Sex and gonadectomy modify behavioral seizure susceptibility and mortality in a repeated low-dose kainic acid systemic injection paradigm in mice

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Summary

Objective:

Sex differences in epilepsy appear driven in part due to effects of gonadal steroids, with varying results in experimental models based on species and method of seizure induction. Furthermore, removing a main source of these steroids via gonadectomy may impact seizure characteristics differently in males and females. Repeated low-dose kainic acid (RLDKA) systemic injection paradigms were recently shown to reliably induce status epilepticus (SE) and hippocampal histopathology in C57BL/6J mice. Here, we investigated whether seizure susceptibility in a RLDKA injection protocol exhibits a sex difference, and whether gonadectomy differentially influences response to this seizure induction paradigm in males and females.

Methods:

Adult C57BL/6J mice were left gonad-intact as controls or gonadectomized (females: ovariectomized, OVX; males: orchidectomized, ORX). At least 2 weeks later, KA was injected i.p. every 30 minutes at 7.5 mg/kg or less until the animal reached SE, defined by at least 5 generalized seizures (Racine stage 3 or higher). Parameters of susceptibility to generalized seizure induction, SE development, and mortality rates were quantified.

Results:

No differences were observed between control males and control females. Gonadectomized mice exhibited increased susceptibility and reduced latency to both generalized seizures and to SE. The effects on generalized seizure susceptibility were more prominent in males, but sensitivity to SE induction was similarly increased in both ORX males and OVX females. However, ORX males, but not OVX females, exhibited strongly increased seizure-induced mortality.

Significance:

The RLDKA protocol is notable for its efficacy in inducing SE and seizure-induced histopathology in C57BL/6J mice, the background for many transgenic strains in current use in epilepsy research. The present results indicate that this protocol may be beneficial for investigating the effects of ovarian hormone replacement on seizure susceptibility and seizure-induced histopathology in OVX female mice, but substantially increased mortality limits its utility in ORX males.

1. Introduction

The existence of sex differences in epilepsy and seizure susceptibility is well-established.[1–4] Many of these differences are likely a result of distinct neural effects of gonadal hormones in males and females.[1,3–5] Females have higher levels of circulating estrogen and progesterone than males,[6,7] whereas males have higher levels of testosterone than females in both rodents and humans.[6,8] Both estrogen and progesterone have proconvulsant and anticonvulsant effects, as documented in a wide variety of studies examining different species and models of seizure and epilepsy induction.[9–23] Testosterone can also have mixed effects on seizures due to metabolism producing both anticonvulsant and proconvulsant compounds.[24]

In previous studies, removal of testes in male rats (orchidectomy, ORX) resulted in decreased severity of, susceptibility to, and duration of kainic acid (KA)-induced seizures, and in reduced susceptibility to bicuculline-induced tonic hindlimb extension.[3,25,26] However, ORX male rats also exhibited increased susceptibility to picrotoxin-induced generalized seizures, whereas ORX male mice had no change in susceptibility to pentylenetetrazole-induced seizures.[27,28] In contrast to the mixed effects of ORX on seizures, removal of ovaries in females (ovariectomy, OVX) has been documented in multiple studies to increase seizure susceptibility in both rats and mice.[16,25,28–34] These studies used a wide range of seizure induction drugs including N-methyl-D-aspartate, pilocarpine, and bicuculline in rats, and pentylenetetrazole and KA in mice.[16,25,28–32,34] Additionally, Wistar Albino Glaxo/Rijswijk female rats, a genetic absence seizure model, exhibit increased spike-and-wave discharges after OVX.[33] By contrast, another study found increased severity of systemic KA-induced behavioral seizures in ovary-intact female rats.[26] Overall, these results indicate that the removal of gonadal hormones via gonadectomy can have differing effects on seizures between males and females, and that these effects are likely shaped by the specific mode of seizure induction and the experimental species.

