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·Original Article·

# Gender difference in acquired seizure susceptibility in adult rats after early complex febrile seizures

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## ABSTRACT

Gender differences are involved in many neurological disorders including epilepsy. However, little is known about the effect of gender difference on the risk of epilepsy in adults with a specific early pathological state such as complex febrile seizures (FSs) in infancy. Here we used a well-established complex FS model in rats and showed that: (1) the susceptibility to seizures induced by hyperthermia, pentylenetetrazol (PTZ), and maximal electroshock (MES) was similar in male and female rat pups, while males were more susceptible to PTZ- and MES-induced seizures than age-matched females in normal adult rats; (2) adult rats with complex FSs in infancy acquired higher seizure susceptibility than normal rats; importantly, female FS rats were more susceptible to PTZ and MES than male FS rats; and (3) the protein expression of interleukin-1β, an inflammatory factor associated with seizure susceptibility, was higher in adult FS females than in males, which may reflect a gender-difference phenomenon of seizure susceptibility. Our results provide direct evidence that the acquired seizure susceptibility after complex FSs is gender-dependent.

**Keywords:** gender difference; complex febrile seizures; seizure susceptibility; epilepsy; IL-1β

## INTRODUCTION

Gender differences are associated with many neurological disorders including epilepsy[1-4], and are involved in its diagnosis and treatment. For example, the prevalence of epilepsy is higher in men than in women[5]; men are more vulnerable to seizure-associated brain damage than women[6]; and gender differences may influence the treatment effect of anticonvulsants[7, 8]. However, these reports only represent a general outline, and little is known about the effects of gender differences in the risk of epilepsy in adults with a specific pathological state in early life.

Febrile seizures (FSs) are the most common type of seizure in childhood, occurring in 2%–5% of children. Most FSs are simple, having a brief duration and a low risk of developing epilepsy[9, 10]. In contrast, one-third of FSs are 'complex', prolonged (>10–20 min) or repeated, and may be associated with a risk of subsequent mesial temporal sclerosis and intractable epilepsy[11-13]. Although some risk factors for developing epilepsy after FSs have been summarized in previous work[14], these retrospective studies could not separate the effect of a FS itself (e.g., severity, seizure duration, and type) from that of pre-existing brain pathology (e.g., brain injury and non-FSs) and the influence of treatment.

To exclude these problems, we used a well-established animal model in which FSs were evoked by exposing rat

pups to a hyperthermic environment[15]. We focused on the gender difference in seizure susceptibility of rats that experienced complex FSs in infancy. Then the maximal electroshock (MES)-and pentylenetetrazol (PTZ)-induced seizure models were used to evaluate seizure susceptibility; these models are considered to be 'gold standards' for the early detection of anticonvulsant effects[16].

## MATERIALS AND METHODS

### Animals

Sprague-Dawley rats (Grade II, Certificate No. SCXK2008-0033, Experimental Animal Center, Zhejiang Academy of Medical Science, Hangzhou, China) were used in this study. Parturition was checked daily, and the day of birth was considered postnatal day 0 (P0). When weaned on P21, rats were housed five per cage according to sex with a 12:12 h light-dark cycle (lights on from 08:00 to 20:00), with water and food *ad libitum*. Experiments were carried out between 10:00 and 17:00 from P10 to P60. All experiments were approved by the Zhejiang University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Attempts were made to minimize the number of animals used and their suffering. Simple randomization was applied for allocation.

In our experiments, three groups of 8 male and 8 female rat pups were each used to evaluate the gender difference of seizure susceptibility to FSs, for PTZ treatment, and for MES. These pups were sacrificed after experiments. In addition, 24 normal male, 24 normal female, 24 FS male, and 24 FS female rat pups were raised to adulthood for further experiments.

### Generation of Experimental Complex Febrile Seizures

Complex FSs were induced in rat pups on P9–P10 according to previous studies[11, 15] with modifications. After weighing, the body temperature of the pups was raised in a chamber at 42–46°C. Core temperature was measured at baseline (33.5–34.8°C) and seizure onset (39.5–42°C). The pups were moved to cool surfaces for 2 min once a seizure was evoked. This process was repeated 10 times and the total behavioral seizure time was ~26 min, which is considered to be prolonged[17]. The latency and temperature threshold to the first onset of hyperthermia-induced seizures were recorded in male and female pups. The behavioral seizures induced by hyperthermia consisted of sudden movement arrest, followed by facial automatisms (chewing), forelimb clonus, and tonic flexion of the body, often associated with a loss of postural control. All pups experienced 10 seizures and the total time in the chamber was ~60 min. The skin of all pups was carefully observed during the FSs, and at 15 min, 6 h, 1 day, 2 days, and 3 days after the FSs, to make ensure there were no burns.

