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Extrasynaptic GABA-A receptor-mediated sex differences in the antiseizure activity of neurosteroids in status epilepticus and complex partial seizures

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Summary

Objective: Sex differences are evident in the antiseizure activity of neurosteroids; however, the potential mechanisms remain unclear. In this study, we sought to determine whether differences in target extrasynaptic δGABA-A receptor expression and function underlie the sex differences in seizure susceptibility and the antiseizure activity of neurosteroids.

Methods: Sex differences in seizure susceptibility and protective activity of three distinct neurosteroids −allopregnanolone (AP), androstanediol (AD), and ganaxolone− were evaluated in the pilocarpine model of status epilepticus (SE) and kindling seizure test in mice. Immunocytochemistry was used for δGABA-A receptor expression analysis and patch-clamp recordings in brain slices evaluated its functional currents.

Results: Sex differences were apparent in kindling epileptogenic seizures, with males exhibiting a faster progression to a fully-kindled state. Neurosteroids AP, AD or ganaxolone produced dose-dependent protection against SE and acute partial seizures. However, female mice exhibited strikingly enhanced sensitivity to the antiseizure activity of neurosteroids compared to males. Sex differences in neurosteroid protection were unrelated to pharmacokinetic factors, as plasma levels of neurosteroids associated with seizure protection were similar between sexes. Mice lacking extrasynaptic δGABA-A receptors did not exhibit sex differences in neurosteroid protection. Consistent with a greater abundance of extrasynaptic δGABA-A receptors, AP produced a significantly greater potentiation of tonic currents in dentate gyrus granule cells in females than males; however, such enhanced AP sensitivity was diminished in δGABA-A receptor knockout female mice.

Significance: Neurosteroids exhibit greater antiseizure potency in females than males, likely due to greater abundance of extrasynaptic δGABA-A receptors that mediates neurosteroid-sensitive,

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DISCLOSURE

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tonic currents, and seizure protection. These findings indicate the potential of developing personalized gender-specific neurosteroid treatments for SE and epilepsy in men and women, including catamenial epilepsy.

Keywords

Neurosteroids; sex difference; epilepsy; GABA-A receptor; kindling; tonic inhibition

1 | Introduction

Sex differences in seizure susceptibility are a widely recognized concern in epilepsy. Clinical evidence shows sex and age-related expression in many seizure syndromes. Generally, the incidence of epilepsy is higher in men than in women, [1,2] with more men being diagnosed with localization-related symptomatic epilepsies and more women being diagnosed with genetic generalized epilepsy.[2–4] However, the neurobiological mechanisms of sex differences in seizure susceptibility are poorly understood.

Susceptibility to brain disorders may arise from sex-linked variations between men and women in factors such as steroid hormones, metabolic enzyme activity, and sexually dimorphic neural circuits in the brain.[5–10] Specifically, estrogens, progesterone, and testosterone are known to distinctly affect seizure susceptibility in males and females. These circulating steroids serve as precursors for the synthesis of neurosteroids in the brain.[11] In females, neurosteroids, such as the progesterone-derived allopregnanolone (3α-hydroxy-5α-pregnan-20-one, AP), are potent positive allosteric modulators of GABA-A receptors and produce powerful antiseizure effects.[12–14] In males, the neurosteroid 3α-androstanediol (5α-androstane-3α,17β-diol, AD) is synthesized from testosterone within the brain. Similar to the mechanism of action of AP [15,16], AD allosterically binds GABA-A receptors, potentiating channel current, and thereby inhibiting neuronal excitability and seizures.[16,17]

Neurosteroids act on both synaptic (γ2-containing) and extrasynaptic (δ-containing) GABA-A receptors to regulate neuronal excitability.[18,19] Neurosteroids exhibit relatively lower sensitivities at synaptic receptors than at extrasynaptic receptors, which are expressed extrasynaptically in the dentate gyrus and contribute to tonic inhibition, promoting network shunting and reducing seizure susceptibility.[18] Neurosteroids are robust anticonvulsants in a variety of animal seizure models, including status epilepticus (SE), [4] with the exception of certain genetic conditions such as absence models where they were shown to cause seizure exacerbations.[20,21] Sex differences are evident in the antiseizure activity of neurosteroids;[5] however, the potential mechanisms remain unclear. It is likely that variations in the production, metabolism, or target receptors of neurosteroids may alter neuronal excitability and thereby play a key role in sex-based differences in seizure susceptibility.[8,10] Therefore, by examining the seizure protection ability of neurosteroids in epilepsy models, we sought to determine whether the differences in target extrasynaptic δGABA-A receptor expression and function underlie the sex differences in the antiseizure activity of neurosteroids.

In this study, we determined the sex-specific, antiseizure effects of three prototype neurosteroids AD, AP and ganaxolone in the pilocarpine model of SE and hippocampus kindling model of complex partial seizures. Our results demonstrate robust sex differences in

antiseizure activity of neurosteroids with greater sensitivity in females than males, likely due to the higher abundance of hormone-sensitive and sexually dimorphic, extrasynaptic δGABA-A receptors in the hippocampus dentate gyrus.

2 | MATERIALS AND METHODS

2.1 | Behavioral seizure studies

2.1.1 | Animals—Adult male and female wild type (WT) mice of the C57BL6 strain weighing approximately 25–35 g were used in the study. GABA-A receptor δ-subunit knockout mice (δKO) were also used.[22,23] All strains were maintained on a hybrid C57BL/6–129SV background. Adult female mice with regular cycles were used in the study. In female mice, estrous cycle was determined by microscopic examination of vaginal smears with eosin staining as described previously.[10] With the exception of the kindling seizure test, all females were tested during the diestrous stage. All animal procedures were performed in a protocol approved by the university's Institutional Animal Care and Use Committee.

2.1.2 | Pilocarpine-induced SE (pilocarpine-SE)—Pilocarpine-SE testing was performed as described previously.[24] Pilocarpine is a widely used cholinergic agonist for producing acute seizures in rodents.[25] AD (10−200 mg/kg, s.c.) and AP (0.5−50 mg/kg, s.c.) were evaluated for protective activity against pilocarpine-induced SE as previously described.[25] In brief, mice were injected with the steroid and 15 min later received intraperitoneal injection of pilocarpine (416 mg/kg, i.p.). At the injection time of the steroid, the animals also received 1 mg/kg scopolamine to block the peripheral cholinergic effects of pilocarpine. The severity of behavioral seizures was rated according to the criterion the Racine scale. Mice failing to show SE or persistent seizures lasting longer than 10 sec were scored as protected.

2.1.3 | Hippocampus kindling model of epilepsy—Experimental procedures for mouse hippocampus kindling were performed as described previously.[26] Kindling stimulation was delivered daily until stage 5 seizures were elicited on 3 consecutive days. Mice were used for drug testing when they consistently exhibited stage 5 seizures after stimulation, which is considered the "fully-kindled" state. In kindling epileptogenic studies, AP was given 15-min before stimulation. In other studies, test drugs were administered at least two weeks after reaching the fully-kindled state. After kindling stimulation, mice were scored for seizure occurrence, severity of behavioral seizures, and afterdischarge duration.

