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ORIGINAL ARTICLE

Aging-associated changes in motor function are ovarian somatic tissue-dependent, but germ cell and estradiol independent in post-reproductive female mice exposed to young ovarian tissue

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Abstract A critical mediator of evolution is natural selection, which operates by the divergent reproductive success of individuals and results in conformity of an organism with its environment. Reproductive function has evolved to support germline transmission. In mammalian ovaries, this requires healthy, active gonad function, and follicle development. However, healthy follicles do not contribute to germline transmission in a dead animal. Therefore, support of the health and survival of the organism, in addition to fertility, must be considered as an integral part of reproductive function. Reproductive and chronological aging both impose a burden on health and increase disease rates. Tremors are a common movement disorder and are often correlated with increasing age. Muscle quality is diminished with age and these declines are gender-specific and are influenced by menopause. In the current experiments, we evaluated aging-associated and reproduction-influenced changes in motor function, utilizing changes in tremor amplitude and grip strength. Tremor amplitude was increased with aging in normal female mice. This increase in tremor amplitude was prevented in aged female mice that received ovarian tissue transplants, both in mice that received germ cell-containing or germ cell-depleted ovarian tissue. Grip strength was decreased with aging in normal female mice. This decrease in grip strength was prevented in aged female mice that received either germ cell-containing or germ cell-depleted tissue transplants. As expected, estradiol levels decreased with aging in normal female mice. Estradiol levels did not change with exposure to young ovarian tissues/cells. Surprisingly, estradiol levels were not increased

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in aged females that received ovaries from actively cycling, young donors. Overall, tremor amplitude and grip strength were negatively influenced by aging and positively influenced by exposure to young ovarian tissues/cells in aged female mice, and this positive influence was independent of ovarian germ cells and estradiol levels. These findings provide a strong incentive for further investigation of the influence of ovarian somatic tissue on health. In addition, changes in tremor amplitude may serve as an additional marker of biological age.

Keywords Menopause · Tremor · Grip strength · Hormones · Longevity

Introduction

The chronological lifespan in humans has been extended dramatically over the last century. However, the timing of menopause or the end of the reproductive life span in women has remained relatively constant over this same period. Reproduction is often thought to negatively influence health and survival. High fecundity has historically been associated with short lifespans among mammals [47]. Opposition to this concept comes from the manipulation of reproduction to increase or decrease exposure to reproductive function and the observations of the influence of these manipulations on health and longevity [29]. Results from these types of experiments suggest that there are still large gaps in our knowledge about the effects of reproduction on health and survival. Reproductive function can significantly influence non-reproductive biological functions and is exquisitely sensitive to aging-related changes.

Reproductive function has evolved to support germline transmission. In mammalian ovaries, this requires healthy, active gonad function, and follicle development. However, healthy follicles do not contribute to germline transmission in a dead animal. Therefore, support of the health and survival of the organism, in addition to fertility, must be considered as an integral part of reproductive function. The survival influence of reproductive effort can be separated from the fertility effort, in the case of the current experiments by removing the influence of the germ cells or follicles, which normally direct the ovary toward the support of germline transmission or production of offspring. Without the follicles present to direct the ovarian somatic tissues toward the production of offspring, the ovarian somatic tissues take on the role of maintaining survival of the organism. This support for survival will continue as long as the organism has the potential to reproduce and/or is able to contribute to the support of existing offspring (the grandmother hypothesis).

Evidence over the past decade indicates that a decline in an individual's reproductive status is commonly associated with an increased risk of developing chronic health conditions (NIH-NICHD Bulletin [34]). This association is particularly striking in women. Prior to reproductive decline, females hold a significant health advantage over males of the same age [10]. However, at the time of reproductive decline, the increase in disease risks for women significantly outpaces those of men. This dependence on reproductive function for the maintenance of health is exemplified in surgically menopausal women and in women with premature ovarian failure, who suffer from a decline in health at a much younger age than in women with traditional menopausal timing [18, 40, 43]. Previous experiments by our group have demonstrated significant health and longevity benefits from manipulation of ovarian function ([14, 25–27, 30, 36–38, 44]) . In the current experiments, we evaluated aging-associated and reproduction-influenced changes in motor function, utilizing two measures of health span; tremor amplitude and grip strength.

Tremors are a common movement disorder, are associated with many disease states, and are commonly correlated with increasing age [7]. Measuring tremors in mice is non-invasive, repeatable, and as outlined in the current manuscript, provides accurate quantitative data [46]. Grip strength as a measure of muscle strength is a more commonly used, non-invasive, and convenient method to evaluate the effects of aging on motor performance and neuromuscular function in rodent models [4, 41]. The well-established supportive role of ovarian hormones in many aspects of female health implicates the loss of hormone production from actively cycling germ cells, as the principal cause of increased disease risks at menopause. While the value of ovarian hormones in female health is unquestionable, efforts to replace the hormonal milieu of actively cycling ovaries in peri- and post-menopausal women have struggled to reliably restore the health benefits enjoyed by young, reproductively

cycling women with young ovaries. Preliminary data from our laboratory suggest that estrogen levels are not changed in post-reproductive recipients of new ovaries, compared with age-matched controls, suggesting a non-estrogenic influence of young ovarian tissue. Here, we specifically examined the influence of young ovarian tissue on tremor amplitude and grip strength in the presence or absence of ovarian germ cell influence.

