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RESEARCH ARTICLE

Differential effects of aging on hippocampal ultrastructure in male vs. female rats

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Abstract Age-related decline in physical and cognitive functions are facts of life that do not affect everyone to the same extent. We had reported earlier that such cognitive decline is both sex- and context-dependent. Moreover, age-associated ultrastructural changes were observed in the hippocampus of male rats. In this study, we sought to determine potential differences in ultrastructural changes between male and female rats at various stages of life. We performed quantitative electron microscopic evaluation of hippocampal CA1 region, an area intimately involved in cognitive behavior, in both male and female adolescent, adult and old Wistar rats. Specifically, we measured the number of docking synaptic vesicles in axo-dendritic synapses, the length of active zone as well as the total number of synaptic vesicles. Distinct age- and sex-dependent effects were observed in several parameters. Thus, adult female rats had the lowest synaptic active zone compared to both adolescent and old female rats. Moreover, the same parameter was significantly lower in adult and old female rats compared to their male counterparts. On the other hand, old male rats had significantly lower number of total synaptic vesicles compared to both adolescent and adult male rats as well as compared to their female counterparts. Taken together, it may be suggested that age- and sex-dependent ultrastructural changes in the hippocampus may underlie at least some of the differences in cognitive functions among these groups.

Keywords Aging · Ultrastructure · Hippocampus · CA1 region · Sex differences · Rat

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Introduction

Aging is a universal phenomenon characterized by progressive changes in all aspects of organism including decreases in general functionality of cells and increased risk of developing diseases including cognitive impairment and Alzheimer's disease (AD) (da Costa et al. 2016; Fulop et al., 2016; Alexander et al. 2020; Azam et al. 2021; Saul and Kosinsky 2021).

Understanding the changes at cellular level may help advance strategies to preserve and improve functional abilities in elderly population (Dumas 2017; Blanchet et al. 2018; Kafri et al. 2019).

Cognition is especially affected with aging as age-related mild cognitive decline may be manifested long before severe cognitive deteriorations that are observed in AD or other neurodegenerative diseases (Li et al. 2020; Wang et al. 2020; Lomidze et al. 2021; Pini and Wennberg 2021; Rizzolo et al. 2021; Fu et al. 2022). Cognitive declines are invariably associated with structural and functional alterations in brain regions and circuits regulating cognitive behavior (Reas et al. 2018; Liang et al., 2020; Lomidze et al. 2021). However, considerable sex-dependent variability may be observed in cognitive decline (Flatt and Partridge 2018; Lovden et al., 2020; Wilson et al. 2020). Thus, women and men differ in disease occurrence and progression. Hence, women have a greater prevalence of AD than men, and that this discrepancy can be at least partly due to differences in biological factors such as sex hormones (Carter et al. 2012; Rocca et al. 2014; Lin et al., 2015; Levine et al. 2021). Moreover, women might have greater cognitive reserve but faster cognitive decline than men, which could contribute to sex differences in late-life dementia (Levine et al. 2021). Preclinical studies have also verified sex-dependent differences in neurophysiology and behavior of various species. In this context, same sensory stimulus, may result in sex-specific neural activity underscored by differential neuroanatomical and molecular bases (van Eijk et al. 2020; Gegenhuber and Tollkuhn 2020; Hansell et al., 2022). In addition, estrous cycle may influence the response in females (Rock et al., 2022; Rock and Kundakovic, 2023).

Regarding aging, however, the influence of sex on cognitive decline and/or neurobiological substrates including ultrastructural changes in areas controlling cognition are far from clear (McCarrey et al. 2016, Zhvania et al. 2021). It is well-established that the hippocampus, one of key brain regions controlling cognition, is influenced by sex. Hence, sex-dependent differences in the size of hippocampal subregions, dendritic organization, microglial reaction or neurogenesis have been reported (Koss and Frick 2017; Nelson et al. 2017; Sharfman and MacLusky, 2017; van Eijk, 2020; Yagi anf Galea, 2019; Zhang et al. 2023). However, so far, there has been no report on the influence of sex on fine/ultrastructural anatomy of the hippocampus during aging.