2.1.4 | 6-Hz model of psychomotor seizures—The 6-Hz model of psychomotor partial seizures was used according to a previously described protocol.[26,27] Mice were manually restrained during the 6-Hz stimulation, and then immediately released into an observation chamber. Animals that failed to exhibit seizure behavior within 10 sec of the stimulation were considered to be protected.

2.1.5 | NSW Model of catamenial seizures—The NSW model of kindling catamenial seizures was used as described previously.[28]

2.1.6 | Drugs—Test drugs AD, AP, and ganaxolone were purchased from Steraloids (Newport, RI). Stock solutions of neurosteroids for injection were made using 30% hydroxyl-propyl-β-cyclodextrin in water. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.1.7 | Data analyses—The dose producing 50% protection (ED_{50}) and the corresponding confidence limits (CL) were determined by the log-probit analysis using the Litchfield and Wilcoxon method as described previously.[29] The significance of differences between steroid dose-response curves in the pilocarpine seizure test was assessed with the Litchfield and Wilcoxon χ^2 test. In all tests, the criterion for statistical significance was $p<0.05$.

2.2 | Biochemical and molecular studies

2.2.1 | Determination of plasma neurosteroid levels—The plasma levels of AP and AD were determined by liquid chromatography-mass spectroscopy (LC-MS) assay as previously described.[29]

2.2.2 | TaqMan real-time PCR—The GABA-A receptor subunit mRNA expression was determined by the TaqMan real-time PCR assay as described.[30]

2.2.3 | Immunocytochemistry and confocal microscopy—The δ-subunit distribution in the hippocampal neurons was determined by immunocytochemistry and confocal microscopy.[10,23]

2.3 | Electrophysiological studies

Recordings in hippocampus slice were performed in whole-cell patch-clamp configuration.[10,23]

Changes in tonic current (I_{tonic}) are expressed in pA of current. For concentration comparisons, currents were normalized to membrane capacitance (pA/pF) as I_{tonic} density. The presence of miniature GABA-A receptor-mediated inhibitory postsynaptic currents (reflecting circuit integration) prior to gabazine application was a criterion for inclusion in analysis.

Additional details of methods and protocols are listed in Supplemental Information.

3 | RESULTS

3.1 | Sex differences in the protective effects of neurosteroids in pilocarpine-induced SE in mice

3.1.1 | Sex differences in susceptibility to pilocarpine-induced SE—To determine the baseline seizure sensitivity of male and female mice, pilocarpine (100–500 mg/kg, i.p.) was tested at various dose levels, and CD_{50} (convulsant dose in 50% animals) was determined (Figure 1A). The CD_{50} of pilocarpine in males (288, 95% CL: 211–419 mg/kg) was moderately greater than females (176, 95% CL: 123 to 251 mg/kg), but there were no statistically significant differences in CD_{50} for convulsive SE, seizure latency, and

SE occurrence between male and female mice (Figure 1B–C). Consequently, the CD_{97} dose (416 mg/kg) of pilocarpine was used to study seizures in male and female mice. Both the measures of latency for occurrence of clonic seizures (stage 3−4) and SE (stage 5 to SE, Figure 1B) and percent occurrence of seizure (Figure 1C) were not significantly different between male and female mice. Thus, the pilocarpine (416 mg/kg)-induced SE paradigm, which causes similar seizure patterns in male and female cohorts, was then further used for evaluation of neurosteroids in mice.

3.1.2 | Sex differences in protective effects of neurosteroids against pilocarpine-induced SE—To determine if there are inherent sex differences in the neurosteroid protection from seizures, AD was administered in the pilocarpine-SE in male and female mice. As shown in Figure 1D, administration of AD (10−200 mg/kg, s.c.) protected mice against pilocarpine-induced SE in a dose-dependent fashion. 3β-AD (5α-androstan-3β,17β-diol), a pharmacologically inactive stereoisomer of AD,[17] however, had no protective effect in the test (6 of 6 mice had SE at 200 mg/kg dose, data not shown), indicating the stereo-selective antiseizure activity of AD. In AD-treated animals, the seizure latencies were markedly delayed according to the dose given (30–50 min at 10–200 mg/kg AD *vs*. control, <20 min). In female mice, the dose-response curve for seizure protection by AD was significantly left-shifted in a parallel fashion from that of male animals ($p<0.05$), indicating that the protective potency of AD was sex-dependent in the pilocarpine-SE test (Figure 1D). The ED_{50} values derived from these curves are listed in Table 1. The antiseizure ED_{50} value of AD was significantly lower (58%) in females than in males (female ED_{50}, 34 mg/kg; male, 81 mg/kg), confirming a greater protective potency of AD in female animals.

The protective effect of AP was also determined in the pilocarpine-SE in male and female mice (Figure 1E). Mice treated with AP (0.5−50 mg/kg, s.c.) were shown to be protected against pilocarpine-induced SE and persistent seizures in a dose-dependent fashion. In female mice, the dose-response curve for seizure protection by AP was significantly left-shifted in a parallel manner from that of male animals ($p<0.05$), indicating that the protective potency of AP was sex-dependent in the pilocarpine-SE test (Figure 1E). The antiseizure ED_{50} value of AP was significantly lower in females (1.8 mg/kg) than in males (8.5 mg/kg) (Table 1). Potency and maximal efficacy were used to quantify the relative antiseizure activity of neurosteroids. AP was about ten-fold more potent than AD in protecting animals against pilocarpine-induced SE. The sex difference due to relative potency of neurosteroids was expressed as the ratio of ED_{50} in females to ED_{50} in males. The sex-dependent protective potency (ED_{50} ratio) of AP (~4.7-fold as female *vs* male mice) was two-fold greater as compared to AD (~2.4-fold as female vs male mice) (Figure 1F). Overall, neurosteroid protection curves were in the opposite direction to that of seizure occurrence curves from untreated cohorts in females as evident from the leftward shift in the direction of antiseizure responses despite exposure to higher levels of pilocarpine, which clearly indicate that females were better protected than males.

3.1.3 | Lack of sex differences in the protective effects of neurosteroids against pilocarpine-induced SE in mice without δGABA$_A$ receptors—To

determine the sex-specific role of δ-containing receptors in the antiseizure activity of AD and AP, we used δKO mice that lack the tonically active, extrasynaptic receptors in the brain.[22,23] The protective effects of AD and AP were determined in the pilocarpine-SE in male and female δKO mice. The protective dose-response curves of AD (Figure 1G) and AP (Figure 1H) against pilocarpine-induced SE were similar between male and female groups. The antiseizure ED_{50} values of AD and AP were not significantly different between gender (Table 1 and Figure 1I), indicating a lack of sex differences in the protective potency of neurosteroids in δKO mice. In quantitative comparison between WT and δKO female mice, δKO mice displayed a significantly reduced potency of both AP and AD in seizure protection (Table 1). Furthermore, in comparing males, there were not significant differences in seizure protection between WT and δKO mice for either AP or AD treatment group. Thus, the targeted deletion of δGABA-A receptors both ablated the sex-specific effects of neurosteroid protection from seizure as well as reduced the potency of neurosteroid in female mice.