Materials and methods

Animals

The CBA/J and DBA mice are common laboratory strains used in aging studies because of the unique loss of ovarian follicles early in life [14, 26, 27]. The present study used the CBA/J mouse strain as an aging model for post-reproductive health. Female CBA/J mice were obtained from the National Institute of Health-National Institute of Aging (Bethesda, MD, USA) and Jackson Laboratory (Bar Harbor, ME, USA). Each mouse was individually housed in ventilated cages (Green Line IVC Sealsafe Plus, Techniplast, West Chester, PA, USA) containing corncob bedding (7097 Corncob, Harlan Teklad, Bartonville, IL, USA). Each home cage had deionized water, laboratory rodent diet ad libitum (2018 Teklad Global 18% Protein Rodent Diet, Harlan Teklad, Bartonville, IL, USA), and added enrichment (4 cm dia × 10 cm L paper tubes, Nestlets®, Kimwipes®). All females were exposed to male mice/bedding throughout the study (female mice were no more than two cages away from a cage containing a male mouse and/or a small amount of soiled bedding from a male's cage was mixed with fresh bedding at cage changes for female mice). The colony environment had fresh filtered air (15 changes/hour), regulated temperatures of 21 ± 2 °C, humidity of 50 ± 20%, and an even light-dark cycle (12:12 h) and was located in the Utah Science, Technology and Research Center (USTAR) at Utah State University. USTAR is an American Association for Accreditation of Laboratory Animal Care (AAALAC) approved facility in accordance with the National Institutes of Health and Animal Use guidelines. Animal care protocols were developed under the National Research Council guidelines found in the Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Utah State University Institutional Animal Care and Use Committee (IACUC-10222).

Surgical procedures

Surgical procedures included anesthetics for both donor and recipient mice and post-operative administration of analgesia with extended administration of analgesia if necessary. Euthanasia of donors and recipient mice occurred by cervical dislocation followed by thoracotomy with rapid exsanguination by cardiocentesis [26, 27]. Mice were monitored twice daily and more frequently if health concerns arose. Mice with acute weight loss were treated with moistened food and subcutaneous fluids. Individuals with acute urine staining or rectal/vaginal prolapse were manually cleaned and treated with Desitin®. Mice were monitored daily and any moribund, aged mice who exhibited overt clinical signs of catatonia were euthanized. Criteria for potential euthanasia was determined in coordination with the attending veterinarian and included, but was not limited to mice found in poor condition with or without crusting around the perineum, diarrhea, urine staining, persistent vaginal prolapse, chronic vulva/rectal swelling, respiratory distress, anorexia, kyphosis, poor coat, and unusual weight loss or gain. An increased rate of weight loss was the most critical factor for determining a moribund state in aged mice. Unexpected deaths were uncommon, but included euthanasia due to neoplastic growths, decubitus ulcers, or uncontrolled cataleptic seizures [25].

Experimental design

Animals were randomly assigned to control or experimental groups (Fig. 1). Control groups consisted of mice with their original ovaries intact (not sham-operated). In previous experiments, sham-operated mice were no different from unoperated mice in terms of lifespan, cardiomyopathy, or osteoarthritis ([25, 27, 30]). Experimental groups consisted of CBA/J females who received ovarian tissue/cell transplants. CBA/J mice at 13 months of age who were assessed as reproductively senescent (acyclic) via vaginal lavage, received germ cell-containing (GC) or germ cell-depleted (GD) donor ovaries or cells from