After hyperthermia, the animals were weighed and moved to a cool surface until the core temperature returned to the normal range for age, and then they were returned to the dams. Control rats were littermates of the experimental group. They were separated from the dams for the same duration, and their core temperatures maintained within the normal range for age.

### Electrophysiological Recording *In Vivo*

To study the concordance of behavioral and electrophysiological seizures provoked by hyperthermia and MES, bipolar electrodes were implanted unilaterally in the dorsal hippocampus of rat pups. Baseline hippocampal electroencephalograms (EEGs) were recorded 24 h later, followed by seizure induction by hyperthermia and MES[18, 19]. In detail, electrodes were implanted under halothane anesthesia (2%–3% halothane inhaled through muzzle), using an infant rat stereotaxic apparatus (512600, Stoelting). Bipolar twisted wire electrodes, enameled except for the tip, were inserted into the dorsal hippocampus through a burr hole at the coordinates AP –1.5, L 1.7, and V 3.0 mm with reference to bregma (wire diameter, 0.1–0.15 mm; vertical inter-tip distance, 0.5–1.0 mm). The electrodes were anchored to the skull with an acrylic cement "cap" also attached to two screws. Recordings were performed in heated, shielded Plexiglas chambers. EEG was recorded *via* long, flexible wires to the freely-moving rat pups, and was continuously recorded throughout the FSs. In total, 5 male and 5 female pups were used for EEG recording.

### Maximal Electroshock Seizure Threshold Test in Rat Pups

The protocols for testing the threshold for MES in P11–P12 pups were modified from previous studies[20]. The threshold was determined using a rodent shocker (Hugo Sachs Elektronik, March-Hugstetten, Germany), which

delivered a constant current (0.2 s, 50 Hz) through a pair of ear-clip electrodes. The endpoint was tonic extension of the hindlimbs. The stimulus current intensity began at 5 mA, was increased in 5-mA steps, and was lowered by 5 mA if the preceding shock caused tonic hindlimb extension. All data were collected and analyzed in a blinded manner. Three stages were used as measures of seizure susceptibility: stage 0: no forelimb extension and no spikes in the EEG; stage 1: complete forelimb extension and a few spikes (<10 s, less than 1 Hz) in the EEG; and stage 2: complete forelimb extension with partial hindlimb extension and a large number of intense spikes (high-frequency rhythmic epileptiform discharges, more than three times the amplitude at baseline) in the EEG.

**Maximal Electroshock-Induced Seizures in Adult Rats**

When rats reached 60 days old, they were subjected to 40 mA or 60 mA MES (0.2 s, 50 Hz) according to the experimental design. The convulsion patterns in adult rats were assigned stages based on the extent of the spread of tonic extension[21]: 0, absence of forelimb extension; 1, complete forelimb extension without hindlimb extension; 2, complete forelimb extension with partial hindlimb extension; and 3, complete fore- and hindlimb extension (with hindlimbs fully extended parallel to the tail).

**Pentylenetetrazole Seizure Threshold Test**

The PTZ seizure threshold test was conducted according to previous studies[22] in P11–P12 pups and P60 adult rats. Briefly, intraperitoneal PTZ injections were started at 20 mg/kg and if necessary, repeated with 10 mg/kg every 10 min, and behavioral seizures were registered after injections. The cortical EEG was recorded in six rats, three males and three females. The seizure severity was evaluated as follows: 0: no response; stage 1: ear and facial twitching; stage 2: convulsive waves throughout the body; stage 3: myoclonic jerks, rearing; stage 4: turning over onto one side; stage 5: turning over onto the back, generalized tonic-clonic seizures; and stage 6: death. In addition, the latencies to the onset of stages1–6 were recorded. In this experiment, the interval between PTZ injections was 10 min, and the total administration frequency was 6–7 times until the rats reach stage 6. Seizures were observed from the first injection to the end of experiments (60–90 min).

**Acute Pentylenetetrazole-Induced Seizure Model in Adult Rats**

Adult rats (P60) were injected intraperitoneally with a single dose of 60 mg/kg PTZ. The animals were observed for 60 min after PTZ administration, and seizure stage, latency to the first minimal clonic seizure (MCS), latency to the first generalized tonic-clonic seizure (GTCS), and mortality rate were recorded[23].

**Western Blotting**

Proteins were extracted from the hippocampus of rat pups (immediately after FSs) and P60 rat adults. The amount of protein sample for each kit was 100 μg. Protein extracts were separated by SDS-PAGE on a 14% resolving gel with a stacking gel and transferred onto nitrocellulose membranes. The membranes were then blocked in 5% non-fat milk for 1 h at room temperature, and incubated with anti-IL-1β (1:500, Cell Signaling Technology) or anti-GAPDH (1:500, Santa Cruz) primary antibody (diluted in TBS/0.05% Tween) overnight at 4°C. Then they were washed and probed with the respective horseradish peroxidase-conjugated secondary antibody (Odyssey, LI-COR, 1:5000 dilution) for 2 h at room temperature. The immunoreactive bands were visualized using the ECL detection reagent (Millipore, Billerica, MA).

