

Brief Communication

Differences in learning and memory between middle-aged female and male rats

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We observed differences in cognitive functions between middle-aged female and male Wistar rats. Both (like youngsters) discriminated new versus familiar objects, showing similar short- and long-term memory (STM and LTM, respectively). Only females show robust LTM for new location of an object. Both successfully form LTM of inhibitory avoidance, though males appeared to be amnesic for memory persistence. Habituation, locomotion, horizontal exploration, "stereotypies," fear, and anxiety-like behavior were similar for both, while vertical exploration was significantly higher in middle-aged and younger females. Therefore, sex-dependent differences in some cognitive functions and behaviors must be considered when designing and interpreting learning and memory studies.

[Supplemental material is available for this article.]

Research studies on learning and memory in animal models often rely on an overrepresentation of males; sometimes they are not discriminated by sex (see Beery and Zucker 2011; Yoon et al. 2014; Karp et al. 2017) or include females only (Paris et al. 2011; Loi et al. 2017; Tang et al. 2020). Nonetheless, differences between males and females could substantially impact the analysis of many traits (Karp et al. 2017), particularly in the neuroscience field (Beery and Zucker 2011). For Wistar rats, a widely used model to investigate learning and memory, literature comparing females and males is sparse and sometimes controversial (Hughes and Beveridge 1990; Alliot and Giry 1991; Temerowski and Van Der Staay 2005; Belviranlı et al. 2012).

The complexity increases with the "age factor." Although progressive age-dependent decline in learning and memory has been partially addressed in Wistar rats, sex influence remains unclear. There are only few reports about dimorphic cognitive changes in rats at the onset of age-related memory decline (Supplemental Table 1; see also Rossetti et al. 2018; McQuail et al. 2021).

Enhanced vascular remodeling and reduced neuronal density were observed in the hippocampus of middle-aged and aged Wistar rats, particularly in male subjects (Jacobson et al. 2008). At 12 mo, there are significant gene expression changes in the hippocampus. There is a down-regulation of steroidogenic-related genes in female Wistar rats compared with males (Rossetti et al. 2018) and an up-regulation of inflammation pathways related to the innate immune response and cytokine signaling in male Sprague-Dawley (SD) rats (Shavlakadze et al. 2019).

Whether the onset of age-related memory difficulties may differ with sex and whether this mainly concerns short- and/or long-term memory and/or specific memory types have not been solved yet. Hence, it seems necessary to assess females and males comparatively.

Twelve-month-old to 13-month-old (middle-aged) female and male Wistar rats (10/group) were sequentially evaluated for 4 wk in spontaneous exploration of an open field (OF), recognition/discrimination of a new object (NOR), novel location for an object (NOL), and inhibitory avoidance (IA) of a footshock (Supplemental Fig. S1). Four-month-old Wistar rats raised and trained under the same experimental conditions were used as age controls (seven to 10/group). Methods are included in the text and figure legends.

Exploration of an open field (OF) is a simple behavioral paradigm in which spontaneous exploration, habituation to the environment, and some emotional conditions can be assessed. For 12-month-old rats during a 5-min training session (Tr) the number of crossings/minute was significantly lower from the second to third minute on (Fig. 1A); the number of rearings progressively decreased also (Fig. 1B). Results were similar with 4-month-old rats (Supplemental Fig. S2A,B). Hence, females and males at both ages recognized the environment and became habituated to it, denoting active working memory and short-term memory (STM). Total number of crossings (Fig. 1C), traveled distance (locomotor activity and spontaneous bidimensional exploration), time spent in the peripheral versus central area, resting time, freezing (levels of behavioral stress, fear, and anxiety-like behavior), and grooming ("stereotyped behavior") were rather similar in middle-aged females and males (Supplemental Fig. S3). Nonetheless, total rearings were significantly lower in males (Fig. 1D; Supplemental Fig. S2D). Rats were evaluated 24 h later in the same OF for long-term memory (LTM) and long-term habituation. The total number of crossings and rearings significantly dropped compared with Tr, evidencing long-term habituation and LTM consolidation (Fig. 1C,D;

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Article is online at <http://www.learnmem.org/cgi/doi/10.1101/lm.053578.122>.