Our previous findings indicated age-related alterations in hippocampal ultrastructure of male Wistar rats (Lomidze et al. 2021). We reported that in comparison with adolescent, old male rats had significantly lower number of synaptic vesicles and synaptic mitochondria (Lomidze et al. 2021). Moreover, we showed that age-related cognitive decline in rats is sex- and context-dependent (Zhvania et al. 2021). In this study, applying electron microscopic (EM) assessments, we sought to determine potential sex-dependent effects on synaptic morphology of the hippocampus in adolescent, adult, and old rats. Thus, by using both male and female rats, we intended not only to verify our previous findings with male rats but also to be able to make a direct comparison between the two sexes. Specifically, we focused on synaptic active zone (AZ), synaptic vesicles (SVs), and presynaptic and postsynaptic mitochondria in hippocampal CA1 region, an area intimately involved in cognitive regulation.

Materials and methods

Animals

The study included adolescent (P30-36), adult (P125-130) and older (P330-340) male and female Wistar rats. Each sex- and age- group consisted of six animals (total 36 animals). The animals were housed three per cage, in open, wire-top polypropylene cages (30 cm width × 30 cm length × 25 cm height). The room was maintained under 12 h light/dark cycle (light on 7 a.m.), temperature (20–22 °C) and humidity (55–60%). Food and water were supplied ad libitum.

Estrous cyclicity is an important aspect that must be taken into consideration when studying female animals as various stages of estrous may have differential effects on brain functions including hippocampal physiology (Rock et al., 2022; Rock and Kundakovic, 2023). While the middle-aged animals are usually in a transition phase where erratic estrous cyclicity and lengthening of cycle may be manifested, old animals have a consistent estrous cycle. Thus, adult, and adolescent females were studied during diestrus stage, a short period of sexual latency between two periods of

estrous - in rats the length of this stage is 48–72 h. The diestrus stage was determined by the recommended visual assessment, which is non-invasive, fast, reliable, and less stressful compared to the vaginal smear in rats (Ajavi and Akhidbe, 2020).

The maintenance of animals and electron-microscopic procedures were conducted in accordance with European Union Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU) and were approved by The Committee of Animal Care at Ivane Beritashvili Center of Experimental Biomedicine.

Tissue preparation

The material for EM study was prepared according to conventional techniques used by our group (Lobzhanidze et al. 2019, 2020; Lomidze et al. 2020; Zhvania et al. 2021). Specifically, after an overdose of sodium pentobarbital (200 mg/ml dissolved in 10% ethanol), animals underwent transcardiac perfusion using ice cold heparinized 0.9% NaCl, followed by 500 ml of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at a perfusion pressure of 120 mm Hg. The brains were removed from skull and postfixed overnight in cold (4 °C) perfusion solution. The regions of the hippocampi between − 2.28 and − 3.48 mm from bregma were identified according to the atlas of Paxinos and Watson (Paxinos and Watson 2006). The tissue was osmicated (1% OsO4 in PB for 30 min), dehydrated in graded series of ethanol and acetone, then in a mixture of acetone and Durcupan resin and flat-embedded in Araldit 502 (Electron Microscopy Sciences, PA, USA). After polymerization, semi-thin Sect. (400 nm) were prepared with an ulltratome Leica EM UC7, stained with toluidine blue and examined under optical microscope Leica MM AF. This was done to facilitate selection of the same subarea of C1 in all animals. Serial ultra-thin Sect. (40-45 nm) were then prepared with an ulltratome Leica EM UC7. In order to avoid duplicate examination of the same presynaptic terminal, we selected every fifth section in each age- and sex group, yielding 7 similar hippocampal samples in each group. The sections were placed on 200-mesh copper grid (Electron Microscopy Sciences, PA, USA), double-stained with uranyl-acetate and lead citrate and examined with JEM 1400 electron microscope (JEOL, Japan).