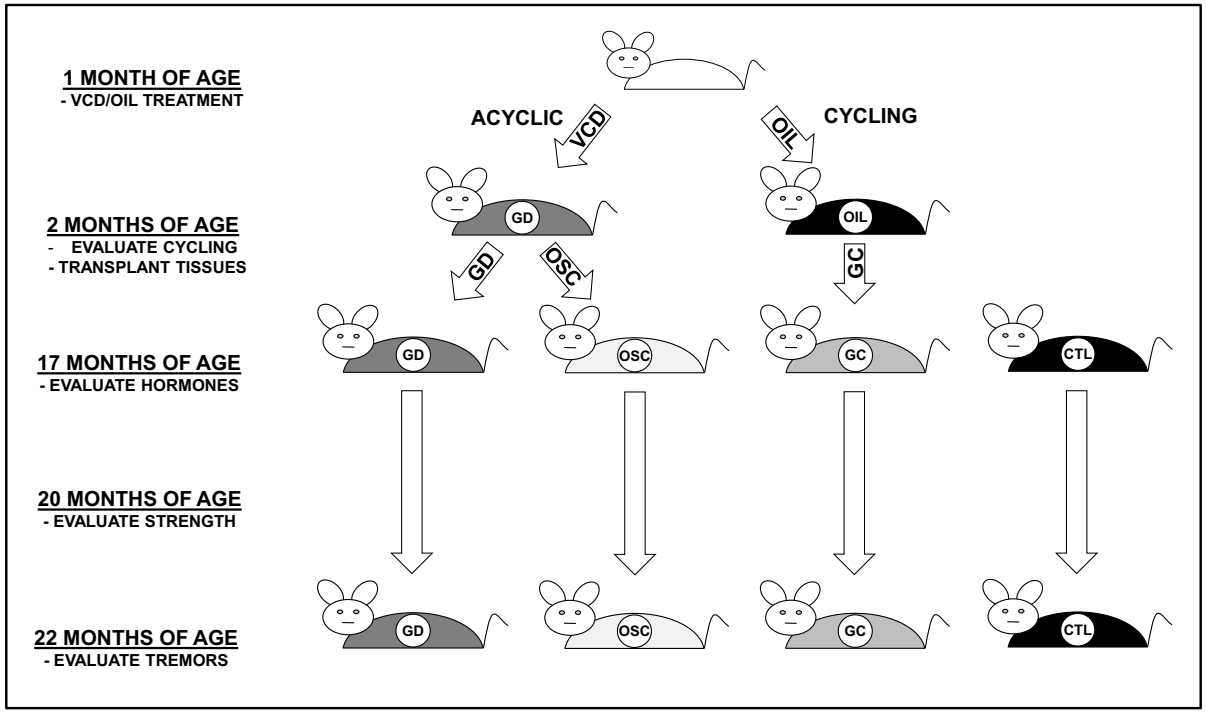


Fig. 1 Experimental design. Donor mice received treatment with 4-vinylcyclohexide diepoxide (VCD) or vehicle for 20 days. Ovarian tissues from 60-day donor mice were collected, processed and transplanted to 13-month recipient mice. Recipients and controls were analyzed for hormone levels (@17 months), grip strength (@20 months), and tremor amplitude (@22 months). Mice were collected at 27 months

2-month-old CBA/J mice (Mean lifespan for CBA/J female mice is ~ 21.2 months).

Ovarian transplantation

CBA/J mice at 13 months of age underwent a bilateral ovariectomy and bilateral ovarian transplantation with a pair of 2-month-old ovaries or ovarian cells from donor mice of the same strain. Bilateral ovarian transplantations were performed as previously described [25, 27, 28]. Briefly, recipient and donor mice were anesthetized using an intraperitoneal injection of an anesthetic cocktail (100 mg/kg ketamine, 20 mg/kg xylazine, and 10 mg/kg acepromazine). The ovaries of the donor mice were exposed starting with a paralumbar incision. Once through the skin and peritoneum, the ovarian fat pad, located distal to the kidney, was placed into the field of view and positioned with a clamp to expose the ovarian bursa containing the ovary. The ovary was then removed by incising the bursa opposite the ovarian hilum. The ovary was gently removed from the bursa and the hilum clamped to prevent bleeding. The excised ovaries were placed in cold saline or digested to a single-cell suspension prior to transfer.

The recipient mice underwent the same process to remove their endogenous, acyclic ovaries. After the endogenous ovaries were removed, young ovaries or isolated somatic cells were placed into the recipient bursa through the original incision. The bursa was closed with sutures using 9-0 Ethicon monofilament (Ethicon, Inc.) and placed back into the body cavity. The abdominal wall was sutured with 6-0 Vicryl (Ethicon, Inc.) and the skin was closed using 9 mm wound clips (MikRon Precision, Inc.). The procedure was repeated on both ovaries in each recipient mouse. Mice were given analgesia and electrolytes and dextrose with continuous post-operative monitoring for any concerns until each mouse was bright, alert, and responsive. Post-operative mortality was less than 5%.

Germ cell depletion

We used 4-vinylcyclohexene diepoxide (VCD) to deplete germ cell-containing follicles in the ovaries of young mice. Treatment with VCD selectively destroys primordial and primary follicles in ovaries of rats and mice (reviewed in [17]). In contrast to ovariectomy, VCD treatment induces gradual ovarian failure through depletion of the germ cell-containing follicles, while leaving the somatic ovary tissue intact. We currently produce germ cell-depleted CBA/J mice in our laboratory following the method of Lohff et al. [20] with minor modifications.

At 28 days of age, CBA/J female mice received daily intraperitoneal (I.P.) injections of 160 mg/kg VCD in sesame oil for 20 days. We treated mice used as GC ovary donors with sesame oil on the same schedule as the VCD-treated mice and stopped VCD and vehicle treatments at 48 days of age. At 60 days of age, ovaries were collected from VCD-treated and vehicle-treated mice for transplantation to 13-month-old, virgin CBA/J mice. Ovaries from 60-day VCD-treated mice were also used for the isolation of young ovarian somatic cells for transplantation. CBA/J mice analyzed at only 37 days after the initiation of VCD treatment already displayed cessation of reproductive cyclicity (persistent vaginal cornification), reduced ovarian weights (1.7 mg in oil-only vs. 0.9 mg in VCD-treated, $P = 0.030$), and depleted primordial ($P = 0.004$) and primary ($P = 0.029$) ovarian follicles compared with controls. Primordial and primary follicle depletion is normally complete by day 46 after the initiation of VCD treatment, as reported previously in other strains of mice [39]. VCD-treated donor mice were superovulated prior to ovary collection to decrease any remaining secondary or later stage follicles.

Somatic cell isolation

VCD-treated ovaries were used for the isolation of young ovarian somatic cells (OSC) for transplant. We used somatic cells collected from one young ovary to inject into each recipient's ovarian bursa. Somatic cells were isolated from young ovaries using mechanical and enzymatic tissue disruption/digestion. Ovaries were collected in PBS+ (with Ca^{+2} and Mg^{+2}) and placed in a depression slide that contained 50 µl of digestion media (Collagenase/Dispase/DNase) and minced with 19ga needles for 5 min. After 5 min, the digestion media/ovaries solution was put into a 1.5 ml Eppendorf tube. The slide was then washed with an additional 450 µl digestion buffer, which was added to the 1.5 ml tube and the solution was incubated at 37 °C for an additional 10 min with frequent pipetting (in a water bath). After a total of 15 min, 500 µl of serum was added to the 1.5 ml tube, mixed well, and the solution was centrifuged and washed 2X with PBS- (no Ca^{+2} or Mg^{+2}). The somatic cells were then isolated from the ovarian cell mixture using a 70 µm filter to remove cellular debris.