3.1.4 | Neurosteroid plasma levels: relationship to sex-specific seizure protection—

Neurosteroid plasma concentrations were determined 30 min after administration of AD (10−100 mg/kg, s.c.) or AP (1−30 mg/kg, s.c.) in mice. As shown in Figure 1J and 1K, neurosteroid plasma levels increased in a dose-dependent manner in both male and female mice. There were no significant differences in the plasma AD levels achieved with corresponding doses of AD in female and male groups (Figure 1J). Likewise, male and female animals exhibited similar plasma AP levels at all tested doses (Figure 1K). These results indicate that the greater protection of neurosteroids in female mice is not due to pharmacokinetic factors leading to increased plasma and brain neurosteroid levels, but may be due to sex-linked differences in the target GABA-A receptors.

3.2 | Sex differences in the protective effects of neurosteroids in kindling epileptogenesis in mice

3.2.1 | Sex differences in susceptibility to kindling epileptogenesis—

To further describe the proposed sex differences in susceptibility to epileptogenesis, we studied the development of hippocampal kindling in male and female mice (Figure 2). Mice reached the fully kindled state with consistent stage 5 seizures after 12 to 20 stimulations (Figure 2A, 2E). Male mice showed significantly more rapid progression to the development of kindling epileptogenesis, as evident in the fewer number of stimulations required to elicit behavioral seizures, in comparison to females (Figure 2A). Measures of evoked electrographic characteristics revealed a greater induction of the afterdischarge duration in male than female mice (Figure 2B). As shown in Figure 2C, the rate of kindling, expressed as the number of stimulations required to induce stage 5 seizures, was significantly lower in female mice. In addition, females required a greater number of stimulations to achieve various seizure stages (Figure 2). There were no significant differences in the current required to trigger the initial afterdischarge. To determine whether neurosteroid treatment affects kindling epileptogenesis in a sex-specific fashion, we administered AP (0.5 mg/kg) to male and female mice 15-min prior to daily stimulations and determined rate of kindling (Figure 2D). AP produced a significant delay in the rate of kindling in male and female mice, as compared to untreated groups (Figure 2D). AP produced a greater extent of kindling

retardation in female than male mice (Figure 2D), which is consistent with higher neurosteroid sensitivity in female animals.

3.2.2 | Sex differences in the protective effects of neurosteroids in kindling seizure test—To determine the sex differences in the protective effect of neurosteroids against limbic seizures, we tested AD and AP in fully-kindled male and female mice. In fully-kindled mice, the afterdischarge threshold (ADT) current for induction of stage 5 seizures, afterdischarge duration, and severity of seizures at 125% ADT were similar between males and females, indicating the lack of significant sex differences in the expression of kindled seizures. In the kindling test, AD administration in female fully kindled mice produced a dose-dependent suppression of behavioral seizure activity (Figure 3A) with more significant effects at 25–100 mg/kg doses than in male mice. The estimated ED_{50} values for suppression of kindled seizures are listed in Table 1. The extent of suppression of afterdischarge duration by AD was similar between sexes (Figure 3B). Similarly, treatment of females with the neurosteroid AP led to significantly greater seizure suppression than males (Figure 3D), but afterdischarge duration profile was similar between sexes (Figure 3E). In addition, we tested the effect of inactive stereoisomers of the neurosteroids AD and AP in the kindling seizure test. 3β-AD failed to produce a protective effect against kindled behavioral seizure activity and afterdischarge duration (data not shown) even at the highest dose (100 mg/kg). Similarly, 3β-AP (10 mg/kg), an inactive stereoisomer of AP, also did not elicit significant suppression of kindled behavioral seizure activity and afterdischarge duration (data not shown). The failures of 3β-AD and 3β-AP, which are inactive in binding and potentiating GABA-A receptors,[17] to suppress behavioral seizure activity and afterdischarge duration provides strong evidence that the antiseizure effects of AD and AP are due to their stereoselective interaction at GABA-A receptors. Taken together, the above results confirm the sex-dependent protective potency of AD and AP in the kindling test, which is a valid model of complex partial seizures.

3.2.3 | Lack of sex differences in the protective effects of neurosteroids in fully-kindled δKO mice—In contrast to fully-kindled WT mice, the antiseizure potencies of AD (Figure 3C) and AP (Figure 3F) were not significantly different between male and female fully-kindled δKO mice. This notion is demonstrated by similar dose-response profiles for suppression of seizures expression and afterdischarge duration and is consistent with the neurosteroid seizure protection profile in δKO mice observed previously in the pilocarpine seizure model. There was no significant sex difference in the antiseizure ED_{50} value of AD or AP (Table 1). These studies suggest that sex differences in the antiseizure effects of neurosteroids may be attributed to differences in extrasynaptic δGABA-A receptors.

3.2.4 | Enhanced antiseizure sensitivity of ganaxolone in catamenial epilepsy via extrasynaptic δGABA-A receptors.—In addition to endogenous AD and AP, we investigated the protective efficacy of the synthetic neurosteroid ganaxolone in kindling model of perimenstrual catamenial epilepsy in female mice.[16,19] We investigated the efficacy of ganaxolone in female mice undergoing perimenstrual-like neurosteroid withdrawal (NSW), which is linked to catamenial seizure exacerbation.[28,31] NSW is

associated with upregulation of δGABA-A receptors in the hippocampus[26] that may contribute to enhanced seizure protection by ganaxolone. Fully kindled control and NSW female mice were tested in the kindling-catamenial model with three doses of ganaxolone (1, 3, and 10 mg/kg, s.c.). Ganaxolone produced dose-dependent suppression of behavioral seizures (Figure 3G) and afterdischarge duration (Figure 3H) in fully-kindled control and NSW mice. As expected, a significant increase in antiseizure effect was observed in the NSW (catamenial) cohort as compared to the non-withdrawal control group (Figure 3G–H). However, such overt seizure protection was not evident in fully kindled δKO mice undergoing similar NSW as compared to control δKO mice (Figure 3I). Taken together, these results indicate that the enhanced antiseizure activity of ganaxolone in catamenial epilepsy is due to relative abundance of extrasynaptic δGABA-A receptors in NSW females. This molecular mechanism is consistent with the sex differences in antiseizure effects of neurosteroids AD and AP.

3.3 | Sex differences in the expression of δGABA-A receptors in the hippocampus

To determine whether there are differences in the expression levels of δGABA-A receptors between males and females, we performed quantitative TaqMan PCR analysis of mRNA in hippocampus tissue samples. We previously used quantitative PCR to demonstrate an ovarian cycle-related plasticity in δGABA-A receptor expression.[10] Expression of δ-subunit and its partnering α_4-subunit mRNA was strikingly greater in the hippocampus in females animals (Figure 4A). There were no significant differences in hippocampal expression of α_2 and β_2 subunits. We further quantified copy numbers of δ- and α_4-subunit mRNA (Figure 4B). The mean mRNA copy number of δ-subunit in females was over 3-fold greater than in males. To further define the role of neurosteroids in δ-subunit plasticity,[10,23] an analysis of δ-subunit expression was performed in mice that were treated with finasteride (50 mg/kg, i.p.), a 5α-reductase inhibitor that blocks neurosteroid synthesis in the brain. Both finasteride-treated, male and female mice experienced significantly reduced hippocampus δ-subunit expression compared to vehicle-injected control (Figure 4C). However, the finasteride-induced reduction of δ-subunit was drastically more pronounced in females (Figure 4C), suggesting that neurosteroids regulate the δ-subunit plasticity in females. To confirm the elevation of δGABA-A receptor protein in the hippocampus, we determined the single cell distribution of δ-subunit by immunocytochemistry using a δGABA-specific primary antibody that we fluorescently tagged. Antibody staining showed broad distribution of δ-subunit on the soma, axon, and dendritic regions of acutely dissociated CA1 pyramidal cells and DGGCs taken from male and female mice (Figure 4D). The mean δGABA-A receptors expressional intensity was significantly greater in DGGCs from female mice (Figure 4F), indicating sex differences in the plasticity of extrasynaptic δGABA-A receptors. A representative Nissl image shows two subfield regions where the neurons were quantified for δ-expression within the hippocampus (Figure 4E). The δ-subunit expression was totally absent in DGGCs from δKO mice (Figure 4D–F), confirming the validity of the methodology for studying δ-subunit expression.