Quantitative EM analysis

In this study, morphometrical analysis of asymmetric axo-spine synapses and synapses on thin dendrites of hippocampal CA1 area were carried out. In the hippocampus such kinds of synapses are mainly considered as the glutamatergic, excitatory types. Synapses were identified based on their pre-synaptic and post-synaptic densities, the presence of round vesicles, and the synaptic cleft between the pre-and post-synaptic membranes in 20,000-times magnified electron micrographs. In presynaptic terminals, the length of AZ, total number of SVs and the number of SVs in different synaptic pools were measured as detailed in our earlier studies (Lobzhanidze et al. 2019, 2020; Lomidze et al. 2020). AZ was identified by rigid opposing of presynaptic and postsynaptic membrane, clustering of SVs at the presynaptic membrane and asymmetric postsynaptic density (Sudhof, 2012). The total number of SVs in presynaptic compartment and number of docking SVs (in the readily releasable pool located at a distance of up to 50 nm from AZ) were quantified (Fig. 1). In total, 90 micrographs per group (15 from each animal) were analyzed. The measurements were carried out in blind manner, using the Image J software (Version 1.44, The National Institute of Mental Health). Quantification result of each parameter consisted of pooled samples from 6 animals. It is important to note that we have consistently applied these methodologies and here also adhered to them strictly so that inter-study comparison of results, especially concerning behavioral and ultrastructural data would be easily tenable.

Statistical analysis

Statistical Computation VassarStats (http://vassarstats.net) was used for quantitative data analysis. To determine the effects of "sex" (two levels - female and male) and "age" (three levels - adolescent, adult and old) on morphometric parameters, two-way ANOVA followed by Tukey's multiple comparisons post hoc test was used. The P-value less than 0.05 was considered as statistically significant. The data is presented as mean ± standard error of the mean (SEM).

