserved in rats aged 28–31 months old. In addition to regulating ovarian blood flow, extrinsic ovarian innervation directly regulates steroidogenesis (Aguado & Ojeda 1984) and follicular development (Lara *et al*. 1993, Moran *et al*. 2000, Rosa *et al*. 2003, Doganay *et al*. 2010, Zhang *et al*. 2010). Both α and β adrenergic receptors are expressed in the ovarian follicles (Aguado *et al*. 1982, Barria *et al*. 1993, Itoh & Ishizuka 2005, Fernandois *et al*. 2012), and direct effects of NE on follicles could be produced by α1 and β2 adrenergic receptor stimulation (Laszlovszky & Erdo 1983, Itoh & Ishizuka 2005). Interestingly, both sympathetic activity and the density of β receptors in the ovary change with the oestrous cycle in the rat (Lara *et al*. 2002). Regarding follicular development, the surgical section of the ovarian superior nerve (SON) in pigs increases the number of small follicles (<3 mm) and decreases the number of large follicles (>7 mm). This change in the follicular population is associated with a decrease in the content of steroid hormones in the follicular fluid (Jana *et al*. 2007). A similar effect is found in rats, where a bilateral denervation of the SON decreases the serum level of oestradiol, progesterone and the number of developing follicles. In addition, when a unilateral denervation of the SON was assessed, a decrease in the number of follicles was observed in the denervated ovary and a compensation (observed as an increase in the number of follicles) was observed in the innervated ovary (Moran *et al*. 2000). In addition, it has been demonstrated that the sympathetic innervation of follicles influences follicular maturation/growth (Mayerhofer *et al*. 1997, Paredes *et al*. 2011), steroidal secretion (Hernandez *et al*. 1988, Barria *et al*. 1993) and ovulation (Kannisto *et al*. 1985). In fact, an increase in ovarian sympathetic activity is observed in rats with an oestradiol-induced polycystic ovary condition. In these rats, alterations in follicular growth and ovulation are observed (Lara *et al*. 1993, 2000, Rosa *et al*. 2003). As reviewed by Lansdown and Rees (2012), polycystic ovary syndrome (PCOS) is associated with an increased noradrenergic tone directly in the ovary. Some PCOS women also display a generalised increase in sympathetic tone activity, which offers a possibility for intervention by lowering the sympathetic outflow using strategies such as drugs, surgery or acupuncture (Lansdown & Rees 2012).

The role of sympathetic innervation in ovarian ageing has been studied by different groups, including us. An early study demonstrated that old Wistar rats (24 months old) have a decrease in the ovarian concentration of NE compared to young (3 months old) rats (Ferrante *et al*. 1990). It is possible that the very low number of follicles present in the ovaries of rats at this age is not enough to maintain an adequate production of neurotrophic factors, principally nerve growth factor (NGF), which is known to participate in the maintenance of noradrenergic fibres (Lara *et al*. 1990*b*). More recent studies have been focused on the transition of young cycling rats through the subfertile period. In these rats, we and others demonstrated an increase in the ovarian concentration of NE with increasing age, without changes in plasma NE levels (Lerner *et al*. 1990, Chavez-Genaro *et al*. 2007, Acuna *et al*. 2009). This increase in the content of NE is accompanied by an increase in the release of NE into the ovary in rats 12 and 14 months old compared to that in 6-month-old rats, demonstrating an increased adrenergic tone with increasing age (Acuna *et al*. 2009). Additionally, we measured a decreasing ovarian density of β adrenergic receptors with age, along with an inverse increase in adrenergic tone, in rats (Fig. 3). Interestingly, in humans, an increase in nerve fibres in the ovary related to ageing has also been demonstrated (Heider *et al*. 2001). More recent studies have confirmed that post-menopausal women have higher nerve activity, baseline plasma NE levels and reduced β-adrenergic receptor responsiveness