3.4 | Sex differences in neurosteroid allosteric activation of δGABA-A receptor-mediated tonic currents in the hippocampus

To directly investigate whether sex differences in δGABA-A receptor expression are associated with functional properties of neurons, we examined the δGABA-A receptor-mediated tonic currents (I_{tonic}) in DGGC from male and female mice. To derive the basal tonic current contributed by extrasynaptic receptors, we examined exogenous GABA responses in patch-clamp recordings from DGGCs in brain slices. Tonic currents were recorded using the GABA-A receptor competitive antagonist gabazine (50 μM) to block all GABAergic current, as previously demonstrated.[21] Non-desensitizing, exogenous GABA (1 μM) was applied to continuously gate extrasynaptic receptors in order to measure allosteric modulation by neurosteroid.[10,23] Rate of change was monitored in real-time, and current recordings were allowed to reach an asymptotic, steady-state level of neurosteroid perfusion before complete block by gabazine to determine total I_{tonic} shift (pA/pF). The mean I_{tonic} response from 0.1 μM AP + 1 μM GABA application (0.86 ± 0.17 pA/pF, n = 6 cells) was greater than, but not significantly different than 1 μM GABA in male DGGCs (0.66 ± 0.22 pA/pF, n = 8 cells; p = 0.312). Increasing concentrations of AP (1.0 μM) elicited a concentration-dependent potentiation of I_{tonic} responses in males and females. Consistent with a greater abundance of δGABA-A receptors in female mice, the neurosteroid AP elicited a significantly greater ($p<0.05$) tonic current as evident from I_{tonic} responses in DGGCs from females as compared to male mice (Figure 5A–B). As EC_{50} could not be determined due to lack of ceiling effect[10,23], we calculated the EF_{100} values to represent the effective functional concentration of drug (nanomolar) required to double the 1 μM GABA response. The EF_{100} responses occurred at significantly lower concentrations of AP in females than in males. However, such enhanced AP sensitivity was not observed in female δKO mice (Figure 5C–D). Thus, these results indicate a greater sensitivity of DGCCs towards the AP in eliciting tonic currents, which are mediated by extrasynaptic GABA-A receptors, especially δGABA-A receptors.

3.5 | Sex differences in the antiseizure sensitivity of ganaxolone in the 6-Hz seizure test in mice

To confirm whether sex-dependent, protective effects of neurosteroids extend to other acute seizure models, we tested the effect of synthetic neurosteroid ganaxolone in the 6-Hz model of partial seizures.[27,28] To test the behavioral sensitivity to 6-Hz stimulation, we first determined incidence of seizures as a function of current intensity in mice (Supplement Figure S1A). Current intensity of 32 mA elicited seizures in 100% of the population of male and female mice. For this reason, further drug studies were conducted at this level of current. Mice were treated with ganaxolone (0.6–20 mg/kg, s.c.)15 min prior to stimulation. After each stimulation, incidence of seizure protection was recorded. Dose-response curves were derived for ganaxolone (Supplement Figure S1B), in term of ED_{50} between male and female mice. Ganaxolone dose-response curves revealed significant sex differences with a greater sensitivity in female (ED_{50}: 1.5 ± 0.1 mg/kg) mice than in males (ED_{50}: 2.9 ± 0.4 mg/kg).

4 | DISCUSSION

There are limited studies on sex differences in seizure protection after neurosteroid therapy. In this study, we demonstrate the robust sex-dependent protective effects of the neurosteroids AD, AP and ganaxolone in experimental SE and partial seizures, but such sex differences are not related to pharmacokinetic factors. Our evidence strongly suggests that sex differences in the antiseizure potency of neurosteroids are due to sexually dimorphic abundance of δGABA-A receptors and tonic currents in the hippocampus. Therefore, neurosteroid sensitivity at δGABA-A receptors confers significant inhibitory control over neuronal excitability and thereby provides robust seizure protection in females than males. These findings underscore the role of neurosteroids in sex-related seizure susceptibility and thereby strengthen the rationale for developing personalized gender-specific, neurosteroid therapy for SE and epileptic seizures in men and women, including catamenial epilepsy.

Our results reveal a dose-dependent antiseizure activity of AD or AP in the mouse pilocarpine model of SE. AD is a neurosteroid synthesized from testosterone within the brain and has been shown to elicit broad spectrum of antiseizure activity in a variety of animal seizure models,[13,32] and AD also protects mice against seizures induced by several chemoconvulsants and electrical stimulation paradigms.[13,33] In the present study, the protective effect of neurosteroids and synthetic analog against seizures in the pilocarpine-SE model, kindling model, and 6-Hz stimulating model is sex-specific, as the AD protective potency is about two-fold higher in females compared to males, and the potency of AP is about four-fold higher in female than in male mice in pilocarpine–SE model (Figure 1). This is highly consistent with the sex differences in the antiseizure effect of progesterone and neurosteroids in the pentylenetetrazol seizure model. [35,36] Sex differences in neurosteroid sensitivity are not related to differences in pharmacokinetics or metabolism of neurosteroids (Figure 1J–K). The sexual dimorphism in brain circuits involved in initiation, synchronization, or propagation of seizures or the differential distribution of steroid receptors could account for the sex-related differences in neurosteroid seizure protection. It is well known that female animals are more sensitive to the neuroprotective actions of progesterone than are males.[35] Since AD and AP potentiate the GABA-A receptor function, it is suggested that AP could contribute to enhanced protective potency of AD in females in part by additive or synergistic effects at GABA-A receptors. Alternatively, testosterone-derived estrogens may possibly dampen the protective effect of AD in males,[37] and thereby reduce its potency. In addition, it is likely that AD and AP might also increase absence seizures in genetic absence models, most likely via their actions at δGABA-A receptors in the thalamus.[38]

Our results suggest that the sex differences in the protective potency of neurosteroids and its synthetic analogs could be related to GABA-A receptor subunit plasticity. The δ-subunit containing GABA-A receptors, located perisynaptically/extrasynaptically, mediate tonic current activated by ambient GABA levels.[19,39] Neurosteroids can activate most GABA-A receptor isoforms, but the δ-containing receptors are more sensitive to neurosteroids.[40,41] The δ-subunit undergoes dynamic plasticity during the ovarian cycle in females with high expression at late diestrous and low expression at other stages.[8,10] Elevated neurosteroid levels associated with physiological conditions (e.g. ovarian cycle and stress) can upregulate