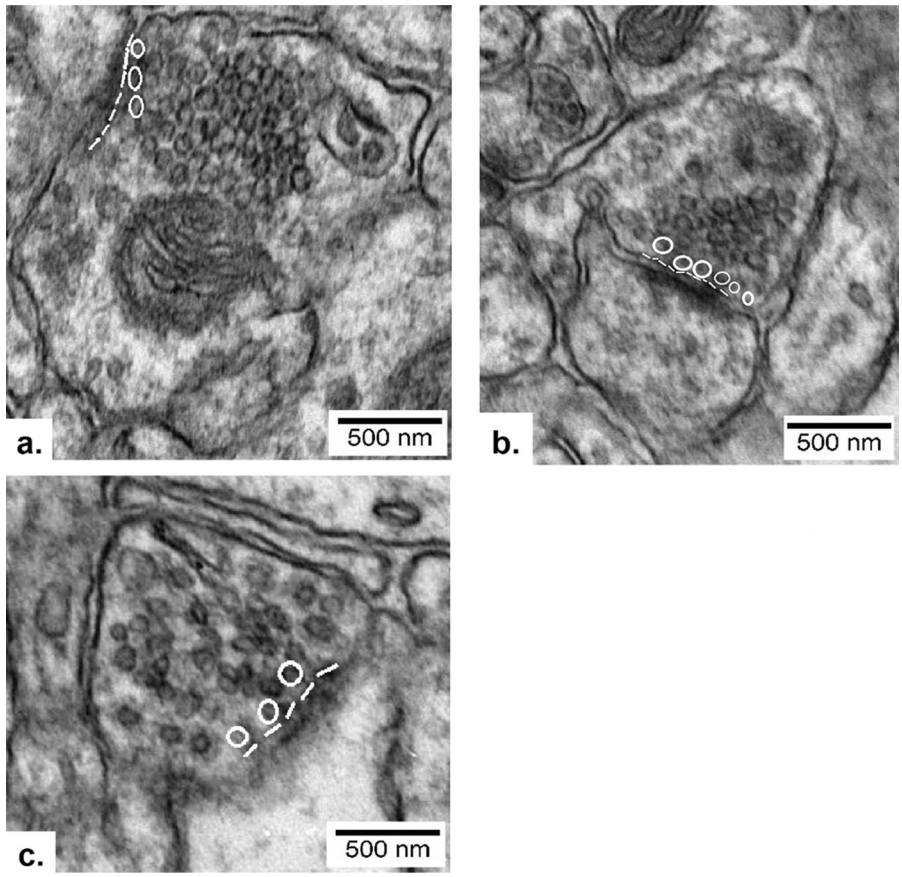

Fig. 1 Representative electron micrographs of synapses on thin dendrites in the CA1 region of the rat hippocampus. Total number of synaptic vesicles, docking vesicles (open circles) in the readily released pool and the length of the active zone of synapse (dashed line) are clearly identifiable. **a** Adolescent male rat; **b** Adult male rat; **c** Old male rat. Scale bar = 500 nm

Results

Length of active zone of synapses (nm)

Significant main effects of sex ($F_{1448} = 21$, $p < 0.001$), age ($F_{2448} = 8.19$, $p = 0.003$) as well as interaction of "sex×age" ($F_{2448} = 8.09$, $p = 0.004$) on the length of active zone were observed (Fig. 2). Thus, in female rats, adults had the lowest measure of synaptic active zone (163.8 ± 7.9 nm) compared to both adolescent (249.2 ± 9.4, $p < 0.01$) and old female (199.0 ± 14.1, $p < 0.01$) rats. Old female rats also had a lower measure compared to adolescents (249.2 ± 9, $p < 0.01$). Although no significant differences between various male groups were noted, both adult (241.1 ± 8.1) and old (258.6 ± 10.95) male rats had significantly higher levels compared to their female counterparts ($p < 0.01$) (Fig. 2b**).**

Total number of synaptic vesicles in the presynaptic terminals

Significant main effect of sex ($F_{1395} = 4.08$, $p = 0.044$), and interaction of "sex×age" ($F_{2395} = 6.6$, $p = 0.0015$) on the total number of vesicles in presynaptic terminals were observed. Thus, although female rats did not exhibit any age-dependent changes, old male rats had significantly lower number of synaptic vesicles (70.7 ± 3.3) compared to both adolescent (92.8 ± 5.5, $p < 0.01$) and adult rats (101.9 ± 5.3, $p < 0.01$). Moreover, adolescent and adult female rats had significantly lower number of synaptic vesicles compared to their male counterparts ($p < 0.05$ and $p < 0.01$, respectively). On the other hand, old female rats had significantly higher levels of synaptic vesicles compared to their male counterpart ($p < 0.01$) (Fig. 2c**).**