We initially lose slightly more of the somatic cells using this procedure compared with differential attachment culture (the ovarian cell mixture is allowed to attach to a culture plate overnight, washed, detached, and reattached, most of the attached cells are somatic in origin). However, attachment to a culture plate can significantly change the phenotype/behavior of the cells. Our procedure is very quick and subjects the cells to very few disruptive environments prior to transplantation. The isolated cells were resuspended in 0.1%F68 surfactant in saline and transplanted to the recipient's ovarian bursa in a 20 µl volume or cryopreserved for future use. Transplantation with other vehicles (i.e., Phytohemagglutinin, [8]) decreases the viability of re-aggregated OSCs (personal communication, Dr. Suzannah Williams, University of Oxford). Preliminary cell transplantations with F68/saline have resulted in the recovery of live cells seven days post-transplantation.

Tremor assay

In our hands, tremor amplitude has provided a reliable and robust measurement of physiological change with age. Tremors were measured using a tremor monitor system (San Diego Instruments, San Diego, CA, USA, [46]). The tremor monitor differentiates tremor events from ambulatory/stereotyped movements. The tremor monitor uses an ultra-sensitive movement sensor to record continuous movement waveforms. Mice (7moCTL-n = 19; 22moCTL-n = 5; 27moCTL-n = 3; 22moGC-n = 4; 22moGD-n = 8) were placed into the tremor monitor for 512 s (the selection of the 512-s recording period was software-dependent). The tremor monitor software allows various interval lengths, and filters to select frequency bandwidth from 1 to 64 Hertz (Hz). Mice

were tested for three consecutive days and the mean amplitude (mV) at each frequency (Hz) was determined for each mouse individually.

Grip strength assay

Grip strength is a widely used method to measure motor performance in vivo in rodents, is a well-accepted functional measure in humans, and studies show that grip strength decreases with age in male and female mice. Changes in grip strength have also been linked to menopause in humans. A grip strength meter with a mesh grid (San Diego Instruments, San Diego, CA, USA, [46]) was used to measure forelimb strength ([5, 31]). Mice (6moCTL-n = 19; 8moCTL-n = 3; 22moCTL-n = 5; 26moCTL-n = 3; 20moGC-n = 7; 20moGD-n = 9; 20moOSC-n = 10) were removed from their home cage, held by the tail, and lowered toward the apparatus. Each mouse was allowed to grasp the horizontal metal grid with both front paws and then pulled gently but consistently in a horizontal plane (approximately 2 cm/s). The grip strength meter determined the maximum force displayed by each animal (grams-force) using a Chatillon (10-LBF) force gauge. Peak force tension was recorded automatically. Untrained mice were tested five times in succession without rest at 0, 5, 10, 30, and 90 min, and the results for each mouse at each period were averaged. Before assessment, each mouse was weighed once, at the same time of day, each day, for three consecutive days to obtain average body weight.

Hormone assay

Serum for estradiol measurements was collected after a 4–5-h fast from mice verified acyclic or in the diestrus stage of the estrous cycle (5moCTL-n = 3; 12moCTL-n = 3; 17moCTL-n = 6; 17moGC-n = 9; 17moGD-n = 12; 17moOSC-n = 7). Samples were collected in gel serum tubes with added protease inhibitor (in-house), allowed to clot for 15 min, spun down, and the serum collected, flash-frozen, and stored at − 8 0 °C until analysis. Estradiol was measured using a Mouse/Rat Estradiol ELISA kit (Calbiotech Inc., cat# Es180S-100, El Cajon, CA).

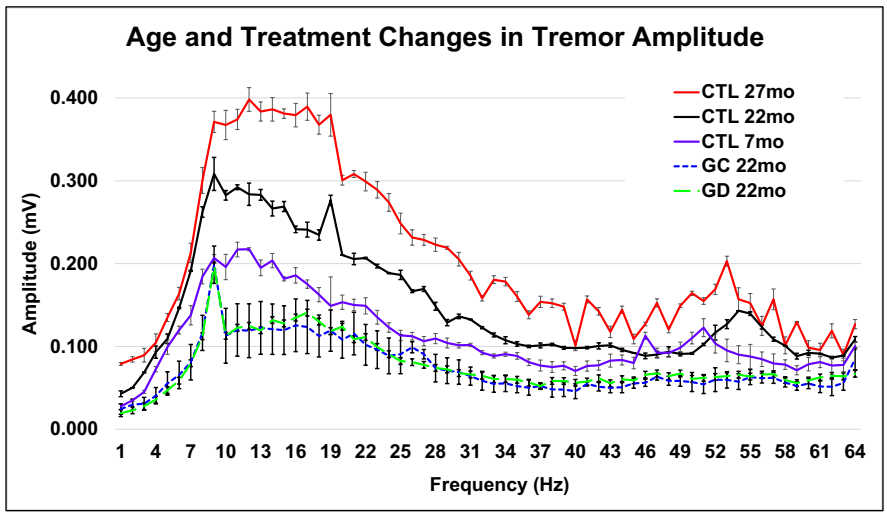


Fig. 2 Age and treatment changes in tremor amplitude. Tremor amplitude increased with age at 9 Hz (7 to 22 months, $P = 0.015$) and 12 Hz ($P = 0.017$) and decreased with transplantation of young ovarian tissue at 9 Hz (CTL 22 months to GC 22 months, $P = 0.009$ and to GD 22 months $P < 0.001$) and 12 Hz (CTL 22 months to GC 22 months, $P = 0.009$ and to GD 22 months $P < 0.001$) at all frequencies. CTL 22mo ($n = 5$) = age-matched controls, GC 22 months ($n = 4$) = received germ cell containing 60-day ovaries at 13 months. GD 22 months ($n = 8$) = received germ cell depleted 60-day ovaries at 13 months. Error bars represent SE