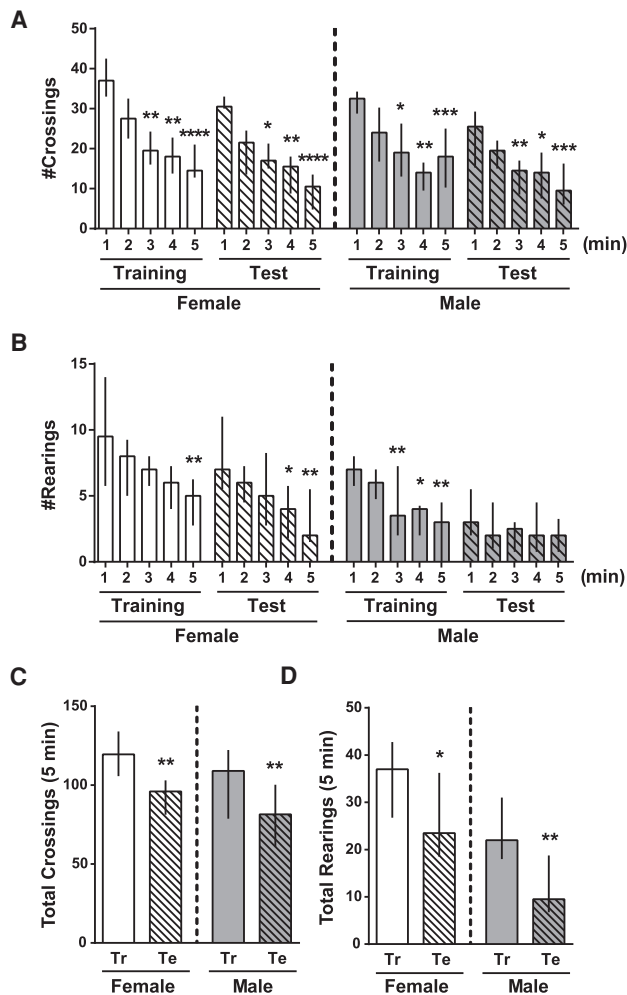


Figure 1. Exploration and habituation to an OF by 13-mo-old female (white bars) and male (gray bars) Wistar albino rats. Animals were trained and tested for short-term (ST; intrasession) and long-term (LT) habituation to an OF. On the first day, each rat was exposed to the OF for 5 min (training session [Tr]). The number of crossings across the lines drawn in the floor (forming 25 identical squares) was recorded as a proxy for “horizontal” exploratory activity and compared within the Tr (minute by minute) to evaluate habituation to the environment. The number of rearings—when the rat was raised on its two hind legs—was the indicator of “vertical” exploratory activity. On the second day, rats were tested in the same OF (test session [Te]) to assess LTM and LT habituation. Animals were considered habituated to the arena when the total number of crossings (and rearings) recorded over 5 min was significantly lower in Te than in Tr. (A,B) The bar diagram shows median and IQR (25;75) for crossings (A) and rearings (B) recorded during each minute of the 5-min session of both Tr (plain bars) and Te (striped bars). Performance in the first minute was compared with the remaining minutes. Friedman paired test followed by Dunn’s post-hoc test; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P \leq 0.0001$; $N_F = 10$, $N_M = 10$. (C,D) LT habituation to an OF (LTM). The bar diagram shows median and IQR (25;75) for the total number of crossings (C) and rearings (D) recorded during both the 5-min Tr and Te. Total crossings and rearings during Te were significantly lower than during Tr. Wilcoxon pairwise signed rank test; (*) $P < 0.05$, (**) $P < 0.01$.

Supplemental Fig. S2C,D). The number of crossings was not different between females and males, while rearings were significantly lower for males (both in Tr and Te). Hence, habituation to the environment was rather similar in the short and long term regardless

of sex and age, though, even when habituated, females showed higher vertical exploratory activity.

Time spent in OF center squares was significantly lower than in the periphery, with no differences between sexes, indicating similar levels of anxiety-like behavior (see Quillfeldt 2015). Resting time was short and similar, suggesting comparable stress and fear levels (Supplemental Fig. S3); none of the rats exhibited freezing behavior. Similar exploratory behavior was reported between sexes in 3- and 23-mo-old Wistar rats (Dorce and Palermo-Neto 1994), though 15-mo-old female Wistar rats displayed higher exploratory parameters than males (Belviranli et al. 2012). Some studies suggest that female rats could rely more than males on available odor trails, even in a water maze (Means et al. 1992; Markowska 1999).