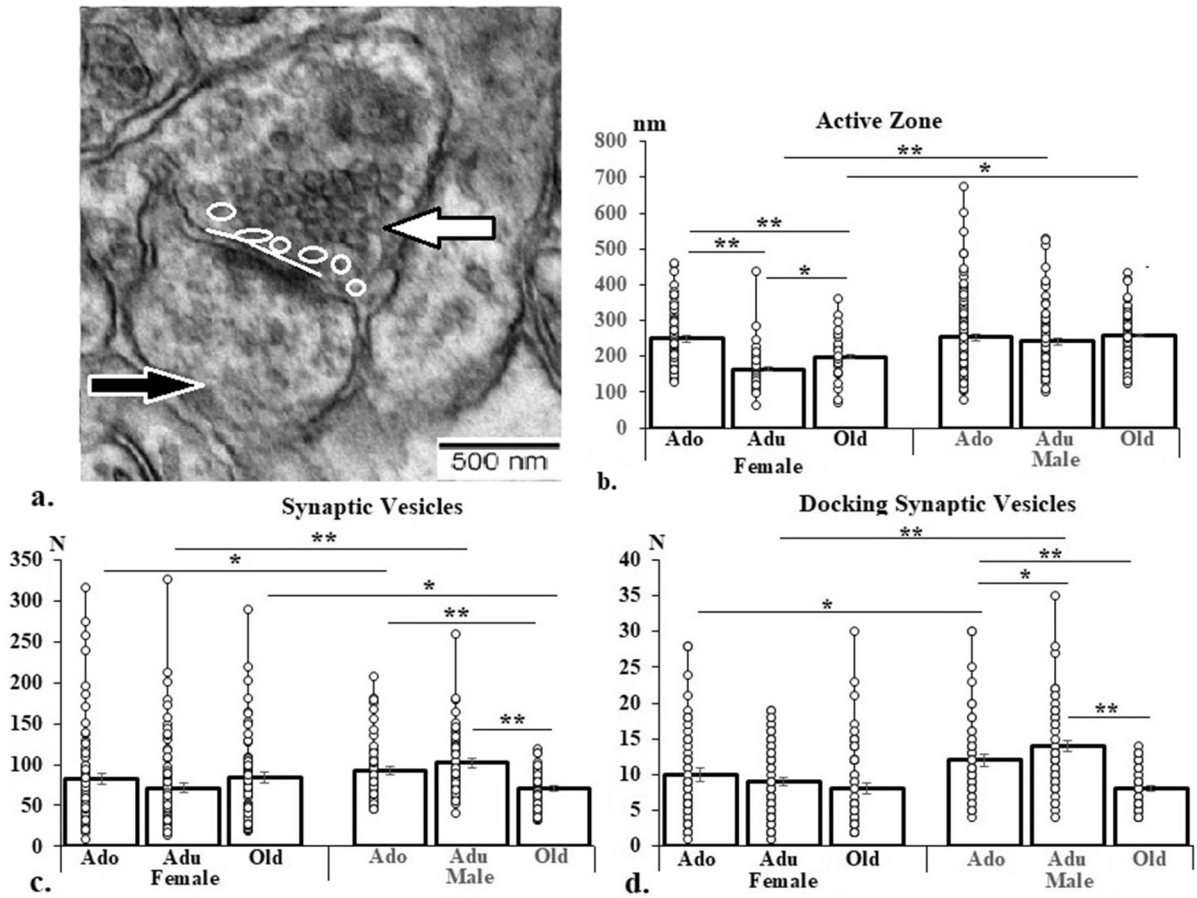

Fig. 2 Morphometric EM analysis of synapses in hippocampal CA1 region in adolescent, adult and old female and male rats. **a** representative electron micrograph of a synapse on thin dendrite in the CA1 region of the adult male rat hippocampus. Pre- and post-synaptic compartments (white and black arrows correspondingly), total number of synaptic vesicles, docking vesicles (open circles) in the readily released pool and the length of the active zone of synapse (white solid line) are clearly identifiable. Scale bar = 500 nm. **b** The length (nm) of active zone, **c** Total number (N) of synaptic vesicles, **d** Number (N) of docking vesicles. *p < 0.05, **p < 0.01. N = 6/group

Number of docking synaptic vesicles in the readily releasable pool (RRP)

Significant main effect of sex (F_{1395} = 11.79, p = 0.007), age (F_{2395} = 5.15, p = 0.006) and their interaction (F_{2395} = 5.76, p = 0.003) in the number of synaptic vesicles in readily releasable pool were observed (Fig. 2b). Thus, although female rats showed no significant differences between various groups, both adolescent (11.75 ± 0.8) and adult male animals (13.8 ± 0.8) had significantly higher levels of synaptic vesicles in RRP compared to the old group (8.06 ± 0.4) (p < 0.01). Moreover, both adolescent and adult female rats had lower synaptic vesicles in this pool compared to their male counterparts (p < 0.01) (Fig. 2d).