Table 1 Age and ovarian influence on tremor amplitude, grip strength, body weight, and estradiol

Treatment group	Young control	Adult control	Middle-age control	Old control	GC transplant	GD transplant	OSC transplant
Tremor amp at 9 Hz		0.21 (7mo) $n = 19$	0.31 (22mo) $n = 5$	0.37 (27mo) $n = 3$	0.20 (22mo) $n = 4$	0.20 (22mo) $n = 8$	
Tremor amp at 12 Hz		0.22 (7mo)	0.28 (22mo)	0.40 (27mo)	0.12 (22mo)	0.12 (22mo)	
Grip strength (g)	97.0 (6mo) $n = 19$	85.0 (8mo) $n = 3$	66.7 (22mo) $n = 5$	63.05 (26mo) $n = 3$	82.2 (20mo) $n = 7$	89.0 (20mo) $n = 9$	75.3 (20mo) $n = 10$
Grip strength (%) a	1.00 (6mo)	0.88 (8mo)	0.69 (22mo)	0.65 (26mo)	0.85 (20mo)	0.92 (20mo)	0.78 (20mo)
Grip strength/BW (%)a	1.00 (6mo)	0.87 (8mo)	0.69 (22mo)	0.69 (26mo)	0.80 (20mo)	0.82 (20mo)	0.73 (20mo)
Body weight (g)	26.1 (6mo)	26.2 (8mo)	26.1 (22mo)	24.6 (26mo)	27.9 (20mo)	29.3 (20mo)	27.6 (20mo)
Estradiol (pg/ml)	16.3 (5mo) $n = 3$	8.7 (12mo) $n = 3$	4.3 (17mo) $n = 6$		4.0 (17mo) $n = 9$	3.8 (17mo) $n = 1$	4.1 (17mo) $n = 7$

[a](%)-percent of values normalized to 6mo control mice

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7.04 (GraphPad Software, Inc., La Jolla, CA, USA). D'Agostino-Person Omnibus test was performed to determine normality. Data were analyzed with a two-factor ANOVA and a Tukey-Kramer post-hoc test was used to determine differences between the groups. Student's two-tailed *t* test was performed on individual treatments assuming unequal distribution of variance. Test results were considered significant for p values < 0.05.