No differences in exploratory behavior were observed between female and male Fisher 344 rats at 6, 12, 18 (Febo et al. 2020), and 21.5 mo old (Bowman et al. 2006), while 15-mo-old Long-Evans (Hughes and Beveridge 1990) and 12-mo-old (Domonkos et al. 2017) and 15-mo-old (Borbélyová et al. 2017) Lewis female rats showed higher spontaneous exploration than male rats (Supplemental Table 1). However, 12-mo-old Lewis females appeared less “anxious” than males (Domonkos et al. 2017), in accordance with previous data showing that females exhibited reduced aversion to the plus-maze’s open arms (Johnston and File 1991).

Interestingly, it has been suggested that differences in anxiety-like behavior may be due to housing conditions, lighting levels, test duration, and prior handling (Rodgers and Dalvi 1997). Rats in our colony were handled 1 min twice per week, plus handling during three cleaning cycles per week, from weaning on, and housed under a light–dark inverted cycle to be mostly awake during experimental sessions.

The NOR task—mainly depending on the cerebral cortex (Wilson et al. 2013)—was used to assess the rats’ capacity to discriminate new from familiar objects. During training (T_{NOR}), each rat was exposed to two similar objects: A and A’. Total exploration time—set at a minimum of 10 sec (Akkerman et al. 2012)—was not statistically different for both sexes and ages, which was relevant to discard a priori differences (data not shown). Preference indexes ($PI-T_{NOR}$) were not significantly different between males and females or from 0.5, indicating no object/side preference (Fig. 2A).

Two test sessions were performed: One hour after T_{NOR} , animals were presented with the familiar (A) and a novel (B) object to assess STM ($Te_{STM-NOR}$); 24 h later, a different novel object (C) was presented instead to assess LTM ($Te_{LTM-NOR}$). Females and males spent a significantly longer time exploring B and C than A. Test session preference indexes $PI-Te_{STM-NOR}$ and $PI-Te_{LTM-NOR}$ were significantly higher than either $PI-T_{NOR}$ or chance, indicating that the animals remembered, recognized, and discriminated the novel from the familiar item, similarly expressing NOR STM and LTM regardless of sex (Fig. 2A).

Accordingly, several studies, mostly done only in males, reported that 12- to 13-mo-old male Wistar rats expressed NOR STM and LTM (Supplemental Table 2). We found only one report on NOR performance in middle-aged female Wistar rats, though the results were not conclusive (Tang et al. 2020).

Here we showed that 4-mo-old male and female Wistar rats (under the same experimental conditions as middle-aged) consistently expressed NOR LTM (Supplemental Fig. S4), as was previously reported in males (Habif et al. 2021). Accordingly, it was reported that young females evidenced STM and LTM of NOR (Mateos et al. 2011; Loi et al. 2017), while both NOR STM and LTM were impaired in older males (17–21 mo old) (Supplemental Table 2).

Briefly, middle-aged female and male Wistar rats, as youngsters, are able to similarly express STM and LTM of objects, discriminating novel from familiar ones, while this capacity is impaired in older males (Supplemental Table 2).