**Data Analysis**

Data are expressed as mean ± SEM. In the PTZ seizure threshold test, two-way ANOVA with Tukey's *t*-test was used to calculate the statistical significance. The $\chi^2$ test was used for the statistics of ratios. For the analysis of MCS and GTCS latencies after PTZ injection, one-way ANOVA with Tukey's *t*-test was used. For the analysis of latency to and threshold of FS generation, and seizure stage after PTZ injection and MES, a nonparametric test was used. Two-tailed tests were used. $P < 0.05$ was considered significant.

## RESULTS

**No Gender Difference in Seizure Susceptibility of Rat Pups**

The latencies and threshold temperatures of febrile seizures in male pups were similar to those of female pups at P10 (Fig. 1A and B). Representative EEGs of seizure discharges are shown in Fig. 1C and D.

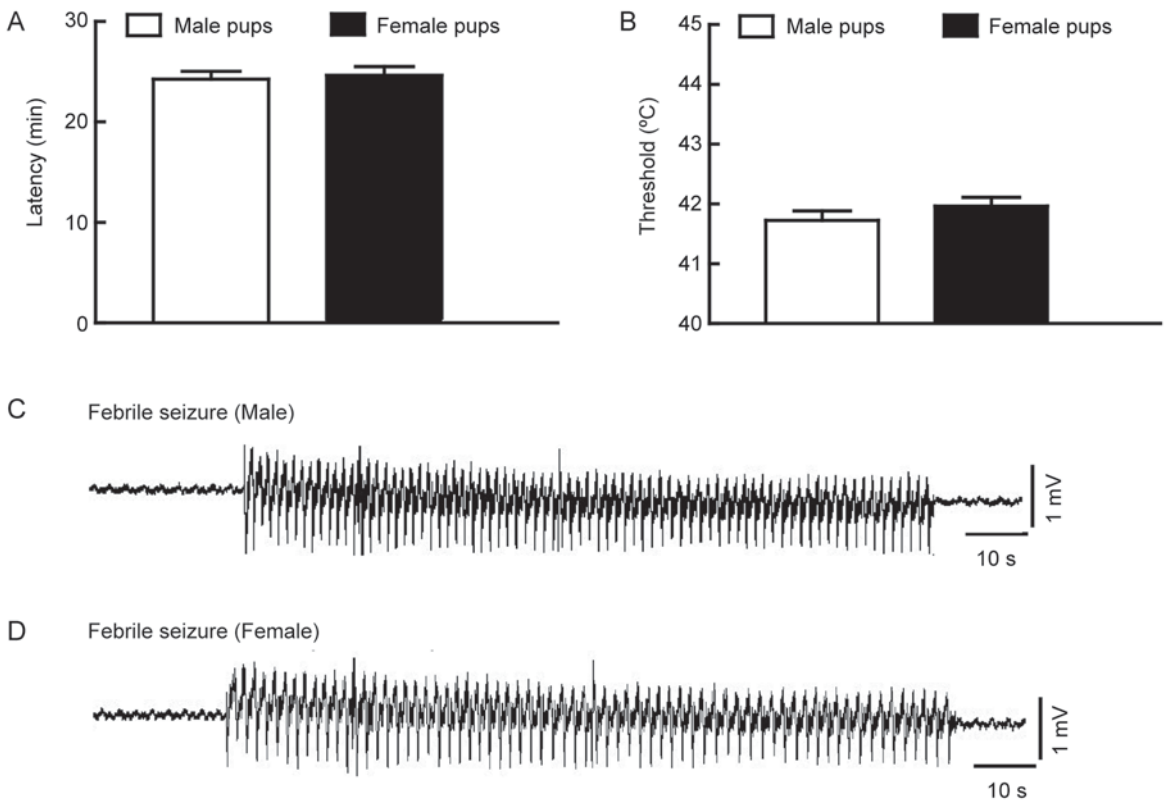


**Fig. 1. No gender difference in the generation of febrile seizures in rat pups. (A) The latencies to the generation of FSs did not significantly differ in male and female pups (*n* = 8 for both groups). (B) Male pups had threshold temperatures similar to female pups for the onset of FSs (*n* = 8 for both groups). (C, D) Hippocampal EEG recordings from freely-moving male (C) and female (D) pups. Normal discharges before FSs were replaced by epileptiform discharges after FSs. A nonparametric test was used for the analysis of latency to and threshold of FS generation.**

The PTZ seizure threshold also did not differ between males and females at P14. The seizure stages after PTZ injection, doses of PTZ to induce stages 1–6, and latencies to PTZ-induced seizures showed no significant gender differences (Fig. 2A–C).