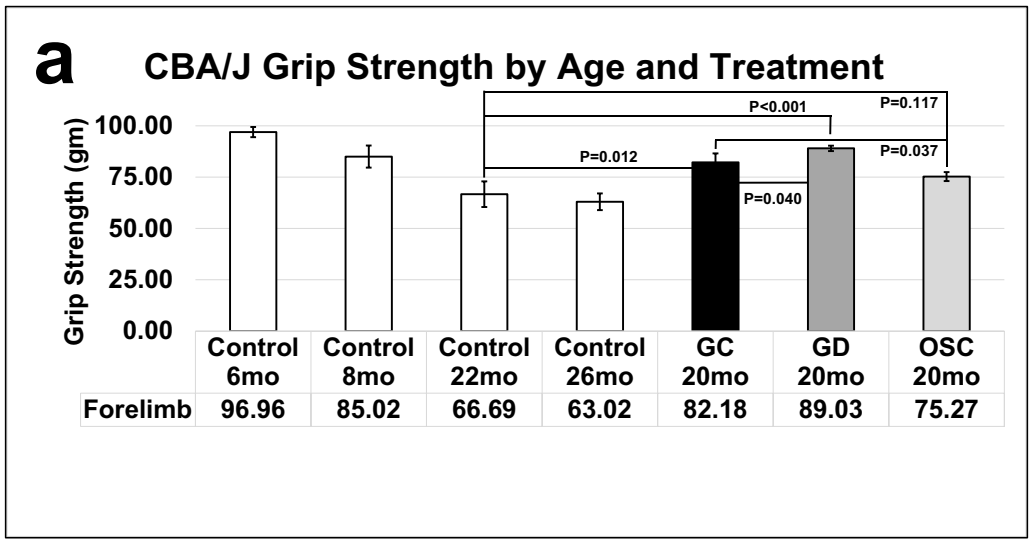
Results

Mice

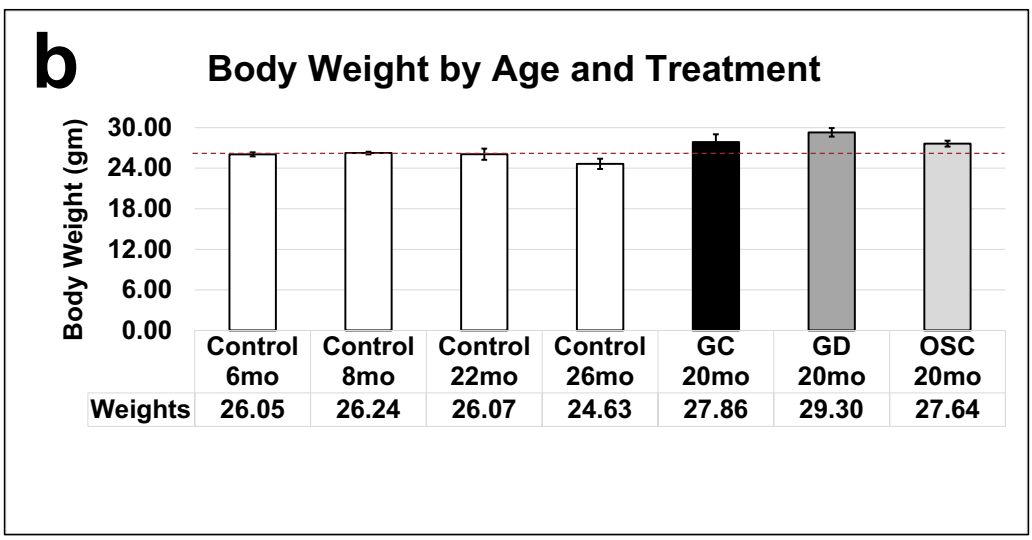
CBA/J female mice displayed regular reproductive cycles until approximately 8–10 months of age. At 11 months, approximately half of the mice were acyclic and by 13 months, most of the mice had stopped cycling. Only acyclic mice were used for subsequent transplant experiments or as controls. Mice that received young, GC ovary transplants at 13 months of age began cycling post-transplantation, but were acyclic by 17 months of age. Mice that received transplanted young, GD ovaries or isolated young, OSCs at 13 months of age remained acyclic.

Tremor amplitude

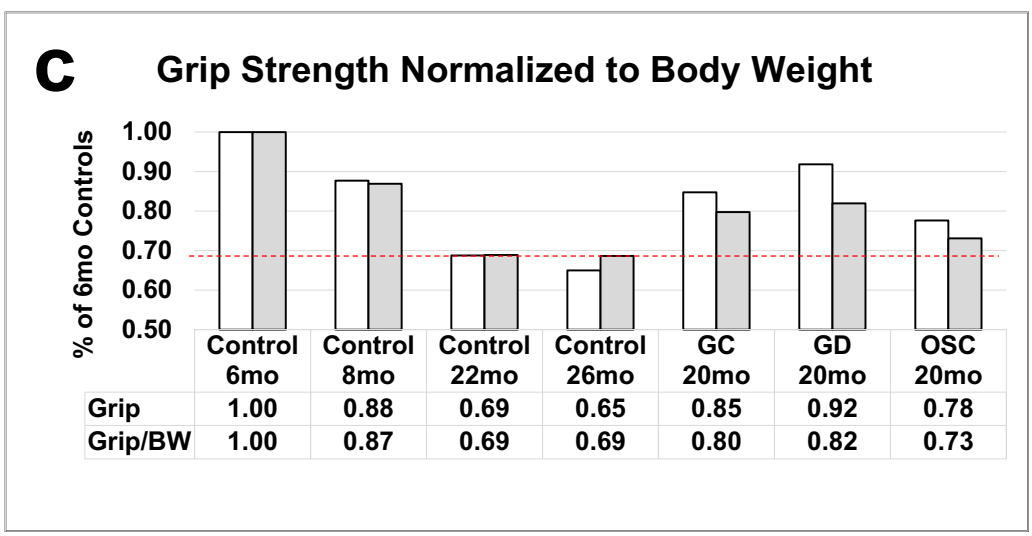
In control mice, tremor amplitude increased with aging at all frequencies measured (1–64 Hz, Fig. 2, Table 1). Tremor amplitude peaked at 12 Hz in young, 7-month-old mice (0.217 mV) and very old, 27-month-old mice (0.398 mV), but at 9 Hz in 22-month-old, age-matched control mice (0.308 mV). Tremor amplitude also peaked at 9 Hz in 22-month-old treated mice. The peak amplitude in mice that received young ovarian tissue transplants reflected that seen in young control mice and was far below the peak amplitude of 22-month-old, age-matched control mice, both mice that received GC tissue (0.199 mV) and in mice that received GD tissue (0.197 mV) transplants. A spike in amplitude at a frequency of 19 Hz followed by a drop at 20 Hz (> 20% change) was present in 22- and



	Control 6mo	Control 8mo	Control 22mo	Control 26mo	GC 20mo	GD 20mo	OSC 20mo
Forelimb	96.96	85.02	66.69	63.02	82.18	89.03	75.27



	Control 6mo	Control 8mo	Control 22mo	Control 26mo	GC 20mo	GD 20mo	OSC 20mo
Weights	26.05	26.24	26.07	24.63	27.86	29.30	27.64



	Control 6mo	Control 8mo	Control 22mo	Control 26mo	GC 20mo	GD 20mo	OSC 20mo
Grip	1.00	0.88	0.69	0.65	0.85	0.92	0.78
Grip/BW	1.00	0.87	0.69	0.69	0.80	0.82	0.73

◂**Fig. 3** Age and treatment changes in forelimb grip strength. **a** Grip strength decreased with age and was improved with transplantation of young ovarian tissue. **b** Body weight was slightly greater in treated mice. **c** After correcting grip strength for body weight, the percent difference between groups decreased, but the trend for increased strength in treated mice remained. GC 20mo (n = 7) = received germ cell containing 60-day ovaries at 13 months. GD 20mo (n = 9) = received germ cell depleted 60-day ovaries at 13 months. OSC 17 months (n = 10) = received transplantation of isolated somatic cells from 60-day ovaries at 13 months. Error bars represent SE

27-month-old controls, but was absent in 7-month-old controls and treated 22-month-old mice. There was also a small rise in amplitude between 50 and 55 Hz in all controls groups, but not in treated animals. (The tremor monitor and grip meter were housed in a Federal facility that was intermittently locked down due to Covid-19 outbreaks during several testing periods. This necessitated shifting some testing periods and eliminating tremor testing for the OSC mice).