The area of presynaptic mitochondria (μm^2)

As depicted in Fig. 3b, the presynaptic mitochondrial area was affected by both sex (F_{1515} = 13.19, p = 0.003) and age (F_{2515} = 10.5, p < 0.001). Thus, in female group, the old rats (0.075 ± 0.008) had significantly higher levels compared to adults only (0.059 ± 0.003 μm^2, p < 0.01). In male group, however, the old rats (0.072 ± 0.006) had significantly higher levels compared to both adolescent (0.053 ± 0.003, p < 0.01) and adult rats (0.047 ± 0.003,

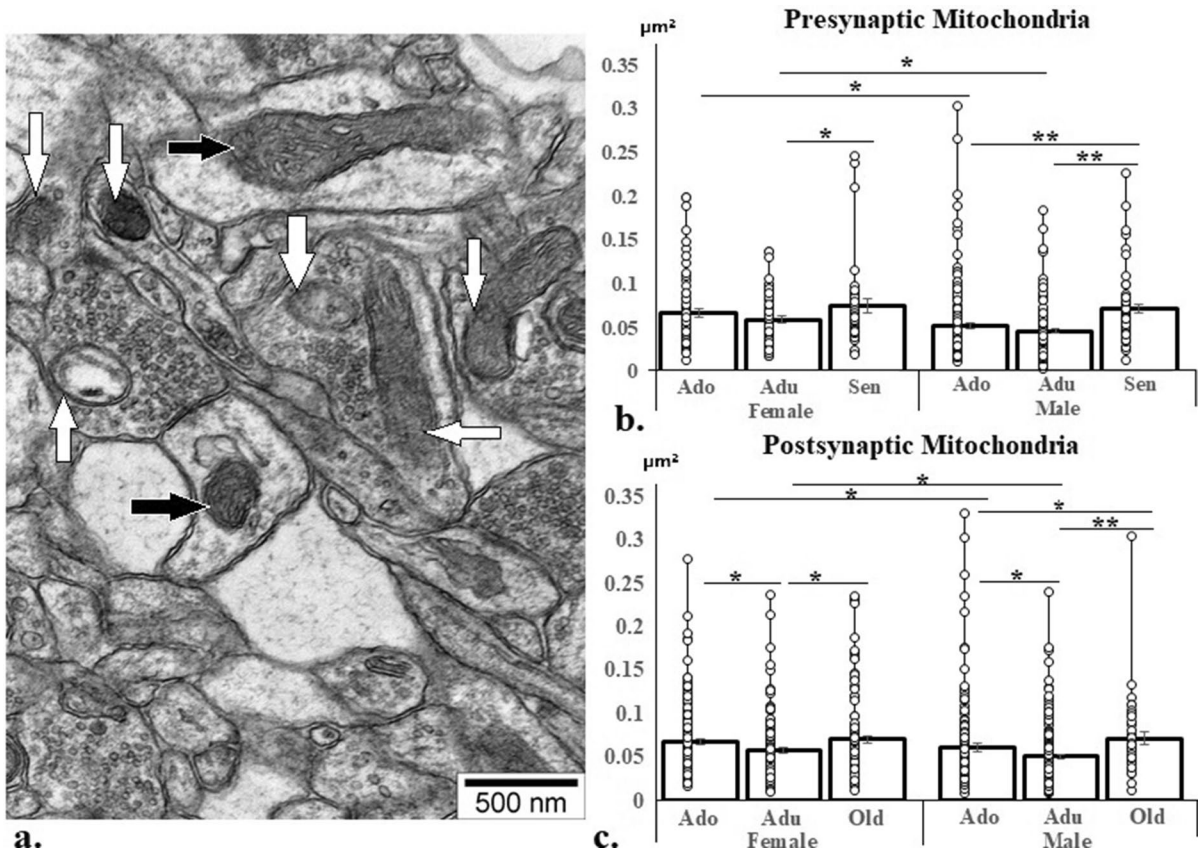

Fig. 3 Morphometric EM analysis of presynaptic and postsynaptic mitochondrial area in hippocampal CA1 region in adolescent, adult and old female and male rats. **a** neuropil of hippocampal CA1 region of adult male rat, white arrow—presynaptic mitochondria, black arrow—postsynaptic mitochondria; **b** Area of presynaptic mitochondria (μm^2). **c** Area of postsynaptic mitochondria (μm2), *p < 0.05, **p < 0.01, N = 6/group