Similarly, the MES threshold to induce stages 1 and 2 had no gender difference (Fig. 2D). Representative EEGs of MES-induced seizures in pups are shown in Fig. 2E.

### Gender Difference in the PTZ Seizure Threshold in Adult Rats

The seizure stages after each subthreshold dose of PTZ in male and female rats are shown in Fig. 3A. In normal rats, the threshold PTZ doses to induce seizure stages 4, 5, and 6 in males were significantly lower than those in females (Fig. 3B), and the latencies to stages 3, 4, 5, and 6 were significantly shorter in males than in females (Fig. 3C). On the contrary, in the FS groups, the threshold PTZ doses to induce seizure stages 4, 5, and 6 in males were significantly higher than those in females (Fig. 3B), and the latencies to stages 5 and 6 were significantly longer in males than in females (Fig. 3C). Interestingly, adult FS females but not males showed more severe seizures than age- and gender-matched normal individuals. This was manifested in two aspects: (1) the doses of PTZ to induce seizure stages 4, 5, and 6 in FS females were significantly lower than those in normal females (Fig. 3B); and (2) the latencies to stages 3, 4, 5, and 6 in FS females were significantly shorter than those in normal females (Fig. 3C).

### Gender Difference in Acute PTZ-Induced Seizures in Adult Rats

We further used acute PTZ-induced seizures to avoid the influence of factors such as the postictal seizure protection

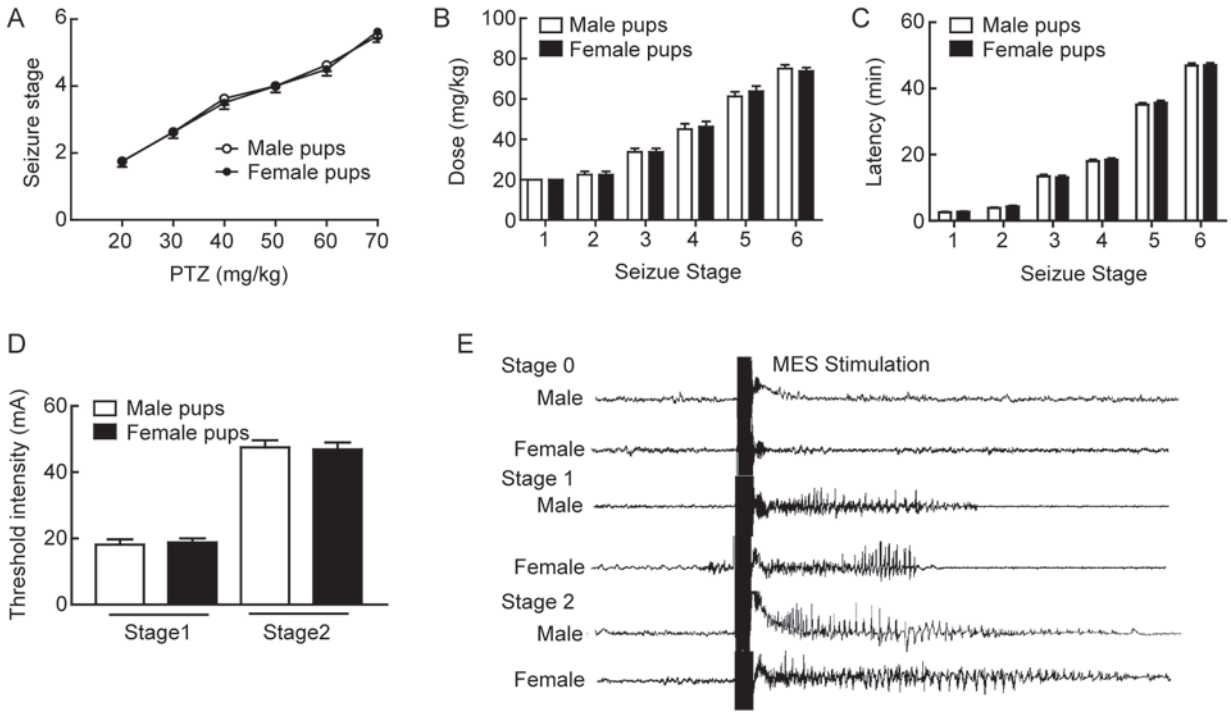


**Fig. 2. No gender difference in the susceptibility to PTZ- and MES-induced seizures in rat pups. (A) Seizure stages after each subthreshold dose of PTZ in normal male and female pups (*n* = 8 for both groups). (B–C) The dose of PTZ (B) and latency (C) for each seizure stage (1–6) in male and female rat pups. (D) In the MES model, the threshold current intensities to induce stages 1 and 2 did not differ between male and female pups (*n* = 8 for both groups). (E) Representative EEGs of MES-induced seizure stages 0, 1, and 2 in rat pups. Two-way ANOVA followed by Tukey's *t*-test.**