Grip strength

Forelimb grip strength decreased significantly with age and was restored in recipients of young ovarian tissue to levels found in 8-month-old control mice (Fig. 3a). Compared with grip strength in GC transplant recipients, recipients of GD ovarian tissue (P = 0.040), and recipients of isolated OSCs (P = 0.037) displayed decreased grip strength. Since grip strength data can be influenced by animals' body weight (Fig. 3b), we normalized grip strength data by body weight and reported the normalized values as a percentage of the value from 6-month-old mice. Treated mice continued to display increased grip strength (Fig. 3c) despite having slightly heavier body weights than control mice (20-month treated mice = 28.3 g vs. 22 month CTL mice = 26.1 g).

Hormones

Estradiol levels decreased with age and were not increased with ovarian tissue transplantation in recipients of transplanted GC 60-day ovaries (from actively-cycling donor mice), GD 60-day ovaries (from acyclic donor mice), or isolated OSCs from 60-day ovaries (from acyclic donor mice, Fig. 4).

Discussion

The CBA/J strain of mice are different from many other strains used for aging or reproductive research in that they prematurely lose their ovarian follicles, often becoming reproductively senescent by 10–12 months of age [3, 9, 19]. Since a reduction of ovarian follicles in the human is normally associated with the onset of menopause, it has been suggested that CBA strain mice may serve as an appropriate experimental model to study age-related changes in the human reproductive system [2, 3, 12]. In the current experiments, all CBA/J female mice used were acyclic at 13 months of age, and at the first evaluation point, 4 months after transplantation (17 months of age) and remained relatively healthy until after the last evaluation point nine months (22 months of age) after transplantation (mean lifespan in GC recipients is 780 days and 880 days in GD recipients).

In humans, the prevalence of tremors increases with chronological aging and is not always associated with a particular neurodegenerative disease [7]. The frequencies used to identify human essential tremors (ET) can be found between 5 and 12 Hz [21, 22, 42]. In the current study, peak tremor amplitudes were found between 9 and 12 Hz of frequency and provided representative data for ET, which are the most common movement disorder seen in humans greater than 65 years of age [1, 7]. The amplitude of tremors across all frequencies increased with age in control mice, but decreased with exposure to transplanted young ovarian tissue and was not different with or without germ cells.

Tremors can have a debilitating effect on an individual's life because they can influence normal daily activities. Several forms of tremors can develop based on different environmental stimuli, changes in muscle contractility, breaks in the neuron pathways, or other influences. Our results suggest that the young ovarian somatic tissue has a positive influence on decreasing the amplitude of tremors commonly associated with aging and that tremor amplitude may serve as a sensitive, estradiol-independent indicator of an age-related reduction in a physiological homeostatic mechanism and as an additional measure to determine biological age/aging.

The Baltimore Longitudinal Study on Aging found that muscle quality is diminished with age [24] and that some of these declines are different between men