$p < 0.01$). No significant difference between adolescent and adult rats was detected in either sex group. However, adolescent and adult female rats had significantly higher presynaptic mitochondrial area compared to their male counterparts ($p < 0.01$) (Fig. 3b**).**

The area of postsynaptic mitochondria (μm^2)

As depicted in Fig. 3b, the postsynaptic mitochondrial area was also affected by both sex ($F_{1852} = 7.49$, $p = 0.006$) and age ($F_{2852} = 9.75$, $p = 0.001$). Thus, in female group, the adult rats (0.058 ± 0.003) had significantly lower level compared to both adolescent (0.068 ± 0.003, $p < 0.05$) and old rats (0.071 ± 0.003, $p < 0.05$). In male group, however, not only the adult rats had lower levels (0.051 ± 0.002) compared to both adolescent (0.062 ± 0.005, $p < 0.05$) and old (0.072 ± 0.008, $p < 0.01$) rats, but the old rats had also significantly higher levels than adolescent rats ($p < 0.05$). Here also, like presynaptic area (Fig. 3b), both adolescent and adult female rats had significantly higher postsynaptic mitochondrial area compared to their male counterparts ($p < 0.01$) (Fig. 3c).

Discussion

The results of this study indicate that aging-associated changes at ultrastructural level of synapses may be influenced by sex differences. It should be noted that although there is a distinction between the term "sex" and "gender", they are often used interchangeably. Sex refers to the biological differences between males and females, such as the reproductive organs

and genetic differences, whereas gender refers to the socially constructed identities and behavior of men, women, or gender-diverse people.

Previously, using a multi-branched maze (MBM) test and the Morris water maze (MWM) test, we had shown sex- and context dependent alterations in cognition during aging (Zhvania et al. 2021). MWM, designed to evaluate spatial memory by using spatial clues to locate the escape platform, and MBM, designed to evaluate short- and long- term memory by measuring escape latency, distance travelled, velocity and some other parameters to find the optimal trajectory, were differentially affected in female vs. male rats (Zhvania et al. 2021). Here also we report that hippocampal CA1 region, an area intimately involved in regulation of cognitive functions (Sakimoto et al. 2021) is differentially affected in different sexes. It may be suggested therefore, that the differences in cognitive behaviors at various stages of life between male and females may at least partially be due to the fine ultrastructural differences in key brain areas. Interestingly, sex-dependent differences were observed during adolescence, adulthood, and old age, confirming distinct sex-dependent effects at various stages of life (Zhvania et al. 2021).

Specifically, our findings indicate that the number of synaptic vesicles, including both presynaptic and postsynaptic, as well as mitochondrial morphology, may be affected by gender at all stages of life. Thus, adult female rats had the lowest synaptic active zone compared to both adolescent and old female rats. Moreover, the same parameter was significantly lower in adult and old female rats compared to their male counterparts. On the other hand, old male rats had significantly lower number of total synaptic vesicles compared to both adolescent and adult male rats as well as compared to their female counterparts. Since synaptic morphology is associated with synaptic strength and/or plasticity, our observation of age-related morphological decline mainly in male rats may suggest a central basis for age-related cognitive decline in this gender.

Various regions of hippocampus control various aspects of cognitive (memory) function. While CA3 area is important for encoding, storage and retrieval of memory; and dentate gyrus serves for pattern separation of the incoming inputs from entorhinal cortex (Balleza-Tapia et al. 2022; Grande et al. 2019; Senzai 2019), CA1 region is critical for acquisition of spatial and nonspatial memory (Asgeirsdottis et al., 2020; Cinalli et al. 2023; Geiller et al. 2023; Sakimoto et al. 2021). Indeed, CA1 "place cells" provide precise representations of specific locations in an environment and code for associations between objects and locations (Stevenson et al. 2018). Therefore, in MWM and MBM tests, CA1 area is actively involved. Moreover, in several neurological and neuropsychiatric disorders (cognitive declines, schizophrenia, major depressive and bipolar disorders) or hypoxic and ischemic damages, the CA1 region seems to be most vulnerable compared to other hippocampal areas (Han et al. 2019; Ota et al., 2016). It is also noteworthy that CA1 area manifests sex-dependent differences (van Eijk et al. 2020). This is likely due to expression of estrogen and progesterone (ovarian hormones) receptors in this area (McEwen et al. 2001; Tozzi et al.,2019; Wang et al. 2021). Additionally, because of commonalities in CA1 region of all mammals, these findings could have translational implications in humans as well. However, this aspect and potential role of other hippocampal regions have to be further explored.