Recent reports describe the advantages of repeated low-dose KA (RLDKA) administration in inducing status epilepticus (SE) and histopathology characteristic of temporal lobe epilepsy in C57BL/6J mice,[35–37] which can be more resistant than other strains to a single bolus injection of KA[38–40] and are the background for many transgenic strains in current use in epilepsy research. However, previous studies using RLDKA injections only examined males or did not differentiate data collected from male vs. female mice,[35–37,41] leaving open the question as to whether sex differences may be observed in response to this type of seizure induction paradigm. In addition, examination of the effects of gonadectomy would clarify the utility of this model for studies examining the effects of gonadal hormone replacement and the selective roles of neural sources of these hormones in modulating seizure susceptibility and excitability. Therefore, here we tested the susceptibility to induction of behavioral generalized seizures and SE, as well as to seizure-induced mortality, in gonad-intact and gonadectomized male and female C57BL/6J mice after RLDKA injection.

2. Methods

2.1 Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois Urbana-Champaign (protocol 20155). C57BL/6J mice (n = 30 females, n = 22 males) were purchased from Jackson Laboratories for delivery at 6 weeks of age. All mice were group-housed (2-5 mice per cage) with others of the same sex and were given a minimum

of 4 days to acclimate to our animal facility prior to experimental handling. Mice were housed on a 14:10 h light:dark cycle with food and water available *ad libitum* and lights-off at 1900 h. On the morning of seizure induction testing, ovary-intact females were assessed for estrous cycle stage using a vaginal cytology protocol as previously described.[42]

2.2 Gonadectomy surgeries

Some females (n=19) underwent OVX and some males (n=10) underwent ORX. Additionally, 3 females and 3 males were included as shams. For OVX/ORX procedures, mice were anesthetized by inhalation of isoflurane (Clipper Distributing Company, St. Joseph, Missouri) with sufficient anesthesia determined by lack of response to tail and toe pinch. Prior to surgery, mice were given the analgesic carprofen (5 mg/kg s.c., Zoetis). For bilateral OVX, small incisions in the skin and abdominal muscle wall were made halfway between the caudal edge of the ribcage and quadriceps muscle, and the ovaries were removed by cautery.[43] For ORX, a small midline incision was made in the skin on the ventral side of the scrotum. The tunica was pierced and each testis was gently exposed and removed by cautery. Bupivacaine (0.25%, 7 ul, Hospira, Lake Forest, Illinois) was applied locally into each surgical site for postoperative analgesia.[43] Surgical sites were closed with wound clips, which were subsequently removed from awake mice 7-10 days later. All OVX and ORX mice were allowed 2 weeks for recovery from the procedure and to allow for full depletion of circulating gonadal hormones before seizure induction testing (described below). Successful OVX status in females was confirmed by visual inspection of the shrunken uterus following euthanasia.[44] Sham surgeries were conducted similarly but without removal of the ovaries for the females and without tunica incision and removal of the testes in males.

2.3 Repeated low-dose kainic acid injection

OVX/ORX, sham, and age-matched gonad-intact control mice (9-11 weeks of age) were tested in a RLDKA seizure induction paradigm using a protocol adapted from a previous report.[37] The first injection was initiated between 1100-1600 h. KA (Tocris Bioscience #0222) was dissolved in sterile 0.9% saline at 2 mg/ml and stored at -20°C for up to 7 days prior to use. On the day of injection, the mice were brought to the experiment room in the laboratory, weighed, and individually placed in fresh cages. All mice were given 30 minutes to acclimate to the experiment room and the clean cages before receiving the first injection. KA was injected i.p. at 7.5 mg/kg every 30 minutes until the animal developed SE, defined by at least 5 generalized seizures of Racine stage of 3 or higher using the following definitions: stage 3 – wet dog shakes, head nodding, and unilateral forelimb clonus; stage 4 – stage 3 with bilateral forelimb clonus or rearing; stage 5 – stage 4 with repeated rearing and falling or tonic-clonic seizures.[45] An additional KA dose was administered if the animal did not have a subsequent generalized seizure for at least 30 minutes after the initial onset of seizures. No further injections were given once a cumulative seizure burden of 30 (calculated by the sum of all Racine scores $\geq$ 3) was reached. Six OVX and one control females with particularly severe seizures that were nearing this cumulative seizure burden score were given half doses of KA, and the data for number of injections were adjusted accordingly. 2 hours after the last KA injection, all mice were injected s.c. with 500 µl Lactated Ringer's solution or 0.9% saline and returned to the mouse facility in single cages. Mortality was quantified based on survival to the end of the injection regimen.