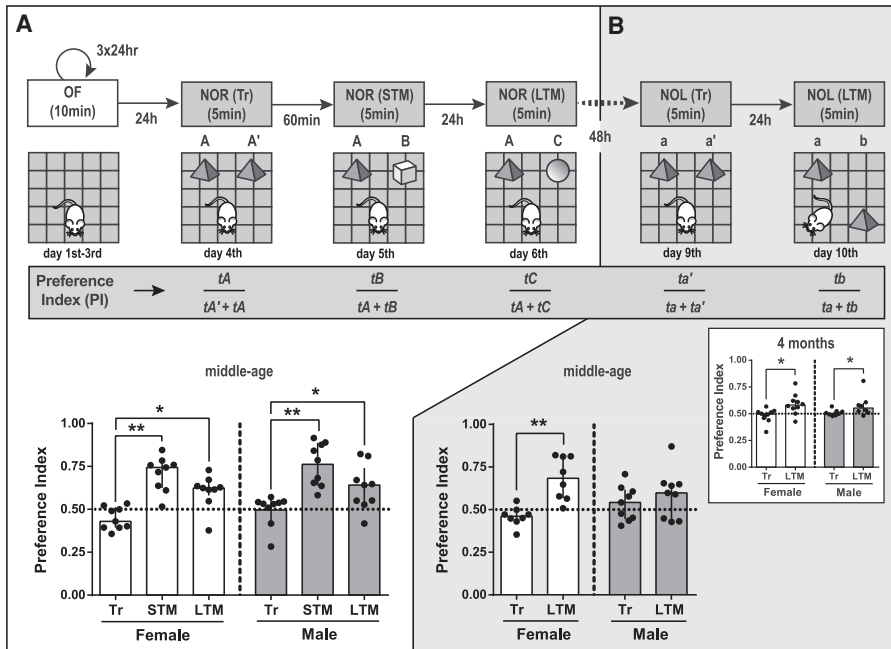


Figure 2. Novel object recognition (NOR) and novel object location (NOL) performance by middle-aged male and female Wistar rats. Schematic representation of the task steps. Rats were first habituated to the OF (5–10 min/session, first through third days). NOR training (Tr_{NOR}): On the fourth day, each rat was left to re-explore the OF for 5 min in the presence of two identical objects (A and A'). NOR STM test ($Te_{STM-NOR}$): One hour after Tr_{NOR} , animals were presented with a familiar (A) and a novel (B) object to assess novel object discrimination and STM. NOR LTM test ($Te_{LTM-NOR}$): On the fifth day, animals were presented with a familiar (A) and a novel (C) object (different from B) to assess object discrimination and LTM. NOL training (Tr_{NOL}): On the eighth day, rats were trained for 5 min in the presence of two identical objects (a and a'), but different from the NOR objects. NOL LTM test ($Te_{LTM-NOL}$): On the ninth day, rats were exposed for 5 min to the already familiar objects in a new spatial configuration (a and b). Preference index (PI) expression formulas are detailed below the scheme (t =exploration time for the given object). The minimum object exploration time to accept a rat performance as appropriate for our records was set to 10 sec (for both objects) per session. (A) Bars represent NOR PI as median and IQR (25;75) for male (gray bars; $N_M=9$) and female (white bars; $N_F=9$) rats. PI was calculated with the exploration time at each object, recorded during Tr_{NOR} (PI Tr_{NOR}), $Te_{STM-NOR}$ (PI $Te_{STM-NOR}$), or $Te_{LTM-NOR}$ (PI $Te_{LTM-NOR}$). For both sexes, both PI $Te_{STM-NOR}$ and PI $Te_{LTM-NOR}$ were significantly higher than PI Tr_{NOR} and significantly different from the chance. Both females and males spent similar amounts of time exploring objects A and A' in Tr_{NOR} , as there were no statistically significant differences either for PI Tr of each object or against the chance ($PI = 0, 5$) for each group of animals. (B) Bars represent NOL PI as median and IQR (25;75) for male and female rats. PI was calculated with the exploration time at each object recorded during both Tr_{NOL} and $Te_{LTM-NOL}$. Females and males spent a similar amount of time exploring objects a and a' in Tr_{NOL} , as there were no statistically significant differences for PI Tr_{NOL} or against the chance. Only female rats evidenced a significantly higher PI for $Te_{LTM-NOL}$ compared with the PI Tr_{NOL} and significantly different from the chance. (Inset) NOL performance by 4-mo-old female and male Wistar rats: Both female (white bars; $N_F=10$) and male (gray bars; $N_M=8$) rats showed a significantly higher PI for $Te_{LTM-NOL}$ compared with the PI Tr_{NOL} and significantly different from the chance. In all of the cases, differences were assessed by Wilcoxon pairwise signed rank test; (*) $P < 0.05$, (**) $P < 0.01$.

A two-object NOL task was used to investigate spatial reference memory, mainly depending on the hippocampus (Broadbent et al. 2004). Rats were trained with two identical objects (a and a') in the familiar OF (Fig. 2B). Total exploration time (set at a minimum of 10 sec) was not statistically different for both sexes at both ages (data not shown). Preference indexes (PI- Tr_{NOL}) were not significantly different between males and females or from chance, indicating no object/side preference. Twenty-four hours later, each rat was presented with the same two objects, but one at a different location (b). PI- $Te_{LTM-NOL}$ was significantly higher than either PI- Tr_{NOL} or chance for both sexes at 4 mo of age and for middle-aged female rats, though not for middle-aged males (Fig. 2B and inset). Hence, middle-aged females, though not males, significantly discriminated the novel location, which requires LTM

consolidation of the previous configuration and its activation at the test phase.