induced by repetitive injection of subthreshold doses of PTZ. All FS and normal rats showed MCSs and GTCSs following PTZ injection of a single dose of 60 mg/kg. Females showed significantly higher seizure stages and significantly shorter MCS and GTCS latencies than males in the FS group, while the normal group showed no gender difference (Fig. 4A–C). Comparing the FS with normal rats, FS males and females both showed higher seizure stages and shorter MCS and GTCS latencies (Fig. 4A–C). In addition, the mortality rate was ~70% in FS females and 35% in FS males, compared to 17% in normal rats (Fig. 4D).

### Gender Difference in Seizure Susceptibility to MES in Adult Rats

At 40 mA MES, the seizure stages of male normal rats were significantly higher than those of female normal rats, while the opposite was found in FS rats (Fig. 5A). Comparing the FS groups with the normal groups, the seizure stages of FS females were significantly higher than those of normal females (Fig. 5A), while there was no difference between male rats (Fig. 5A). At 60 mA MES, no gender difference was found in either the FS or normal groups (Fig. 5B), but the seizure stages of both males and females in the FS group were significantly higher than those of normal rats (Fig. 5B). The percentages and numbers of female and male rats with seizures at stages 1–3 in each group are shown in Fig. 5C.

### IL-1β Contributes to the Gender Difference in Seizure Susceptibility

Using western blot, we found that the protein levels of IL-1β were similar in normal male and female pups (Fig. 6A). After complex FSs, the protein expression of IL-1β increased in all rat pups compared to the normal pups (Fig. 6A). In adult rats, the protein level of IL-1β in normal males was higher than that in normal females, while the opposite was found in FS rats (Fig. 6B).

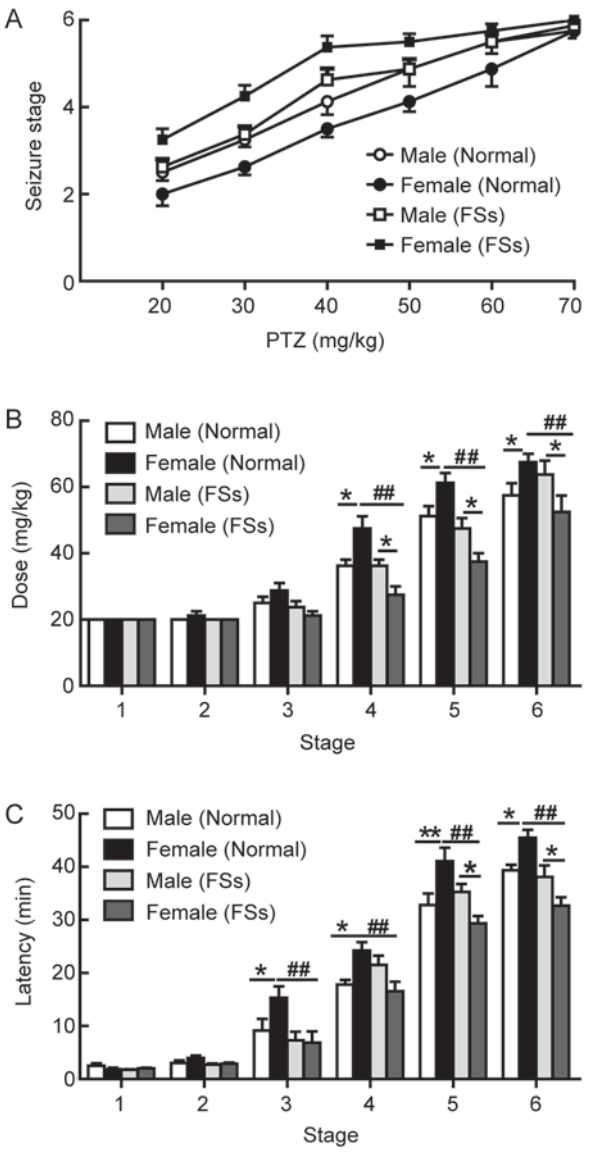


**Fig. 3. Gender difference in the PTZ seizure threshold in adult rats. (A) Seizure stages after each subthreshold dose of PTZ in all rats (*n* = 8 for each groups). (B, C) Dose of PTZ (B) and latency (C) for each seizure stage (1–6) in normal and FS rats. **P* <0.05, ***P* <0.01 compared with normal or FS males, ##*P* <0.01 compared with normal females. Two-way ANOVA followed by Tukey's *t*-test.**

## DISCUSSION

The major findings of the present study are as follows: (1) the susceptibilities to FSs, PTZ, and MES were similar in male and female rat pups; (2) males were more susceptible to epilepsy than age-matched females in normal adult rats in the PTZ and MES seizure models; (3) FS rats acquired higher seizure susceptibility compared with normal rats; (4) the acquired seizure susceptibility after complex FSs differed with gender as males were less susceptible than females; and (5) IL-1β may be involved in this gender difference. Thus, our results provide direct evidence that complex FSs lead to higher epileptic susceptibility in adult female than male rats, suggesting that gender difference may also be a risk factor for epilepsy in patients who experienced complex FSs in infancy.