2.4 Statistical analyses
Statistical comparisons were done using R software or OriginPro (OriginLab, Northampton, MA). Chi-square tests of independence and independent samples t-tests were used to test if sham surgical mice differed from the gonad-intact mice (i.e., those that did not undergo sham surgery). As no statistical differences were found between these groups on any measure for either sex, the sham mice were combined with the intact mice as the control male and female groups in analyses. All estrous cycle stages were represented in the control female group, and the data do not indicate any trends in this regard. However, the study was not designed or powered to test for differences dependent on cycle stage.

To test for differences in the number of injections needed to produce generalized seizures and SE, and the latency in minutes to first generalized seizure or SE, two-way ANOVAs and Tukey's *post hoc* pairwise comparisons tests were used with sex and gonad status as the independent variables. Data normality was determined using Shapiro-Wilks tests. In the absence of a reliable non-parametric test equivalent to a two-way ANOVA, data transformations (e.g., log or square root) were used where specified to allow for use of parametric statistics. Choice of log vs. square root transformation was based on accuracy of the normalizing effect for the given dataset. Fisher's exact tests and *post hoc* Bonferroni-corrected pairwise comparisons were used to test for differences in the proportions of mice in each group developing generalized seizures, SE, and seizure-related mortality. A power analysis demonstrated that the final sample sizes achieved statistical power for 2-way ANOVA over 84% at $\alpha = 0.05$ for a medium effect size (Cohen's f) of 0.3. Data are presented as means ± SD. Statistical significance was set at $p < 0.05$.

Results

3.1 Effect of gonadectomy in increasing susceptibility to generalized seizures is larger in males
To determine whether generalized seizure induction in response to RLDKA injection is influenced by mouse sex and/or gonad status, male (control n = 15, ORX n = 10) and female (control n = 14, OVX n= 19) mice were tested. The data for the number of injections needed to produce generalized seizures and latency to the first generalized seizure were log- and square root-transformed, respectively, to account for non-normal distributions. The number of injections needed to produce generalized seizures in mice varied significantly by gonad status ($F_{1,54} = 20.544$, $p < 0.001$) but not by sex ($F_{1,54} = 0.041$, $p = 0.841$), and with no interaction between sex and gonad status ($F_{1,54} = 1.38$, $p = 0.245$) (**Figure 1A**). Pairwise comparisons indicated that fewer injections were required to induce generalized seizures in ORX males compared to controls (controls 3.67 ± 0.82, ORX 2.30 ± 0.48 injections, $p = 0.0022$), and that there was a trend towards significance between control and OVX females (controls 3.50 ± 1.29, OVX 2.65 ± 0.79 injections, $p = 0.061$). There were no sex differences observed within control ($p = 0.89$) or gonadectomized groups ($p = 0.778$).

There were also differences between groups in the proportion of mice who had at least one generalized seizure by 2 injections ($p = 0.005$) and by 3 injections ($p = 0.002$) (**Figure 1B**). *Post hoc* Bonferroni tests revealed that a greater proportion of ORX males than control males had at least one generalized seizure within both 2 injections (control, n = 1 of 15, ORX n = 7 of 10; $p = 0.010$) and 3 injections (control, n = 6 of 15, ORX, n = 10 of 10; $p = 0.017$). No differences were found in rate of generalized seizure development between OVX and control female groups at

either 2 injections (controls n = 3 of 14, OVX n = 7 of 19; p = 1) or 3 injections (controls n = 7 of 14, OVX n = 16 of 19; p = 0.34).

Similarly, there was an effect of gonad status ($F_{1,54} = 15.488$, $p < 0.001$; **Figure 1C**) on the latency to the first generalized seizure but no effect of sex ($F_{1,54} = 0.151$, $p = 0.699$) and no interaction between sex and gonad status ($F_{1,54} = 1.917$, $p = 0.172$). Furthermore, pairwise comparisons indicated that the effect of gonadectomy was primarily driven by the difference within the male group, as ORX males exhibited shorter latency to the first generalized seizure than corresponding controls (controls 93.13 ± 23.92, ORX 56.90 ± 9.33 min, p = 0.0049). In females, however, there was no difference between OVX and control groups (controls 88.00 ± 37.45, OVX 69.11 ± 21.42 min, p = 0.219). These results indicate that gonadectomy increases susceptibility to generalized seizure induction in response to RLDKA injection, with a larger effect in males.