Accordingly, NOL STM was impaired in 13-mo-old male Wistar rats (Cechella et al. 2014), while 3- to 4-mo-old female Wistar rats performed well 1 and 24 h after Tr (Loi et al. 2017), and 70-d-old females and males performed equally well in the NOL test after 10 min (Mateos et al. 2011).

NOR and NOL STM impairment was reported in 21.5-mo-old female and male Fisher 344 rats, revealing deficits in object and spatial reference memory formation, attributed to the aging process (Bowman et al. 2006); furthermore, both sexes at 12 and 18 mo did not reach the criteria for NOL STM either (Febo et al. 2020).

Age-related impairment of spatial reference and working memory was observed in MWM performance for 12-mo-old Fischer 344 males compared with females (Febo et al. 2020), and for both 12-mo-old males (Bizon et al. 2009) and females (Markowska 1999) compared with their 6-mo-old counterparts.

Jacobson et al. (2008) reported that 4- and 9-mo-old male and female Wistar rats performed well in the spatial MWM, with shorter latencies to the platform by males. Although performance was notably impaired at 18 mo, males achieved the criteria. Interestingly, neuronal density was reduced in the hippocampus of 9- and 18-mo-old males, though not in females, compared with 4-mo-old counterparts (Jacobson et al. 2008).

According to literature and our experience, LTM for NOR and NOL is successfully achieved by younger rats (Fig. 2B, inset; Supplemental Fig. S4; see Mateos et al. 2011; Loi et al. 2017; Febo et al. 2020) but is impaired with age, particularly in NOL LTM in middle-aged males (Fig. 2B) and older males and females (Supplemental Table 2). Hence, middle-aged males were not able to form/retrieve an appropriate spatial reference LTM to accomplish NOL, evidencing a sexual dimorphism in the establishment of NOL LTM, at variance with NOR LTM.

Inhibitory avoidance (IA) of a footshock is an associative task involving inhibition of an innate tendency with aversive and spatial components. IA is useful to evaluate associative learning as well as memory consolidation and persistence after one training step (Tr). The rat's Tr latency to enter a dark (preferred) compartment was recorded; there, the rat received a mild footshock (0.5 mA). Tr latencies did not significantly differ among sexes (Fig. 3A,D). This, together with the amount of time spent in corners plus periphery compared with the center in the OF for males and females (Supplemental Fig. S3), corroborates similar levels of anxiety-like behavior.

LTM was assessed by comparing Tr latency with test latency at the first test session (Te_1) performed 24 h later. Te_1 latencies were significantly higher than Tr latencies and similar for both sexes, suggesting similar LTM consolidation and expression. Memory