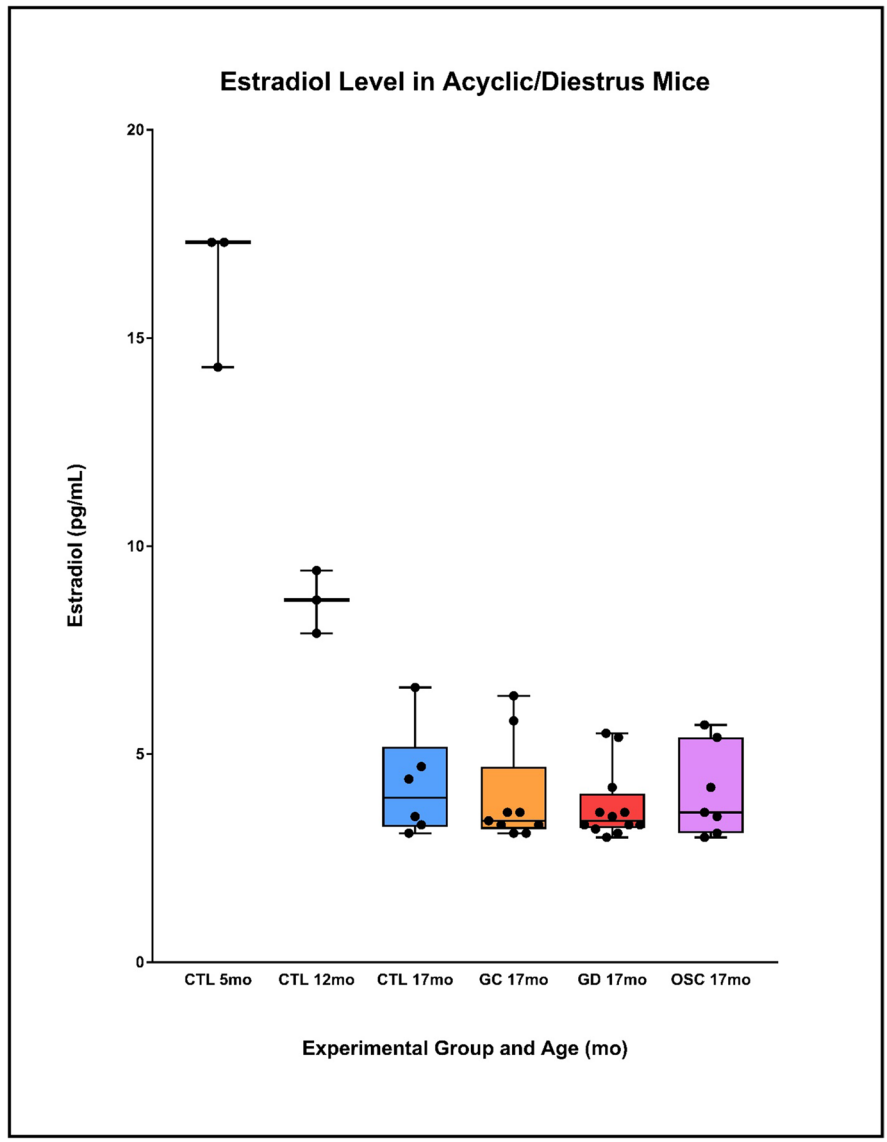


Fig 4 Age and treatment changes in estradiol levels. Estradiol levels decreased with age and were not changed from age-matched control mice with transplantation of young ovarian tissue. CTL 17 months (n = 7) = age-matched controls, GC 17 months (n = 9) = received germ cell containing 60-day ovaries at 13 months. GD 17 months (n = 12) = received germ cell depleted 60-day ovaries at 13 months. OSC 17mo (n = 7) = received transplantation of isolated somatic cells from 60-day ovaries at 13 months. Error bars represent SE

and women. In the current study, a classic grip test was performed to assess musculoskeletal function, by evaluating the strength of the forelimbs. Control mice demonstrated an aging-associated physiological decline in forelimb strength as expected. Increased exposure to young ovarian tissue/cells improved/restored grip strength to that seen in much younger mice, suggesting a strong connection between motor function and ovarian age. However, this effect appeared to be independent of germ cells and independent of any change in circulating estradiol levels. In mice, it has been reported that the force generated by soleus muscle declined by ~ 25% with age and did so around the age at which ovaries fail in the strain of mice used [33]. In other studies, leg muscles from ovariectomized mice were 10–20% weaker than corresponding muscles from ovary-intact and 17β-estradiol replaced mice [23, 32, 33, 45]. However, most of the mice in these studies were young (5–6 months). An additional confounding factor in these studies was the constant delivery (implant) rather than cyclic delivery of estradiol to the treated mice.

Circulating hormone levels change throughout life and reproductive status. Estradiol is the principal endogenous estrogen produced cyclically by ovarian follicles during the reproductive years [6, 15]. After

menopause, total estrogen levels are reduced [15]. As expected, the CBA/J control mice in the current study showed a decrease in estradiol from 5 to 17 months of age (measured in the diestrus phase of the estrous cycle in cycling mice). Estradiol levels in mice that received increased exposure to young ovarian tissue, either through tissue or cell transplantation, were not significantly different from the age-matched control group. Therefore, observed changes in tremor amplitude and grip strength were not due to increased levels of estradiol upon ovarian tissue/cell transplantation, as might have been expected.

Based on our observation with our ovarian tissue transplantation model and observations from studies with primitive species, the mechanisms of the observed protective effects, which include neuromuscular function, may be based on mechanisms to promote overall organismal survival. Work in primitive species has identified FoxO pathway signaling as a potential contributor to the gonadal somatic tissue-dependent enhancement of survival. Aging can induce the loss of the protective functions of FoxOs, which in mammals, can exert their protective effects through the regulation of superoxide dismutases (SODs) and have roles in the regulation of GSH-mediated detoxification by inducing the transcription of GSTM1 [13, 35]. When considering neuromuscular function specifically, aging may increase oxidative stress in motor neurons [11]. Preliminary analysis of liver proteins by our group has revealed that both GSTM1 and SOD2 are increased with aging (unpublished observations), which may be reflecting the degree of oxidative stress in the local environment [16]. An increase in both proteins was also observed with aging in human ovaries (unpublished observations). Both GSTM1 and SOD2 were decreased with transplantation of young ovarian tissue to the levels found in young mice. An additional pilot study revealed that, while FoxO1 protein was abundant in ovarian transudate from 60-day-old ovaries, FoxO1 protein was absent in ovarian transudate from 18-month-old ovaries (unpublished observations).

In conclusion, in female CBA/J mice, tremor amplitude increased and grip strength and estradiol levels decreased with chronological and ovarian aging. Increased exposure of post-reproductive females to young ovarian tissue improved/restored tremor amplitude and grip strength parameters to reflect measures found in much younger mice and did so independent of ovarian germ cells and circulating estradiol. In addition, while grip strength is well established as a measure of aging-related health span change, the current results suggest that tremor amplitude, which is independent of subject collaboration, may serve as an additional measure to determine biological age/aging and health span. The current results suggest the presence of a germ cell- and estradiol-independent positive ovarian influence on aging-associated changes in motor function. Longevity is not favored by natural selection unless living longer results in higher reproductive success. Reproductive success is dependent on elevated levels of gonadal hormones (compared with pre-and post-reproductive hormone levels). However, lower levels of reproductive hormones do not appear to be detrimental to pre-reproductive survival, and exogenously elevated hormones post-reproductively have inconsistent survival benefits at best. There is ample evidence for the role of ovarian tissue in survival and longevity, and an unquestionable role for follicle-driven hormone production in reproduction. However, the role of follicle-driven hormone production in survival and longevity is unclear. Particularly in view that women can live half of their lifespan in a post-reproductive state. The results presented here bring into question the role of follicle-driven hormone production in survival, and support a hormone-independent role for the ovary in female health and survival.

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Declarations

Competing interests The authors declare no conflict of interest.

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