δ-subunit expression in the hippocampus, and thereby augment tonic inhibition.[10,31,42] Because AP and AD are present in substantial quantities in serum, cerebrospinal fluid, and brain,[43–46] they act as endogenous regulators of neuronal excitability by activating δGABA-A receptors. Indeed, we observed a relatively greater δGABA-A receptor expression in the hippocampus in females than males, confirming the role of extrasynaptic δGABA-A receptors in sex-specific response to neurosteroid actions (Figure 4). This observation is consistent with the ovarian cycle-linked regulation of the plasticity and function of extrasynaptic GABA-A receptors.[10,23,31] This in turn resulted in functional increase to neurosteroid potentiation of tonic currents (Figure 5). Previous reports attest such GABA-A receptor changes within hippocampal neurons in females. Moreover, sex-related differences in binding affinity to certain ligands is observed at GABA-A receptors.[47,48] Consistent with our findings, δ-subunit expression in the hippocampus of female mice a diestrous stage, assessed by western blot analysis, is significantly higher than in control male mice.[42] The δ-subunit expression is also markedly higher in females at estrous as compared to males.[8] This data is highly consistent with the possibility that the greater δGABA-A receptors that mediate tonic inhibition may underlie the greater potency of neurosteroids in females. Our earlier studies have demonstrated a significantly reduced susceptibility to kindling epileptogenesis in mice at diestrus relative to estrus phase.[10] Likewise, the AP-treated mice in diestrus showed significant suppression of seizure activity compared to those in estrus, which is consistent with the increased expression of δGABA-A receptors during diestrus. Similarly, neurosteroids were highly effective in catamenial seizures due to diestrous-like upregulation of δGABA-A receptors in the hippocampus. Overall, potential differential expression of δGABA-A receptors in males and females or their greater plasticity in females may contribute to the greater protective potency of neurosteroids.

In conclusion, our results demonstrate a robust sex-specific antiseizure activity of neurosteroids AD, AP, and ganaxolone in females compared with male mice. Consistent with a greater expression of extrasynaptic δGABA-A receptors, AP produced an enhanced potentiation of tonic currents in hippocampal granule cells in normal wildtype but not in δKO female mice. Thus, sexually dimorphic, GABAergic tonic inhibitory circuits in the hippocampus may contribute to sex differences in seizure susceptibility and neurosteroid protection. These findings have broader implications in designing personalized sex-specific, neurosteroid therapies for epileptic seizures, such as SE, complex partial seizures, and catamenial epilepsy.[49]

Supplementary Material

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Key Points

- Sex difference in seizure susceptibility is a widespread concern in epilepsy
- Neurosteroids exhibit robust antiseizure activity in a sex-specific fashion
- High abundance of extrasynaptic δGABA-A receptors confers greater tonic inhibition in females
- Sexually dimorphic δGABA-A receptors plasticity contributes to sex differences in seizure susceptibility and neurosteroid sensitivity

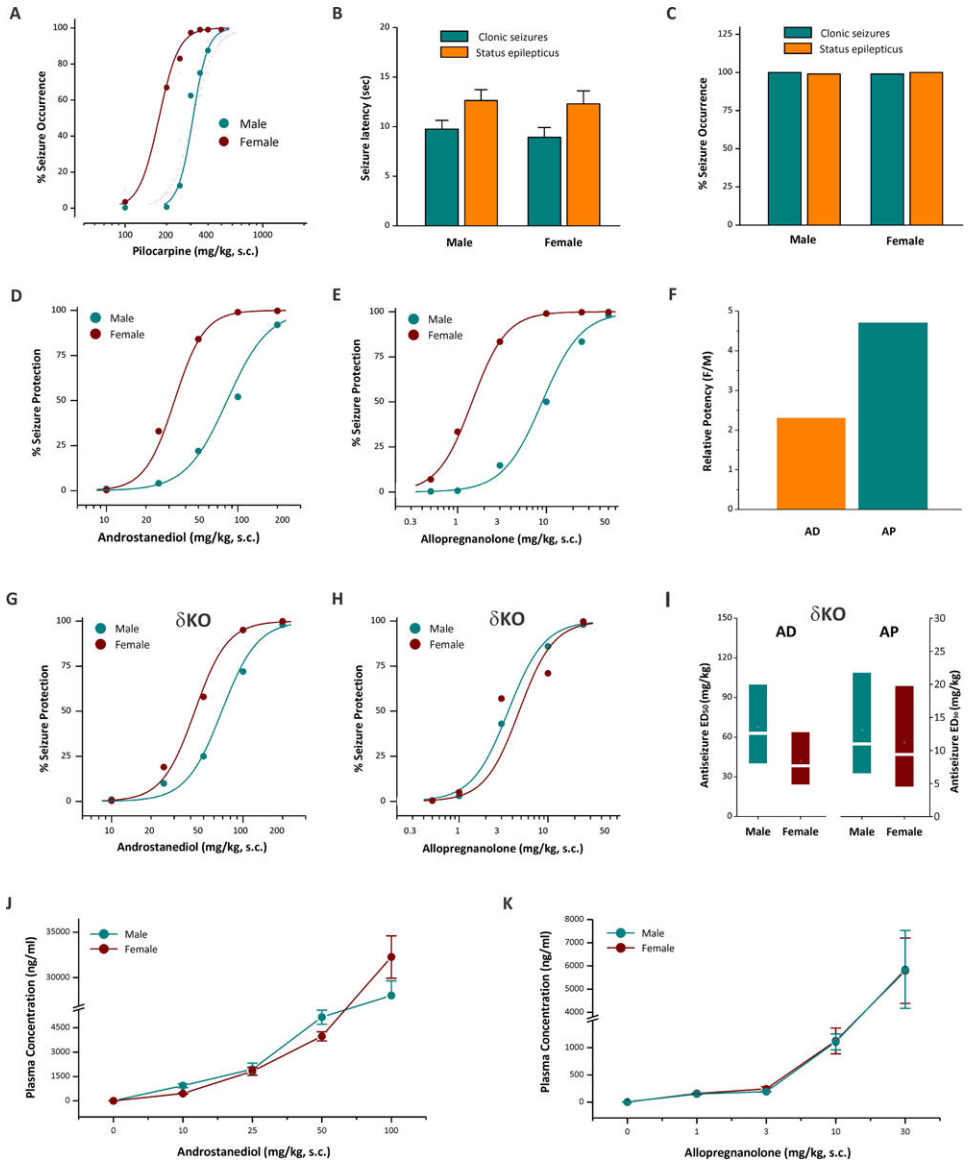

FIGURE 1.