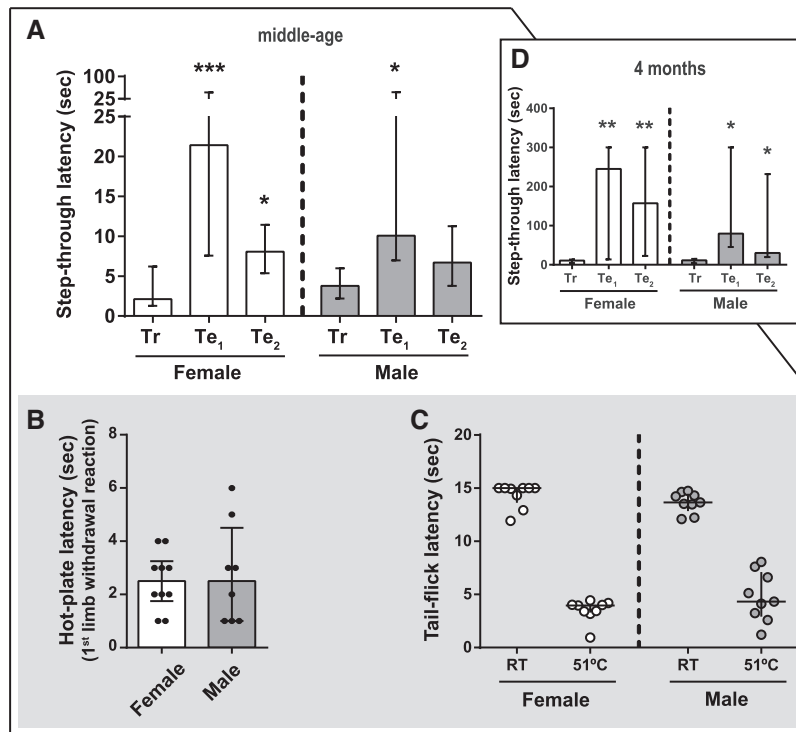


Figure 3. LTM of inhibitory avoidance (IA) in 12- to 13-mo-old male and female Wistar rats. (A) IA task. The bar diagram represents the median of latencies and IQR (25;75) of male (gray bars) and female (white bars) littermates. Training (Tr): Rats were individually left inside a lighted compartment, while a sliding door—separating it from a dark compartment—remained closed. After 15 sec, the door was opened, and the latency to escape from the light to the dark (preferred) side was recorded. The door was closed, and a 5-sec mild footshock (0.5 mA) was delivered to the grid floor. Test sessions (Te) were carried out 24 h (Te₁) and 14 d (Te₂) later, though without shock. Animals reached the learning criteria when latencies to enter the dark compartment were significantly higher during the Te than during the Tr, and the latency difference (Te–Tr) was significantly higher than zero. Te₁ latencies were significantly higher than Tr latencies for males and females. However, only female rats showed a significant difference between Te₂ and Tr latencies. Friedman paired test followed by Dunn's post-hoc test; (*) $P < 0.05$, (***) $P < 0.001$. No significant differences were found in the comparison of latency differences (Te–Tr) between male and female groups, considering neither Te₁ nor Te₂ (unmatched Kruskal–Wallis test with Dunn's post-hoc test, $P > 0.05$). For Te₂, latency difference was significantly higher than zero for females (Mann–Whitney test [****] $P < 0.0001$), though no significant difference was found for males ($N_F = 9$, $N_M = 9$). (B,C) Hot plate response (B) and tail flick latencies (C) were used to evaluate sensitivity and response to a painful stimulus. In B, the animal was habituated by placing it into a transparent square-shaped chamber on top of an aluminum thermostatic platform at room temperature (RT) three times, 10 min apart. The platform was then heated to $55.0^\circ\text{C} \pm 0.5^\circ\text{C}$. Latency to first limb/footpad withdrawal reaction within a 45-sec exposure time was recorded and is represented as median values in the bar/scatter plot. (C) The animal was habituated to the procedure by introducing its tail (three times, 10 min apart) into a $20^\circ\text{C} \pm 1^\circ\text{C}$ water bath, until tail withdrawal or up to 20 sec. Twenty minutes later, the tail was introduced into a $51^\circ\text{C} \pm 1^\circ\text{C}$ water bath. Tail withdrawal latency from the hot water bath is represented in the scatter plot. No significant differences were observed between males and females in either B or C (unpaired Mann–Whitney test). (D, inset) LTM of IA in 4-mo-old male and female Wistar rats: Te₁ and Te₂ latencies were significantly higher than Tr latencies for both males and females. $N_F = N_M = 9$; Friedman paired test followed by Dunn's post-hoc test; (*) $P < 0.05$, (**) $P < 0.01$. Animals were subjected to the same pain sensitivity tests as in B and C (data not shown).

persistence was evaluated in a second test (Te₂) performed 14 d later. Te₂ latencies were significantly higher than Tr latencies for middle-aged females and for both sexes at 4 mo of age (Fig. 3A, D). To address possible differences in tail and footpad sensitivity between sexes, tail flick and hot plate assays were performed. Females and males revealed similar latencies for tail withdrawal from a hot water bath and similar latencies for first limb/footpad withdrawal reaction from a hot plate, denoting similar tail and footpad sensitivity to a painful stimulus (Fig. 3B,C). Hence, there was a tendency toward an amnesic effect on IA memory persistence

in middle-aged males likely due to deficits in memory processing (Fig. 3A).

Cognition can be modulated by ovarian hormones like estradiol and progesterone. It was reported that ovarian hormones induce facilitation of some cognitive functions in 2- to 4-mo-old (Frye et al. 2007), 3- to 5-mo-old (Paris and Frye 2008), and 6-mo-old (Pawluski and Galea 2006) Long-Evans rats, and increased dendritic spine density in 3- to 3.5-mo-old Long-Evans rats (Kinsley and Lambert 2006) as well as 2-mo-old (McLaughlin et al. 2008) and 4-mo-old (Woolley et al. 1990) SD rats. NOR and NOL performance typically improves when young ovariectomized SD rats received estradiol (see Cost et al. 2012). In Fischer 344 ovariectomized rats, administration of estradiol from adulthood to middle age led to enhanced water radial-arm-maze performance only when working memory was taxed (Koebele et al. 2020).