Increasing evidence from both clinical and experimental studies shows that gender is an important factor in many diseases, including epilepsy[24, 25]. For example, male patients with mesial temporal lobe epilepsy usually have more secondarily generalized tonic-clonic seizures and suffer more seizure-induced damage than females[6, 26]. Male adult rats are also more susceptible to seizures induced by PTZ, kainic acid, or pilocarpine[27]. In this study, we extended these findings by showing that the susceptibility to seizures induced by hyperthermia, PTZ, and MES were similar in rat pups, while in normal adult rats, the susceptibility to seizures was higher in males than in females. Thus, these results confirm the gender difference of susceptibility to seizures in normal adults, and indicate that this difference is development-related. In accord with our observations, it has been reported that the incidence of complex FSs in children is not gender-related[14]. Recently, the FEBSTAT (Consequences of Prolonged Febrile Seizures in Childhood) study also noted no significant differences in seizure duration and MRI abnormalities between boys and girls[28]. Meanwhile, Hosseini *et al.* found that sex hormones are associated with the sex-dependent differences in PTZ-induced seizures in normal adult rats[29].

Retrospective studies have shown a relationship between a history of early FSs and susceptibility to temporal lobe epilepsy. Many risk factors for developing epilepsy after FSs have been reported, such as a family history of epilepsy and the occurrence of complex FSs, however it was still unclear whether gender is also a risk factor[10, 30, 31]. In this study, we found that adult rats that experienced early-life complex FSs were more susceptible to seizures induced by PTZ and MES than normal rats. This finding is consistent with previous reports that FSs in infancy lead to long-term enhanced excitability[12].

