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Sex differences in sensitivity to seizures elicited by pentylenetetrazol in mice

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Abstract

Sex differences in sensitivity to seizures elicited by intraperitoneally injected pentylenetetrazol (PTZ) were studied in 240 (120 males and 120 females) adult Swiss mice. Animals were separated into four groups according to the dose that was injected: 40, 50, 60 and 70 mg/kg. Seizure severity was expressed by the following scoring scale: (0) no abnormal behavior; (1) myoclonus; (2) running bouncing (RB) clonus; (3) tonic hind limb extension (THE). The analyses of the dose–response curves indicated that females were more susceptible than males when the 50- and 60-mg/kg doses were used. Specifically, females often displayed RB clonus, while males frequently displayed only myoclonus or no abnormal behavior. No significant sex differences were demonstrated when either the 40- or the 70-mg/kg doses were used. These data indicate that, for a specific range of doses, sex differences in seizure susceptibility can be clearly demonstrated with the use of intraperitoneally injected PTZ. In this sense, this method could be used as a tool to investigate the role played by sexual hormones in regulating the sensitivity of the γ-aminobutiric acid ($GABA_A$) receptor complex (GRC).

Keywords: Sex differences; Pentylenetetrazol (PTZ); γ-Aminobutiric acid (GABA); Seizures; Mice

1. Introduction

Sex differences in behavior have been extensively demonstrated both with (Jung et al., 1999; Pericic et al., 1986) and without (Schmidt et al., 1999) the use of drugs as part of the experimental design. The former can be illustrated by dependence to alcohol (ethanol) in both humans (McGue et al., 1997) and animals (Lancaster et al., 1996). Ethanol acts on the γ-aminobutiric acid ($GABA_A$) receptor complex (GRC) (Crews et al., 1996), which is also known to be the site of action of many convulsants, such as picrotoxin, bicuculline, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and pentylenetetrazol (PTZ) (Macdonald and Olsen, 1994; Olsen, 1981).

The effects of gender and sexual hormones on incidence and severity of seizures are evident in humans. Such effects can be illustrated by the influence of the estral cycle on seizures in women (Backstrom et al., 1990). In addition, some types of epilepsy are clearly affected by gender. For instance, it is well known that childhood absence epilepsy affects more girls than boys (Holmes et al., 1987). On the other hand, epilepsy with myoclonic absences affects more boys than girls (Commission on Classification and Terminology of the International League Against Epilepsy, 1989). Several lines of research now point to the fact that seizure susceptibility is intimately related to the modulation of the activity of the GRC by sexual hormones (Finn and Gee, 1994; Pericic et al., 1996; Wilson, 1992).

The study of sex differences in animal models of epilepsy in which the chemoconvulsant acts on the GRC is complicated by the fact that the aforementioned receptor presents distinct binding sites for different convulsants (Macdonald and Olsen, 1994; Olsen, 1981). In fact, several reports suggested that each binding site of the GRC could be affected by sex in a way that favors males in some cases and females in others. For example, Pericic et al. (1986), using both rats and mice, have studied sex and species differences with $GABA_A$-related convulsants, such as bicuculline and picrotoxin. These authors demonstrated that female mice are more sensitive to intraperitoneal administration of bicuculline, while males are more sensitive to

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intraperitoneal administration of picrotoxin. On the other hand, male and female rats were equally sensitive to bicuculline, while female rats were more sensitive to picrotoxin.

PTZ, which also acts on the GRC (Macdonald and Olsen, 1994; Olsen, 1981), is the most frequently used chemoconvulsant in experimental models of epilepsy (Löscher et al., 1991). However, studies concerning sex effect in response to PTZ specifically used rats in their experimental design. For instance, Kokka et al. (1992) reported "a small but significant sex difference" in intravenously injected PTZ seizure threshold, males being considered more susceptible than females. On the other hand, using the same route of injection, Finn and Gee (1994) showed that male rats had a similar response to females and, additionally, these authors demonstrated that the estrus cycle did not significantly affect susceptibility to PTZ. Likewise, Kubová et al. (1993), using PTZ (100-mg/kg dose) subcutaneously injected, did not observe a significant difference between sexes.