Ovarian hormones are markedly influenced by aging in rats, as estrous cycle frequency decreases and variability increases (Lu et al. 1979; Wise and Ratner 1980). In gonad-intact female rodents, Tuscher et al. (2015) reported that object memory appears to be better when estradiol and progesterone levels are elevated during estrous cycle and pregnancy, while cognitive decline onset has been linked to a decrease in steroid levels.

During perimenopause, progesterone levels slightly diminish while estradiol remains relatively unchanged compared with young animals (Wise and Ratner 1980; Wise 1982; Gore et al. 2000; Bimonte-Nelson et al. 2003). Thus, it is feasible to hypothesize that ovarian hormones could be protective against the loss of dendritic spine density and memory decline at middle age. However, which hormone or derived metabolite is mainly involved remains controversial. Paris et al. (2011) reported that reproductively maintained 12-mo-old Long-Evans females outperformed those in reproductive decline on amygdala-mediated tasks involving aversive stimuli, like inhibitory avoidance. However, neither estradiol nor corticosterone supplementation significantly improved their performance, though high levels of progesterone metabolite $3\alpha,5\alpha$ -tetra-hydroxyprogesterone were associated with object memory improvement (Paris et al. 2011). Interestingly, a higher expression of allopregnanolone synthesis enzymes in middle-aged female Wistar rats appeared concomitant with a better performance in object recognition but an impairment in episodic-like memory (Rossetti et al. 2018).

Concerning anxiety-like behavior, fewer entries were reported into the center zone of an OF by 12-mo-old ovariectomized female Wistar rats, while estradiol supplementation returned them to control (nonovariectomized) levels (Rencz s et al. 2020).

Rat strain, age, reproductive stage, and memory type often have been taken into account when assessing cognitive functions, but sex scarcely has been compared; a few longitudinal studies have confirmed some age- and sex-related differences in rats (Markowska 1999; Rossetti et al. 2018; Febo et al. 2020; McQuail et al. 2021).

Our results show that spontaneous exploration of the environment is rather similar for middle-aged female and male Wistar rats, except for a higher vertical activity/exploration by females. Moreover, anxiety-like behavior levels, short-term and long-term habituation to the environment, and STM and LTM for objects (mainly depending on the cerebral cortex) were similar.

However, middle-aged males showed clear cognitive impairments (1) for NOL, lacking spatial reference LTM (involving the hippocampus) compared with females and with 4-mo-old males (since acquisition and STM of objects are preserved, NOL amnesia suggests serious deficits in spatial reference memory consolidation/activation), and (2) for IA (involving the hippocampus and the amygdala), tending to be amnesic for memory persistence compared with females.

Hypothetically, the relatively stable level of estradiol in 12-mo-old middle-aged female rats might protect them from aging-related hippocampal spine density loss and from memory impairment observed in males. Also, changes in gene expression and vascularization might be compensatory responses to the onset of neuronal degeneration (Jacobson et al. 2008; Shavlakadze et al. 2019).

Our results clearly point out that male and female rats should be compared when assessing spatial memory and associative LTM involving spatial reference and/or aversive stimulus, particularly from middle age on. This must be emphasized in learning and memory studies with wild-type animals and when using them as control to assess cognitive functions in transgenic, surgically, or pharmacologically treated rats.

Acknowledgements

The D.A.J. laboratory acknowledges financial support from Agencia I+D+i (National Agency for the Promotion of Science and Technology, Argentina; PICT no. 2016-2513), UBA (University of Buenos Aires, Argentina; UBACyT no. 20020170100745BA), and CONICET (National Scientific and Technical Research Council, Argentina; PIP2015-2017 no. 11220150100136CO). We acknowledge the technicians' excellent work in taking special care of the rats at the Instituto de Biología Celular y Neurociencia animal house.

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Received March 3, 2022; accepted in revised form March 23, 2022.



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Learn. Mem. 2022, **29**:

Access the most recent version at doi:[10.1101/lm.053578.122](https://doi.org/10.1101/lm.053578.122)

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