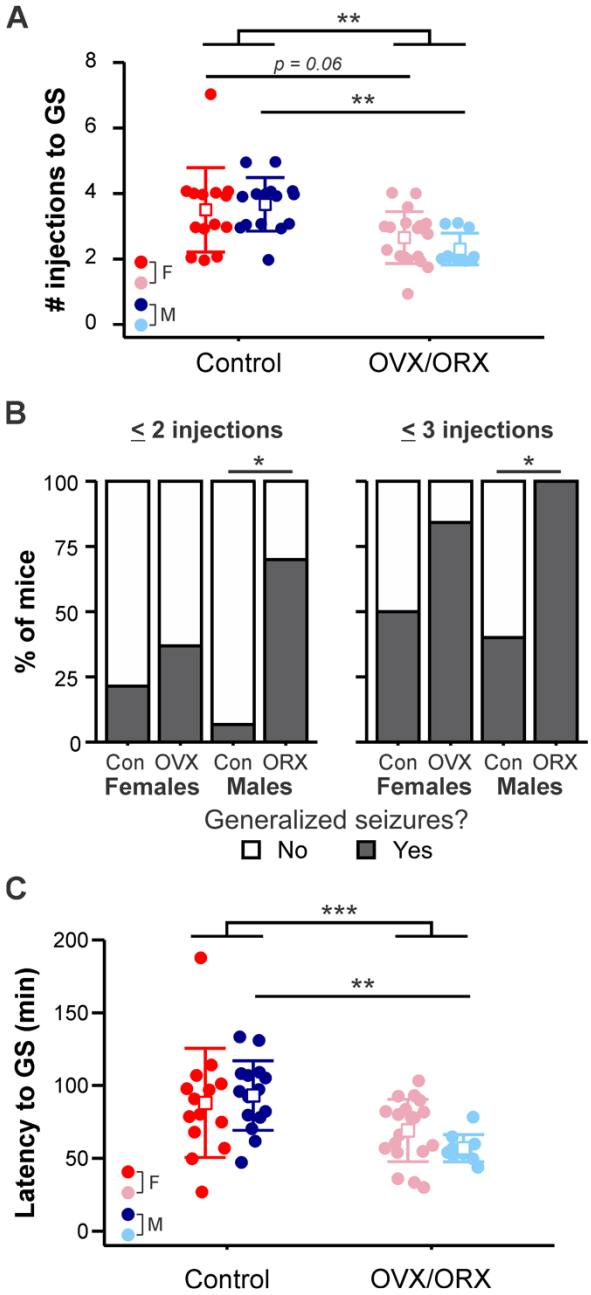

Figure 1. Effect of gonadectomy on susceptibility to generalized seizures in most prominent in males. A) Individual values (circles) and mean ± SD (squares and lines, respectively) for the number of i.p. kainic acid injections needed to reach generalized seizures (GS). Raw data are presented; for statistical analysis, the data were log-transformed to improve normality. B) Percentage of mice in each group with at least one generalized seizure by two (left) or three (right) KA injections. C) Individual values and mean ± SD for the latency in minutes to reach generalized seizures. Raw data are presented; for statistical analysis, the data were square root-transformed to improve normality. Blue = control males (M); red = control females (F); light blue = ORX males; pink = OVX females. *p<0.05, **p<0.01, ***p<0.001

3.2 Gonadectomy increases SE susceptibility in both males and females

We also determined whether sex and/or gonadectomy impact susceptibility to SE induction in this chemoconvulsant paradigm. Four mice (1 from each group) did not survive to SE and thus were not included in these analyses. The number of kainic acid injections needed to reach SE differed by gonad status ($F_{1,50} = 29.452$, $p < 0.001$) but not by sex ($F_{1,50} = 1.184$, $p = 0.282$) and with no interaction between sex and gonad status ($F_{1,50} = 0.079$, $p = 0.779$) (**Figure 2A**). As with susceptibility to initial generalized seizures, fewer injections of KA were required to induce SE in ORX males compared to controls (controls 4.14 ± 0.66, ORX 2.67 ± 0.71 injections, $p = 0.0025$). Furthermore, OVX females also displayed an increased susceptibility to SE induction (controls 4.35 ± 1.07, OVX 3.02 ± 1.05 injections, $p = 0.0013$).