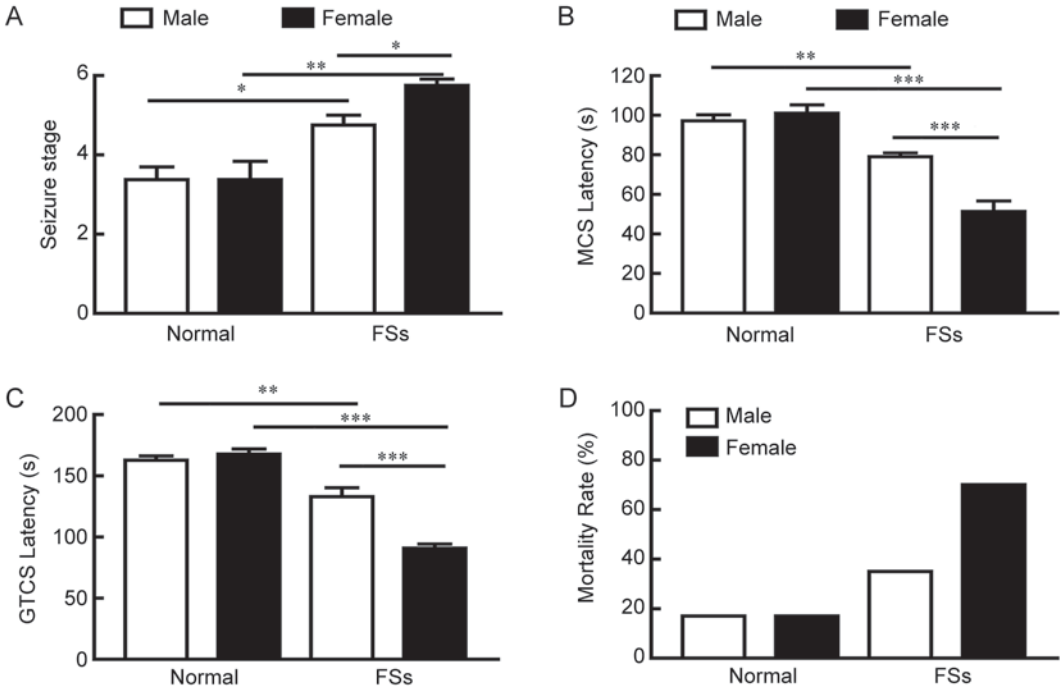


**Fig. 4. Gender difference in acute PTZ-induced seizures in adult rats. (A) Seizure stages of FS rats were higher than normal rats after 60 mg/kg PTZ injection (*n* = 8 for all groups). Seizure stages of FS females were higher than those of FS males. (B) MCS latency in FS males was longer than that in FS females. MCS latency in normal males was equal to that in normal females. (C) GTCS latency in FS males was longer than that in FS females. GTCS latency in normal males was equal to that in normal females. (D) The mortality rate differed between FS males and females but not between normal males and females. Error bars indicate SEM. **P* <0.05, ***P* <0.01, ****P* <0.01. The $\chi^2$ test was used for the statistics of ratios. One-way ANOVA with Tukey's *t*-test was used for the analysis of MCS and GTCS latencies after PTZ injection. A nonparametric test was used for seizure stage.**

Furthermore, the seizure susceptibility of males was lower than that of females in adult FS rats, which was contrary to that of normal adult rats. It has been reported that the gender difference in susceptibility to picrotoxin-induced seizures is seizure- and stimulation-dependent[32]. Here, we speculate that the gender difference in susceptibility is variable and can be reversed in some cases. As in our study, the gender difference in susceptibility is reversed by infant FSs. These results provide direct evidence that the acquired seizure susceptibility after complex FSs is gender-dependent, suggesting that early complex FSs might result in more epileptic susceptibility in adult females than males. In addition, when given a subthreshold dose of PTZ or a low current intensity of MES, adult FS females but not males showed more severe seizures than age- and gender-matched normal individuals. These results further support the gender-dependent phenomenon of acquired seizure susceptibility after complex FSs, and we propose that, clinically, FS females may be more easily affected by epileptic attacks.

The mechanisms underlying the enhanced seizure susceptibility in adults after complex FSs are still unclear. Early-life inflammatory factors such as IL-1β have been associated with FS generation and seizure susceptibility in the adult brain[19]. It has been reported that IL-1β increases seizure susceptibility to kainic acid in adult rodents[33]. In fact, significantly increased IL-1β has been reported in children with a history of FSs[34]. Here, we found that the expression of IL-1β increased immediately after FSs in infancy. More interestingly, this increase was long-lasting and more marked in adult FS females than males, and was strongly associated with the gender difference in seizure susceptibility induced by FSs. Although further data are needed, these results at least suggest that IL-1β contributes to the gender difference in seizure susceptibility induced by FSs. A possible interpretation for the long-term increase in