Sex differences in the antiseizure activity of neurosteroids androstanediol (AD) and allopregnanolone (AP) in the pilocarpine model of SE in wild type (WT) and δGABA-A receptor knockout (δKO) mice. A, Dose-dependent seizure induction of pilocarpine (100 – 500 mg/kg, i.p.) in male and female mice. B, The time to clonic seizures and status epilepticus onset in male and female (at diestrous) mice following injection of pilocarpine (416 mg/kg, i.p. used to study seizures). C, The percentage of animals showing clonic seizures and status epilepticus in male and female mice following injection of pilocarpine. Clonic seizures and SE were quantified as described in *Methods section*. Mice failing to show SE or persistent seizures as defined in *Methods section* were scored as protected. D and E, Dose-response relationship for AD (10 – 200 mg/kg, s.c.) and AP (0.5 – 50 mg/kg, s.c.) protection against pilocarpine-induced SE in females was significantly ($p < 0.05$, Litchfield & Wilcoxon's χ^2 test) shifted to the left from that of males. F, Both neurosteroids

are relatively (2–5 fold) more potent in females than males. Sex-linked relative potency was derived from the relative potency ratio from the ED_{50} values (i.e. the dose producing 50% protection) in female to value in male (F/M). Neurosteroids were administered 15 min before pilocarpine SE test. ED_{50} values are given in Table 1. G and H, Dose-response curves for AD and AP protection against pilocarpine-induced SE are similar between δKO males and females. I, Summarized data of antiseizure ED_{50} values of AD and AP were similar different between δKO genders. n = 6−8 mice per group for panels of *A*−*I*. J, LC-MS measured AD plasma concentrations in male and female mice treated with AD (10–100 mg/kg, s.c.). K, LC-MS measured AP plasma concentrations in mice treated with AP (1–30 mg/kg, s.c.). Plasma samples were taken 30 min after administration of the indicated dose of neurosteroids. Values represent the mean ± SEM (6–8 mice per group).

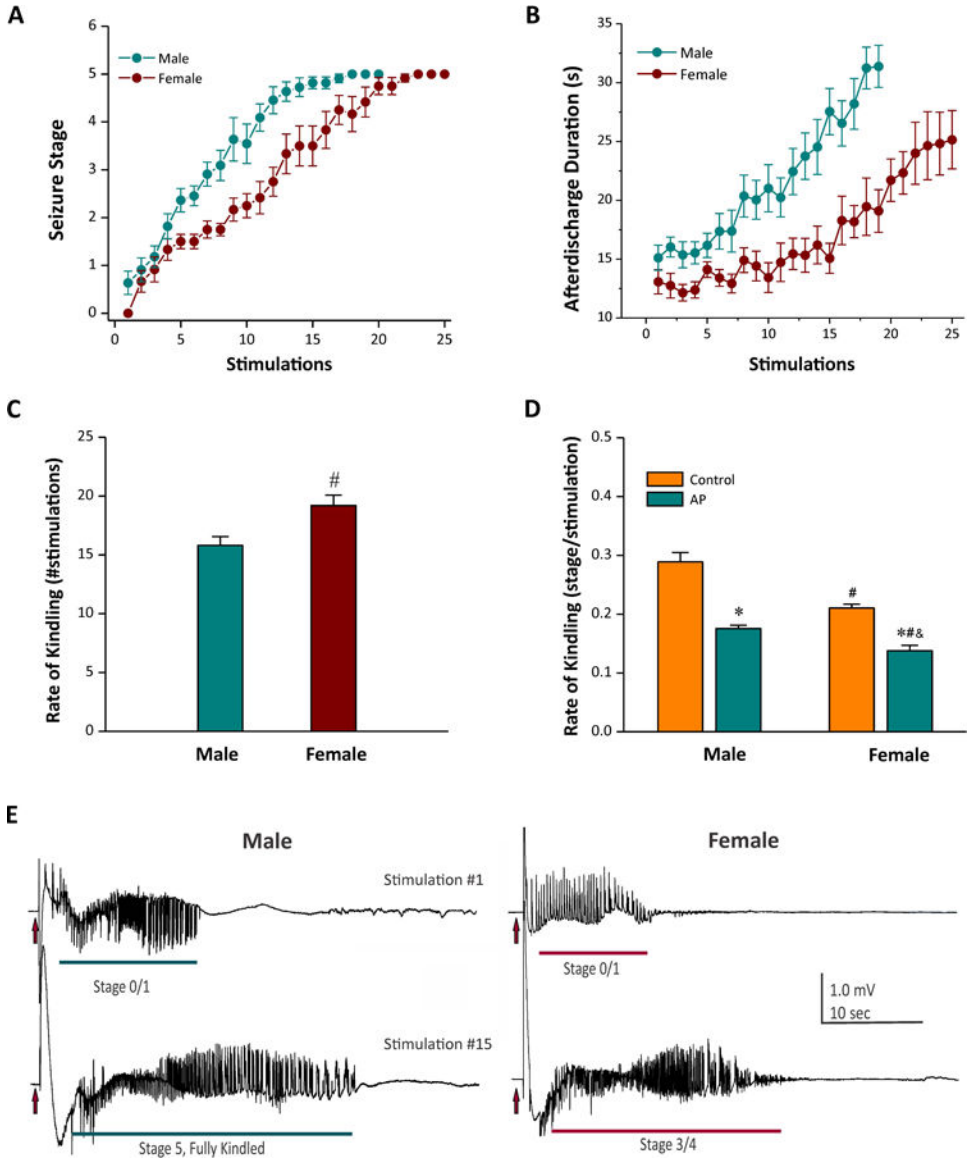

FIGURE 2.

Sex differences in the development of hippocampus kindling epileptogenesis and disease-modifying effect of the neurosteroid AP in mice. A, Female mice displayed marked retardation of kindling development as expressed by a lower mean seizure stage at the corresponding stimulation session ($p < 0.05$). B, Afterdischarge duration was markedly lower in females than in male mice ($p < .05$). C, Rate of kindling development (number of stimulations for stage 5 seizures) was significantly higher (i.e. inhibited) in female mice. D, Rate of kindling development (seizure stage/stimulation) was significantly inhibited by AP (0.5 mg/kg, s.c.) in male and female mice; the protective effect of AP for inhibition of kindling epileptogenesis was statistically significant in females compared to males. **E,** Representative traces of electrographic afterdischarge at stimulation 1 or 15 in male and female mice during kindling development. Values represent the mean ± S.E.M. (n = 6−13

mice per group). *p<0.05 versus control within sex group; #p<0.05 versus male in panel *C* and male (control group) in panel *D*; &p<0.05 versus male AP group.