In addition, the proportions of mice that reached SE by 2 ($p = 0.002$) or 3 ($p < 0.001$) injections varied significantly between groups (**Figure 2B**). Although no two groups differed significantly in the rate of SE induction within 2 injections by pairwise testing, a larger proportion of ORX males (n = 8 of 9) reached SE by 3 injections compared to both control males (n = 2 of 14) ($p = 0.004$) and control females (n = 2 of 13) ($p = 0.009$), with a trend approaching significance in comparison to OVX females (n = 11 of 18) ($p = 0.069$). The rates in OVX and control female groups were not different from each other ($p > 0.15$).

As the data for latency in minutes to SE was not normally distributed, this variable was square root-transformed. Gonad status ($F_{1,50} = 20.854$, $p < 0.001$), but not sex ($F_{1,50} = 2.236$, $p = 0.141$), was found to have a significant effect on the latency to SE, with no interaction ($F_{1,50} = 0.23$, $p = 0.63$) (**Figure 2C**). Gonadectomized mice of both sexes exhibited shorter latency to SE than corresponding controls (males: controls 114.86 ± 22.17, ORX 79.89 ± 9.03 min, $p = 0.0089$; females controls 123.46 ± 34.68, OVX 92.67 ± 24.62 min, $p = 0.014$). These results indicate that susceptibility to SE in response to RLDKA injection is increased following gonadectomy in both male and female mice.