Our data is also consistent with reports of sex-dependent differences in molecular architecture of brain regions involved in cognitive functions (Bundy et a., 2017; Ocañas et al. 2022). Sex-dependent differences in age-related neurodegenerative diseases and associated cognitive declines due to alterations in hippocampal function are amply documented (Gurvich et al. 2020; Kilpi et al. 2020; Guo et al.,2022). Although our quantitative measure of synaptic vesicles in presynaptic terminals cannot be considered a direct measure of neurotransmitter levels, these vesicles represent the storage of neurotransmitters and a decrease in their number may be considered an indirect indication of a decrease in neurotransmitter release (Kim et al. 2017, 2019; Kusick et al. 2020; Tran et al.,2022). It is noteworthy that after fusing with the plasma membrane and expelling the neurotransmitter, new vesicles supplied by the recycling and reserve pools replace the consumed vesicles. Thus, the observed decrease in the number of vesicles in old male rats might be indicative of a decrease in functional capacity of the CA1 region in this particular group.

In contrast to male rats, both adult and old female rats showed significant decrease in the length of the active zone of synapses, the main function of which is to convert presynaptic action potential into

neurotransmitter signal (Watanabe et al. 2020). In addition, this specialized area participates in short- and long-term plasticity (Südhof 2012; Monday et al. 2018; Sigrist et al.,2018). Hence, a reduction in the length of the active zone in adult and old female rats may reflect decreased activities in specific hippocampal circuitries. However, whether estrogen fluctuations, particularly during menopause may contribute to these outcomes and whether these changes have a bearing on cognitive performance has yet to be determined.

Finally, a very similar pattern of changes was noted in pre- and post-synaptic mitochondria in both female and male rats across all ages. Presynaptic mitochondria provide the energy needed for neurotransmission and play an essential role in synaptic plasticity (Subramanian and Jonas 2021; Li and Sheng 2022). Postsynaptic mitochondria, on the other hand, generate the energy used to maintain downstream events associated with neuronal function (Mendelsohn et al. 2022). The intensity and the size of both mitochondrion types are directly related to their activity and the metabolic status of the neurons (Freeman et al. 2017; Thomas et al. 2019). The increase in volume of pre- and post-synaptic mitochondria is usually associated with higher firing rates (Justs et al. 2022; Li and Sheng 2022). Therefore, it may be suggested that the significant increase in synaptic mitochondria in old animals of both sexes may reflect a compensatory mechanism towards maintaining the energy requirement of the cell. However, further verification of this hypothesis is necessary.

In summary, our EM morphometric analyses of the hippocampal CA1 region in adolescent, adult and old male and female rats reveal sex-dependent ultrastructural changes at both pre-and post-synaptic levels. Although further verifications are required, such modifications may underlie differential cognitive changes between the two sexes at various stages of life including aging.

Author contributions Data collection were performed by NP and NL electron microscopic imaging was performed by FR and EG statistical analysis of data was performed by NJ and YT The first draft of the manuscript was written by MZ, YT prepared the final version of manuscript. All authors reviewed the manuscript.

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Data availability The datasets generated and analyzed during current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval This study was performed in line with the Declaration and recommendations of the National Institute of Health (NIH) Guide for the Care and the Use of Laboratory Animals and was approved by the Research Ethics Committee of Ivane Beritashvili Center of Experimental Biomedicine.

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