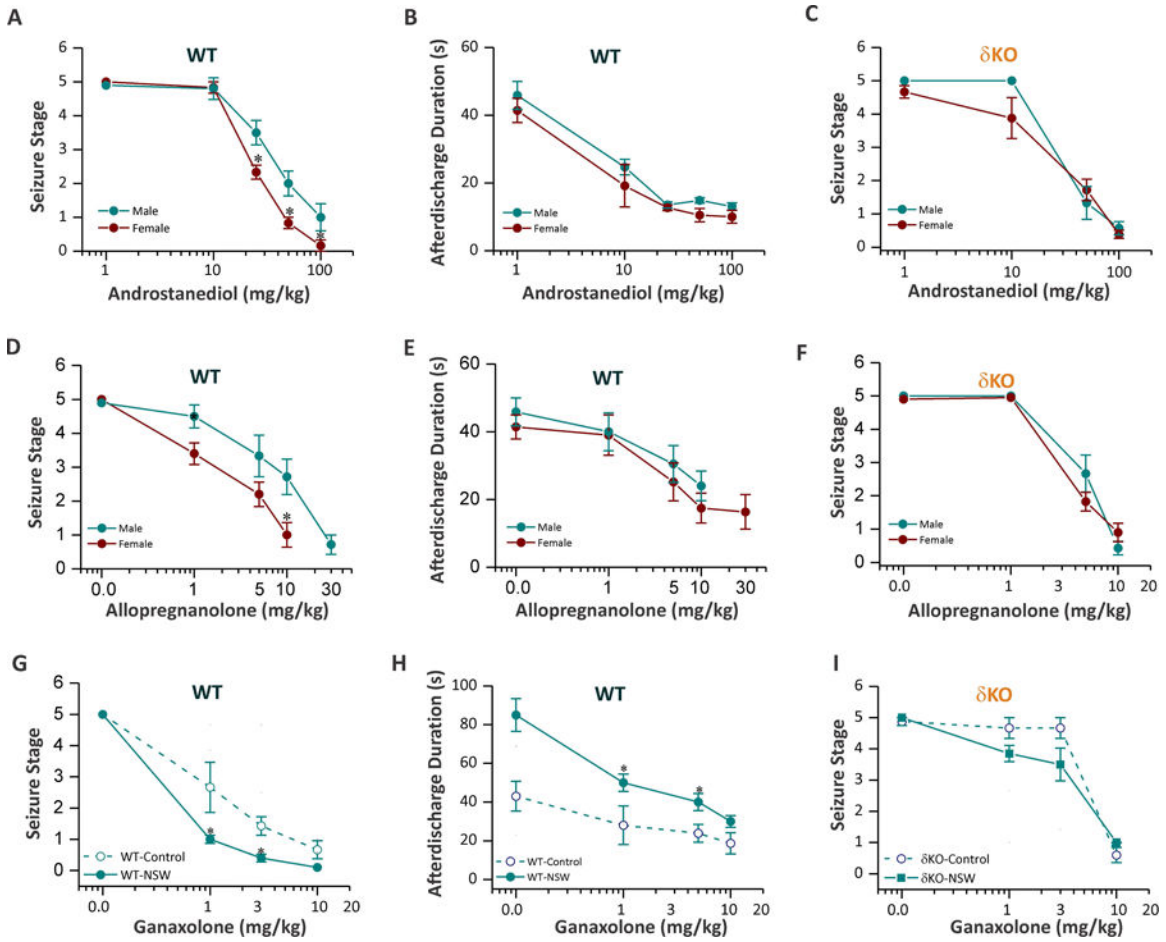

FIGURE 3.

Sex differences in the antiseizure effects of the neurosteroids AD, AP and ganaxolone in fully-kindled mice. Fully kindled mice with stage 5 seizures were utilized for testing the effect of neurosteroids on seizure activity. A and B, Dose-response curves for AD-induced (10–100 mg/kg, s.c.) suppression of behavioral seizure stage and afterdischarge duration in mice. C, Lack of sex differences in antiseizure activity of neurosteroids AD in fully- kindled δKO mice. D and E, Dose-response curves for AP-induced (1–10 mg/kg, s.c.) suppression of behavioral seizure stage and afterdischarge duration in mice. F, Lack of sex differences in antiseizure activity of neurosteroids AD in fully- kindled δKO mice. The behavioral seizure protection curves for AD and AP were significantly different between males and females ($p < 0.05$). *$p < 0.05$ *vs* male group at panels of *A*−*D*. G and H, Dose-response curve for ganaxolone (1–10 mg/kg, s.c.)-induced suppression of behavioral seizure stage and afterdischarge duration in female WT non-withdrawal control and neurosteroid withdrawal (NSW) groups, a condition associated with upregulation of δGABA-A receptors in the hippocampus. I, Lack of sex differences in antiseizure activity of ganaxolone in δKO non-withdrawal control and NSW group. Test drugs were given 15 min before kindling stimulations. *$p<0.05$ *vs* WT-control (non-withdrawn) group at panels of *G*−*H*. Data represent mean ± S.E.M. (n = 6−13 mice per group).

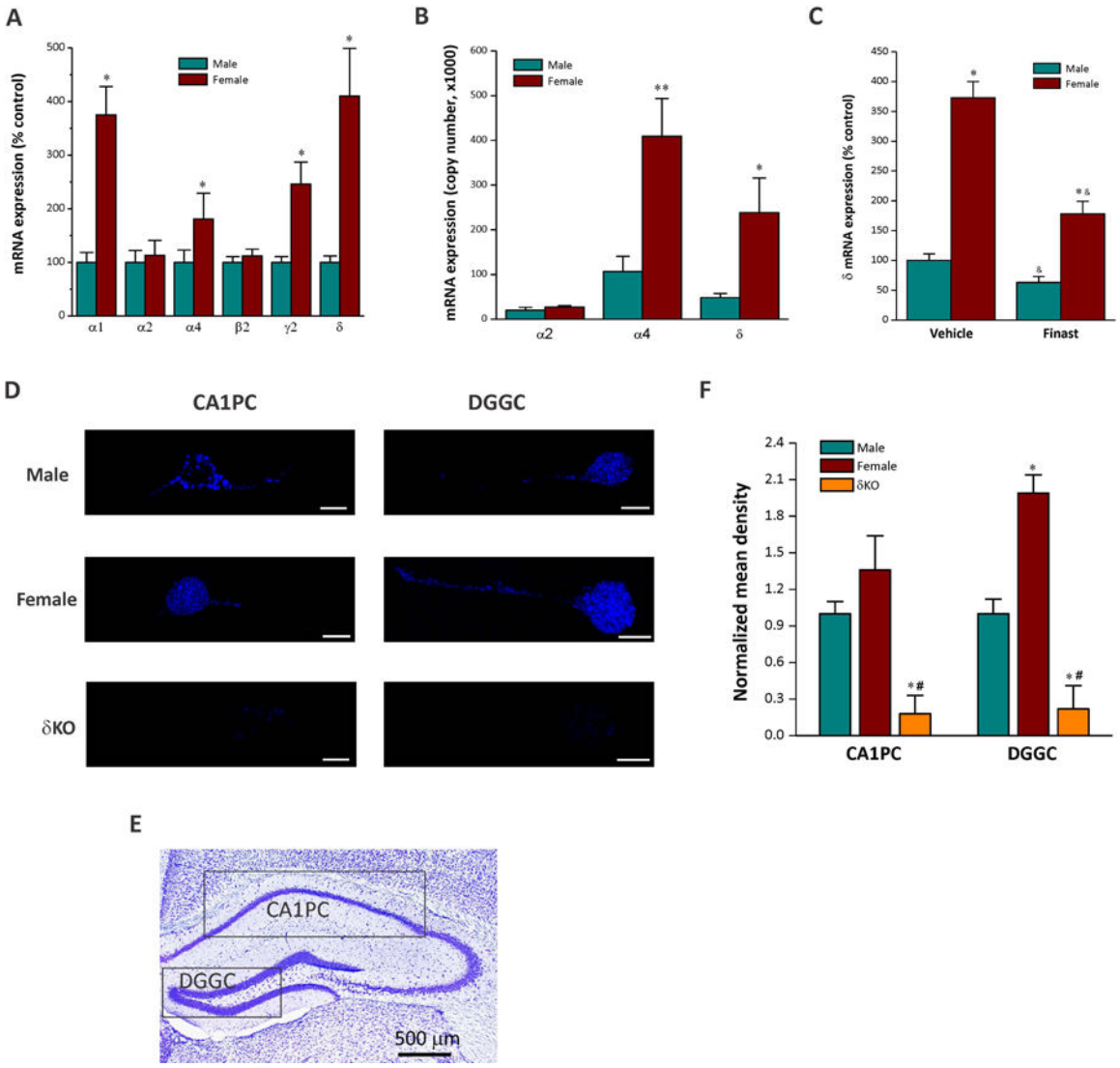

FIGURE 4.