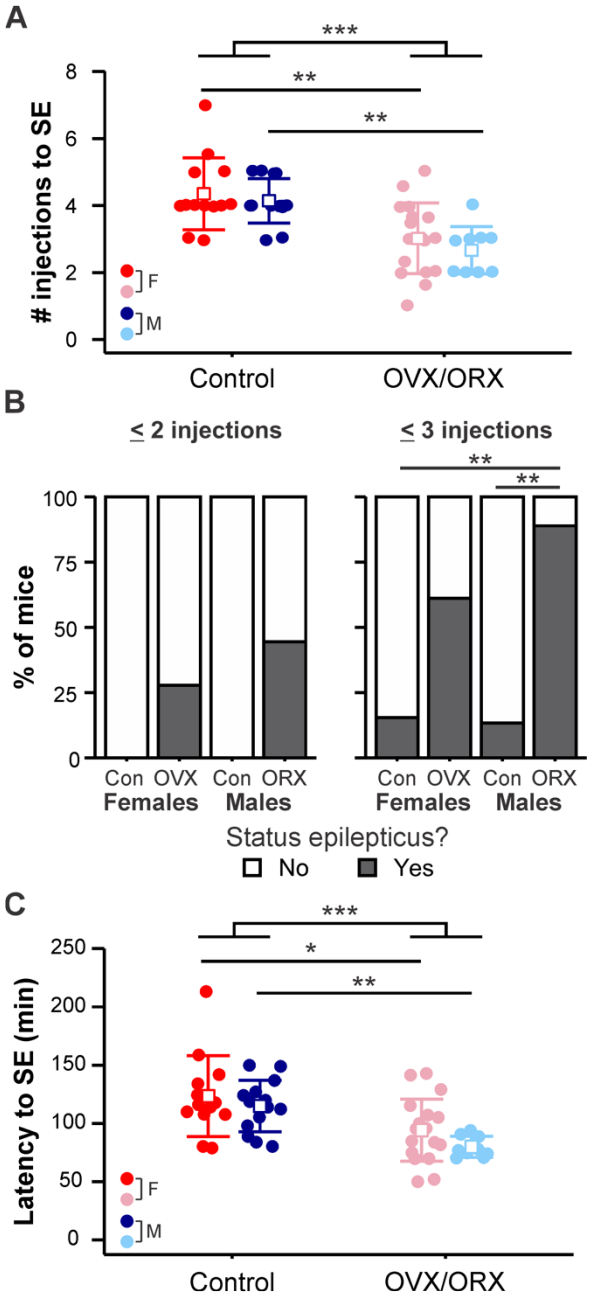

Figure 2. OVX females and ORX males exhibit increased susceptibility to status epilepticus (SE). A) Individual values (circles) and mean ± SD (squares and lines, respectively) for the number of kainic acid injections needed to reach SE. B) Percentage of mice in each group that developed SE by two (left) or three (right) KA injections. C) Individual values and mean ± SD for the latency in minutes to reach SE. Raw data are presented; for statistical analysis, the data were square root-transformed to improve normality. Blue = control males (M) ; red = control females (F); light blue = ORX males; pink = OVX females. *p<0.05, **p<0.01, ***p<0.001

3.3 Gonadectomy increases susceptibility to seizure-induced mortality in males but not females
Although previous studies indicate high survival rates following RLDKA injection,[36,37] not all mice in the present cohort survived the injection regimen and the induced SE. To determine if this mortality reflected differences based on sex and/or gonad status, we used a Fisher's Exact Test, which revealed that the rate of mortality observed within the time course of the RLDKA injection series and acute seizure monitoring differed by group ($p < 0.001$). Further analysis with Bonferroni *post hoc* tests showed that ORX males (n = 8 of 10 died) had significantly higher mortality than both intact males (n = 1 of 15 died, $p = 0.002$) and OVX females (n = 2 of 19 died, $p = 0.002$; **Figure 3**). The difference between OVX females and ORX males persisted when the OVX female group was adjusted to only include mice that received full doses of KA in each injection (n = 2 of 13 died) ($p = 0.021$). The rates for OVX and control females (n = 4 of 14 died) were not different, both with and without the mice that received half doses included. These results indicate that ORX males, but not OVX females, exhibit greatly increased susceptibility to mortality in response to RLDKA injections.

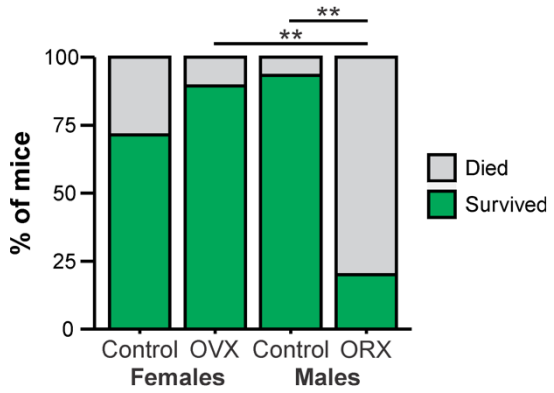

Figure 3. ORX males exhibit increased seizure-related mortality. The percentage of mice in each group that survived (green panels) and died (gray panels) within the time course of the RLDKA injection series. **p<0.01

Discussion

The RLDKA paradigm was recently found to be highly effective in inducing seizures, SE, and post-SE histopathology in C57BL/6J mice.[35–37] However, it is unclear whether mouse sex and/or gonad status influence susceptibility to seizures and SE in this model. Such information would indicate whether this model may be useful in studies of sex differences and effects of gonadal hormones in seizure activity. The present results demonstrate that gonadectomy had a detrimental effect, driving increased susceptibility to generalized seizures and SE. With respect to susceptibility to generalized seizures, the effect of gonadectomy was largest in males, whereas both ORX males and OVX females exhibited increased susceptibility to SE. In addition, ORX males, but not OVX females, exhibited increased seizure-related mortality compared to controls. These studies thus indicate that gonadectomy increases sensitivity to seizure and SE induction in this model, with more prominent effects in males.