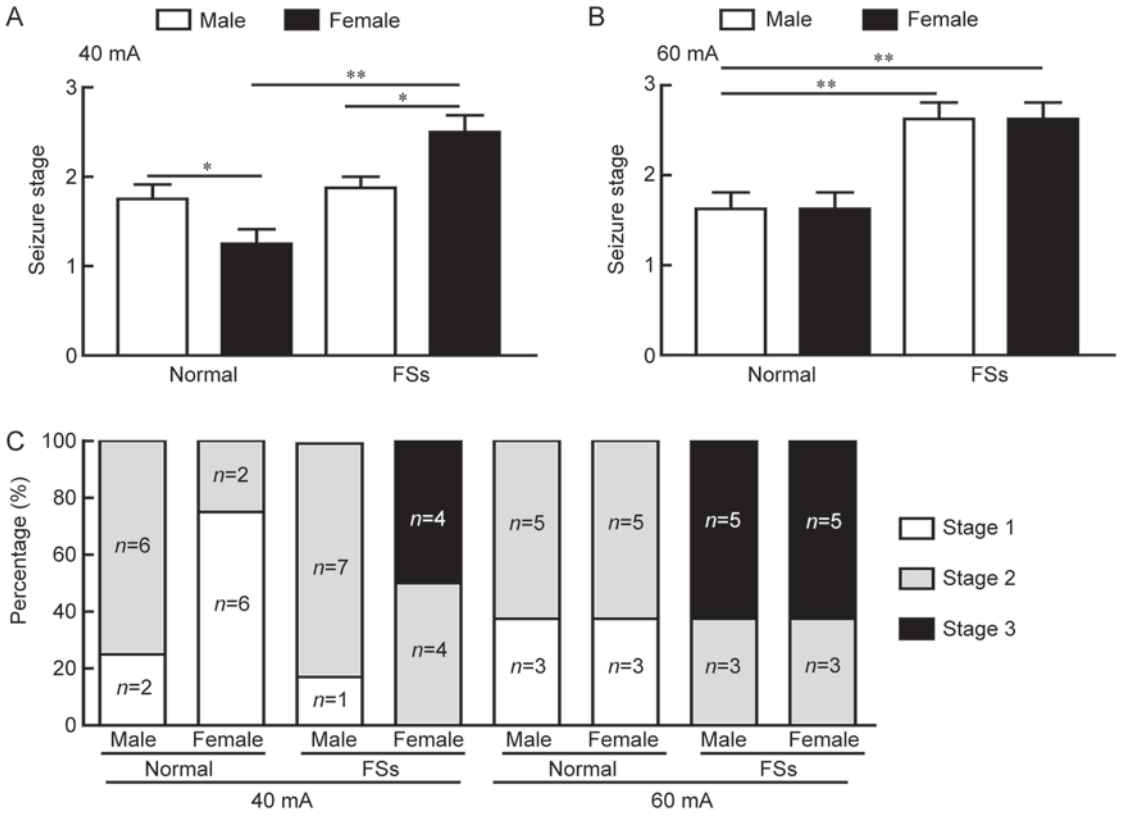


**Fig. 5. Gender difference in MES-induced seizures in adult rats. (A) Seizure stages of normal males were higher than those of normal females with 40 mA MES. Conversely, the seizure stages of FS males were lower than those of FS females ($n$ = 8 for all groups). (B) Seizure stages of FS rats were higher than those of normal rats without a gender difference ($n$ = 8 for all groups) with 60 mA MES. (C) Percentages of stages 1–3 in the four groups after 40 mA or 60 mA MES. $^{*}P$ <0.05, $^{**}P$ <0.01. A nonparametric test was used for the statistics of seizure stage.**

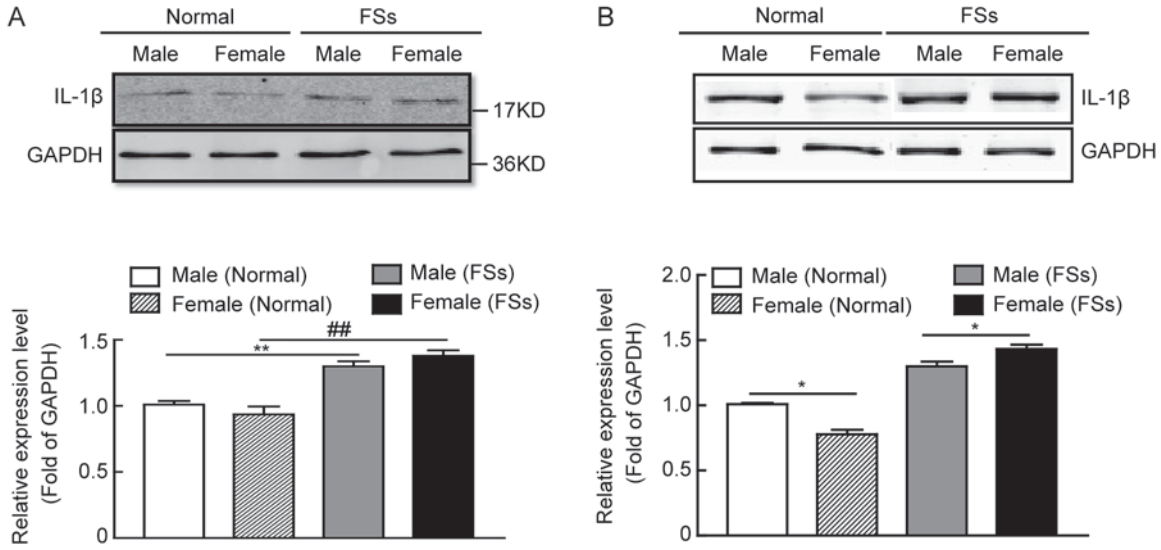


**Fig. 6. Western blots of IL-1β and quantitative analysis. A: Protein levels of IL-1β in the hippocampi of normal and FS rat pups immediately after FSs. B: Protein levels of IL-1β in the hippocampi of normal and FS P60 rats.**

IL-1β may involve epigenetic mechanisms such as histone acetylation that could promote the expression of IL-1β. Certainly, more experiments are needed to determine the exact mechanism. Interestingly, we also found that in adult normal rats, the protein level of IL-1β was higher in males than in females. This finding may be partly interpreted on the basis of a previous study showing that estradiol and progesterone inhibit the production of IL-1 by human peripheral monocytes[35]. In addition, there is increasing evidence showing that sex hormones are associated with neuronal development, neuronal excitability, and epileptic susceptibility[36-38]. Since sex hormones may be influenced by seizures, fever, and stress, they may be involved in the gender difference of seizure susceptibility after complex FSs.

In conclusion, here we found that males were more susceptible to seizures induced by PTZ and MES than females in normal rats. Conversely, when complex FSs were experienced in infancy, females were more susceptible to seizures than males. IL-1β may be involved in this gender difference of seizure susceptibility induced by FSs. These results demonstrated that the acquired seizure susceptibility after complex FSs is gender-dependent, which may assist the diagnosis and treatment of epilepsy in the clinic.

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