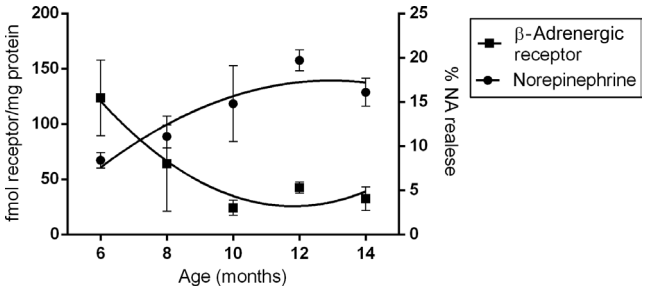

Figure 3 Concentration of ovarian β-adrenergic receptors and NE release according to age. Data of β-adrenergic receptors are unpublished work, and data of ovarian NE release were obtained from Acuna and coworkers (2009). The ovarian β-adrenergic receptor content was determined by a radio-ligand binding technique and is expressed as fmol dihydroalprenolol bound/mg of membrane protein. Ovarian NE release was determined as the percentage of ^{3}H-NE released after 1 min of electric stimulation from the total of ^{3}H-NE previously incorporated into ovaries (NE dpm ×1000/ovary). The data are plotted as the mean ± S.E.M.

compared to premenopausal women (Sherwood *et al*. 2010, Hogarth *et al*. 2011).

As the adrenergic tone of the ovary is increased during the subfertile period, we believe that NE contributes to the changes in follicular development observed in the ovary during the transition through 8–12 months old until the infertile period. As discussed previously, there is also an increase in the adrenergic tone of the ovary in the model that uses oestradiol valerate administration (Lara *et al*. 1993). In this model, it is possible to observe the same changes in follicular dynamics that are seen in the ovaries of ageing rats (Lara *et al*. 2002, Acuna *et al*. 2009, Cruz *et al*. 2012). Within these changes, we observe a decrease in preantral and antral follicles, a decrease (or absence) of corpora lutea and the appearance of type III and cystic follicles. In this model of polycystic ovary, the denervation of the ovary by sectioning the SON leads to a partial recovery of follicular development (Rosa *et al*. 2003). As around the 8–10-month-old stage the ovary still has enough primordial follicles capable of developing into preovulatory ones, we believe that the development of abnormal structures (i.e., type III follicles and cysts) is due to a deviation of antral or preovulatory follicles under the influence of increased noradrenergic tone. Using this hypothesis, we tried to reverse the polycystic condition associated with low follicular development and low plasma steroidal hormones by administering the β adrenergic blocker propranolol and hence, increase fertility within the subfertile period. We found that daily administration of propranolol (5 mg/kg of body weight) for 2 months in 8- and 10-month-old rats increases the number of healthy antral follicles and increases the number of corpora lutea in the ovary. Along with this improvement in follicular development and ovulation, propranolol decreased the number of follicular cysts. These changes in follicular development were functionally associated with an increase in serum progesterone, androgens and oestradiol. Additionally, the β adrenergic blocker improved the pattern of the oestrous cycle by increasing the number of 4–5-day cycles during the treatment (Fernandois *et al*. 2012). However, denervation with guanethidine for 7 days increased the proportion of healthy antral follicles, but not ovulation (number of ova shed) in 12- and 18-month-old rats (Chavez-Genaro *et al*. 2007). In this study, guanethidine was administered, and the rats were immediately killed. However, 7 days (less than 2 oestrous cycles) was not enough time to permit the healthy antral follicles to reach the ovulation stage. This explains the differences in the results with those of the previously described study.

In young rats, β-adrenergic stimulation of the ovary increases follicular development (Mayerhofer *et al*. 1997), whereas pharmacological denervation delays the development of follicles (Lara *et al*. 1990*a*). This effect of adrenergic stimulation on follicular development is, in part, produced by NE stimulation on FSHR expression (Mayerhofer *et al*. 1997). If this also occurs in ageing rats, which are under a hyperadrenergic tone, we would expect an increase in follicular development. However, this does not seem to be the case if we consider the net number of growing follicles in rats during the subfertile period. Recently, we found that sympathetic nerves regulate the paracrine factor kisspeptin (KP) in the ovary and that KP modulates the effects of sympathetic nerves on ovarian function, which could explain why the increase in the sympathetic tone is not reflected in higher follicular growth (Fernandois *et al*. 2016).