Sex differences in the expression of δGABA-A receptors in the hippocampus in mice. A, Upregulation of δ mRNA expression in female (at diestrous) mice compared to male mice as measured by TaqMan real-time PCR. B, Significantly greater δ mRNA copy number in female hippocampus. C, Finasteride treatment (Finast, 50 mg/kg, i.p. for 1 week) significantly decreased δGABA mRNA expression in female mice. (for panels of *A*−*C*: n = 6–8 mice per group). D, Immunocytochemical distribution of δGABA-A receptors in hippocampal CA1 pyramidal neurons (CA1PC) and dentate gyrus granule cells (DGGCs) from female (diestrous) and male mice. E, The Nissl image depicts two different regions where neurons were quantified for δ-subunit expression within the hippocampus. F, Relative quantification of the abundance of δGABA-A receptors in CA1PCs and DGGCs from male and female mice. Note the complete absence of δGABA-A receptor staining in DGGCs from δKO mice. Bar = 10 μm (Panel D). Data represent mean ± S.E.M. (for panels of *D and F*: n = 6−9 cells from 3–4 mice per group). *p<0.05 *vs* male; **p< 0.01 *vs* male; #p<0.05 *vs* female group at panel *F*; &p<0.05 *vs* same sex in the vehicle group at panel *C*.

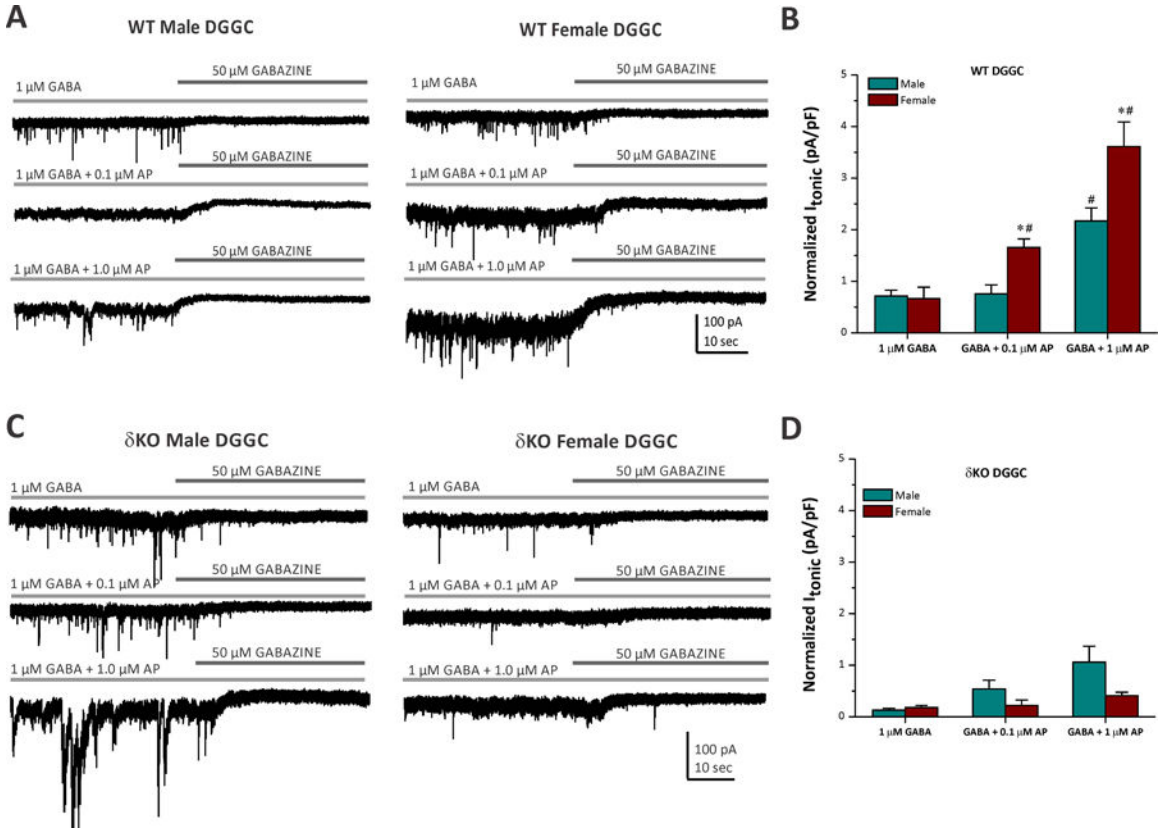

FIGURE 5.

Sex differences in the AP potentiation of extrasynaptic δGABA-A receptor-mediated tonic currents in the hippocampal DGGCs in WT and δKO mice. A, Representative tonic current recordings from DGGCs in slices from WT male and female mice. AP (0.1 – 1.0 μM) was applied to the bath in addition to 1 μM GABA to measure allosteric enhancement of tonic current. 1 μM GABA, AP, and gabazine were sequentially applied in perfusion to measure tonic current shift. B, Summarized data of AP concentration-response from male and female DGGCs in WT mice. Female DGGCs displayed significantly greater tonic current potentiation by AP compared to males. C, Representative tonic current recordings in the presence of AP or/and GABA from DGGCs in slices from δKO mice. D, Summarized data of AP concentration-response from male and female DGGCs in δKO mice. Hippocampal slices were from diestrous female or male mice, recorded in whole-cell mode, voltage-clamped at −70 mV. Averaged amplitude of tonic current shift was measured. The GABA-A receptor tonic current was expressed as the outward shift in holding current after the application of gabazine (50 mM). Tonic current was measured and averaged in 100 milliseconds for each epoch with 1-second intervals between epochs for 30 epochs. The measurements were taken 30 seconds before and 2 to 3 minutes after application of a drug. Tonic current was normalized to cell capacitance (pA/pF) as a measure of current density. *p<0.05 *vs* males; #p<0.05 *vs* 1 μM GABA baseline current in the same sex (n = 6−8 cells per group).

TABLE 1

Antiseizure ED_{50} values (mg/kg) of neurosteroids in the kindling model and pilocarpine model of status epilepticus (SE) in male and female WT and GABA-A receptor δ-subunit knockout (δKO) mice.

	Androstanediol		Allopregnanolone	
	WT	**δKO**	**WT**	**δKO**
Pilocarpine SE				
Male	81 (45−143)	63 (40–101)	8.5 (3−25)	12 (6–22)
Female	34 (22−51) *	38 (22–65)	1.8 (0.9−3.3) *	9.4 (4–20) #
Kindling seizures				
Male	54 (39–70)	18 (8.2–43)	8 (4.5–13.2)	4.7 (2.5–8.6)
Female	30 (23–41) *	1.6 (7.8–42)	3 (2.1–4.8) *	4.2 (2.3–7.8)

Numbers in parentheses are 95% confidence limits.

* $p<0.05$ vs. male group

# $p<0.05$ vs. WT group (Litchfield and Wilcoxon χ^2 test. n=6–13).