In females, the depletion of ovarian progesterone may drive the increased susceptibility to SE and generalized seizures after OVX. Progesterone primarily exerts anticonvulsant effects through its metabolite allopregnanolone, which potentiates $GABA_A$ receptor-mediated synaptic inhibition.[10–13,17,23,46,47] However, progesterone actions via nuclear progesterone receptors can

also increase excitatory firing and exacerbate seizures.[15,20,21] Removal of the ovaries also depletes circulating estradiol, the primary ovarian estrogen. Although estradiol has largely been found to have proconvulsant effects, it is important to note that anticonvulsant effects have also been reported depending on the dose, receptors activated, brain region, and whether acute or chronic seizures were studied.[9,12,14,16,18,19,26,48–51] Therefore, it is possible that reduction of estradiol may at least partially underlie the observed seizure-promoting effects of OVX in the present studies, which are in line with several previous results.[16,25,28–33] The present findings indicate that the RLDKA paradigm may be useful in studies examining the effects of physiological replacement of circulating progesterone and/or estradiol on acute seizure and SE susceptibility, and in studies examining the brain itself as a source of these hormones.[26,52]

Previous results on the effect on seizures of ORX in male rodents have been mixed. One study documented increased susceptibility to picrotoxin-induced generalized seizures in ORX male rats,[27] in line with the present results in ORX mice. By contrast, others found the reverse effect on KA-induced seizures, with ORX in rats resulting in a decreased number of generalized seizures, and increased latency to seizures of both low and high severity.[3,26] Of note, rats and mice exhibit some species differences in responses to seizure-inducing drugs, including KA.[53,54] One possible explanation for these discrepant results could be differential rates of metabolism of testosterone to estradiol (via aromatase) vs. androgens such as 5α-dihydrotestosterone or 3α-androstanediol (via 5α-reductase and 3α-hydroxysteroid dehydrogenase, respectively)[24,55] in different species or strains. Alternatively, differences between the present study and past work may reflect differences in the mode of KA administration. While the present work tested a systemic RLDKA paradigm, the other studies that contrast with these results used single bolus injections of larger doses of KA.[3,26]

In line with previous findings using RLDKA paradigms in C57BL/6J mice,[36,37] the present results demonstrate high survival rates for control males and females. However, although both control and OVX females had mortality rates comparable to intact males, ORX males had a strikingly higher mortality rate, with over 80% dying within the acute time frame of the injection series. As a result, the RLDKA protocol may be of limited utility in studying post-SE sequelae, such as epileptogenesis and histopathology, in ORX males. These results suggest that testis-derived factors provide critical neuroprotection in male mice. The finding of higher mortality in ORX males also suggests that sex steroids made outside of the gonads, such as those produced in the brain, or other factors may perhaps yield greater compensation for the loss of gonadal steroids in females than in males. Indeed, gonadectomy can differentially impact levels of sex hormones, and the corresponding synthesizing enzymes, in males compared to females.[56–58] For example, gonadectomy decreases estradiol levels in the preoptic area of male, but not female, rats.[58] In females, ovarian hormones, in addition to modulating seizure susceptibility, also produce prominent neuroprotective effects.[59–62] In this regard, it is important to note that in the present study, six OVX females were given decreased doses of KA at later time points in the injection series due to early expression of very severe seizures, which may have provided some degree of protection against mortality. However, the number of injections to SE, a proxy for cumulative KA dose in the present experimental design, was not different between gonadectomized males and females, and the findings regarding rates of mortality are similar with and without inclusion of mice that received partial doses. Therefore, the increased mortality in ORX males cannot be ascribed solely to a difference in the amount of KA administered. Future

studies are needed to further resolve any sex differences in compensatory effects of brain-derived sex hormones or other factors in gonadectomized animals.

Of note, no differences in seizure or SE susceptibility were observed between control males and females. Therefore, in many studies using this model, inclusion of mixed groups of males and females should be highly feasible. This paradigm is thus advantageous for meeting the requirements of granting agencies, such as the U.S. National Institutes of Health,[63] for incorporation of both male and female animals in experiments.

A limitation of the present work is that seizure evaluation was done solely by screening for behavioral seizures in the absence of electroencephalography (EEG). It thus remains possible that more subtle differences in seizure susceptibility, particularly early on in the injection series, might be observed between males and females and between gonadectomized and gonad-intact mice. The present studies indicate that follow-up work using EEG and examining the effects of selective gonadal hormone replacement in the RLDKA paradigm would be fruitful avenues of future investigation.

Author contributions

C.A.C.-H. designed research; N.V.L. and E.A.C. performed research; E.A.C., N.V.L., and C.A.C.-H. analyzed data; E.A.C., N.V.L., and C.A.C.-H. wrote the paper.

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

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