Role of ovarian kisspeptin in ovarian ageing and its relation to sympathetic innervation of the ovary

The kisspeptins are a family of peptides resulting from differential proteolytic processing of a single precursor (Pinilla *et al*. 2012). In humans, four biologically active peptides have been described, whereas in rats, only two peptides have been detected (UniProt 2015). All these peptides share the last 10 amino acids of the C-terminal region, the region responsible for binding to the Kisspeptin receptor (KISS1R or GPR54) (Kotani *et al*. 2001, Muir *et al*. 2001, Messager *et al*. 2005). Hypothalamic KP is considered a master regulator of the gonadotropic axis and is critical for the onset of puberty in mammals (Pinilla *et al*. 2012). Knockout (KO) mice of the kisspeptin receptor (GPR54$^{-/-}$) (Colledge 2009) and the novel *Kiss1* KO rats (Uenoyama *et al*. 2015) do not reach puberty and are infertile due to the absence of gonadotropin secretion. KP and its receptor KISS1R are expressed in the ovary of different species including humans, rats and mice, and their expression and immunolocalisation are in theca cells (TCs) (Castellano *et al*. 2006, Gaytan *et al*. 2009, Zhou *et al*. 2014) and granulosa cells (GCs) (Ricu *et al*. 2012, Laoharatchatathanin *et al*. 2015).

In addition, KP mRNA levels have been demonstrated to change throughout the oestrous cycle in the rat ovary, being at their highest during proestrus (Castellano *et al*. 2006, Laoharatchatathanin *et al*. 2015).

We recently found that intraovarian KP increases as age increases during the subfertile period in the rat (Fernandois *et al*. 2016). Along the same lines, a very recent study confirmed our results demonstrating that ovarian mRNA levels of *Kiss1* and its receptor *Kiss1R* increase in aged mice compared to that in younger mice. Although the authors failed to measure ovarian kisspeptin mRNA in women GCs, they found that ovarian *Kiss1R* mRNA levels increase according to age (Merhi *et al*. 2016). Interestingly, ovarian KP has a positive correlation with ovarian NE release during the subfertile period in the rat (Fernandois *et al*. 2016). This is in agreement with a previous study from our group demonstrating that KP expression of the ovary increases after β adrenergic stimulation *in vitro* (Ricu *et al*. 2012). Considering that chronic administration of propranolol improves the development of ovarian follicles (Fernandois *et al*. 2012) and that ovarian KP is under the control of sympathetic innervation, we thought that KP could be decreasing the follicular development during ageing. To test this hypothesis, we designed experiments using *in vivo* stimulation with KP or its antagonist P234 directly in the ovary in rats during 28 days within the subfertile period. The results showed that KP administration decreases the number of antral follicles, whereas P234 increases it (Fernandois *et al*. 2016). Moreover, both the sympathetic denervation of the ovary and propranolol administration decreases KP in the ovary and accelerates follicular development (D Fernandois, G Cruz, EK Na, HE Lara and AH Paredes 2016, manuscript accepted). Consistent with these findings, *in vitro* experiments demonstrated that KP prevents the induction of FSHR expression by the β adrenergic agonist isoproterenol (Fernandois *et al*. 2016). Therefore, it is understandable that *Kiss1R* haplo-insufficient mice ($Kiss1r^{+/-}$) present an early loss of oocytes, primordial follicles and antral follicles by 8 months old, showing that these mice present a premature ovarian failure despite maintaining circulating gonadotropin levels (Gaytan *et al*. 2014). Likewise, *Kiss1R* KO mice with reinsertion of the Kiss1R gene in GnRH neurons show premature ovarian ageing, even when there is a normally functioning hypothalamic kisspeptin system (Leon *et al*. 2016). This could mean that the lack of inhibitory action of KP on FSHR expression induced by the sympathetic system leads to an enhanced loss of follicles through life and could cause premature ovarian senescence. This hypothesis, however, must be confirmed.

Conclusion

In the present review, we show evidence attributing a role to sympathetic innervation on follicular dynamics during the subfertile period in the rat. The mechanisms underlying this sympathetic control are complex and still should be fully elucidated. In particular, it would be interesting to observe which follicular structure present in the ovaries of senescent rats expresses adrenergic receptors and responds to NE modulation of KP secretion. The capacity of a β blocker and a KP antagonist to increase follicular growth during the subfertile period in rats offers a new possibility to pharmacologically intervene in the pool of developing follicles during ageing, with the aim of improving fertility and its outcome.

Declaration of interest

The 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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