To our knowledge, sex differences in sensitivity to PTZ have not been systematically studied in mice. The high genetic variability of the mouse allows the development of many epilepsy-prone and epilepsy-resistant strains (Kosobud and Crabbe, 1990; Löscher and Schmidt, 1988). Furthermore, several Mendelian traits have been related to susceptibility to seizures, indicating that genetic factors play an important role in seizure disorders (Kosobud and Crabbe, 1990; Puranam and McNamara, 1999). In addition, the investigation of sex differences in response to chemoconvulsants in rodents can be used to understand how sexual hormones and brain sexual dimorphisms influence seizure susceptibility. Here, we study the effect of sex on the susceptibility to PTZ seizures in the mice.

2. Methods

2.1. Animals

Male and female Swiss mice were bred and maintained in our laboratory on a 12:12 light/dark cycle (lights on at 2:00 a.m.). Animals were housed at a constant temperature (22°C), and had unrestricted access to food and water. They were not habituated to intraperitoneal injection of PTZ before the beginning of the experiments. Experiments were carried out between 8:30 a.m. and 12:30 p.m. in a noise-free room. Both sexes were used in all experimental sessions. Each animal was tested only once. On average, 6.1 ± 0.4 (mean ± S.E.M.) animals were tested per day.

2.2. Drug

PTZ (Sigma) was freshly prepared and was dissolved in saline solution (0.9%).

2.3. Experimental procedures

Initially, we injected 50 mg/kg of PTZ intraperitoneally in 80 adult Swiss mice (40 females and 40 males). A previous study (Stone, 1970) demonstrated that this dose is the CD_{50} for the mouse. Immediately after injection, the animals were individually placed in a glass cubicle ($12 \times 24 \times 17$ cm), which had a transparent front wall. The behavior displayed by each animal was, then, recorded for 10 min. These recordings were subsequently used to evaluate the severity of the seizures.

Seizure severity was expressed by the following scale:

(0) no abnormal behavior;
(1) myoclonic twitches (sudden muscle jerk, sometimes accompanied by tail movements and head twitch);
(2) running bouncing (RB) clonus (violent whole body clonus, with or without loosing of righting reflexes);
(3) tonic hind limb extension (THE; extreme rigidity with fore and hind limbs extended caudally).

Each animal received a final score that corresponded to the most severe seizure it presented during the test. For all animals that presented scores of 1 or above, the latency to the first seizure, regardless of severity, was also measured (in seconds).

Considering the results of the first experiment, we decided to extend our study to include lower and higher doses. To that end, in a second experiment, animals were divided into four groups according to the dose that was injected: 40, 50, 60 or 70 mg/kg of PTZ intraperitoneally. Each group consisted of 40 adult Swiss mice (20 females and 20 males). The testing procedure and behavioral analysis were the same used in the first experiment.

2.4. Statistics

Initially, Kendall's coefficient of rank correlation (τ) was calculated in order to determine if seizure scores were significantly affected by sex and dose. Subsequently, statistical analysis of the scores was performed separately on the data from each dose group. The average scores for male and female groups were calculated (results are presented in terms of means ± S.E.M.). Differences in the average scores between sexes were evaluated by means of Mann–Whitney *U* tests (M-W). Overall differences in location, dispersion and skewness between the distributions of scores were evaluated by performing the Kolmogorov–Smirnov two-sample test (K-S). When there was identity in distribution for the two samples, the Wilcoxon two-sample test (Wilc) was applied. It should be mentioned that the Wilc test is sensitive to the number of interchanges in rank necessary to separate the two samples, whereas the K-S test is less sensitive to differences in the location only. Specific differences between males and females in the percentage of animals presenting a given score were compared by means

of chi-square tests. In the first experiment, mean latencies for males and females were compared using the Student's *t* test. The effects of dose and sex on latencies, in the second experiment, were evaluated by using a general factorial ANOVA. Results were considered significantly different at $P<.05$.

3. Results

3.1. First experiment

The average score of female mice (1.85 ± 0.08) was significantly higher (M-W; $Z=3.9$, $P<.001$) than that exhibited by males (1.05 ± 0.15). The K-S test revealed a significant difference between the distribution of scores of males and females ($P<.01$, two-tailed). The analysis of the distribution of the scores showed that 17 male mice (42.5%) did not present abnormal behavior, whereas only one female mouse did not react to PTZ. This difference was found to be highly significant ($\chi^2=18.4$, $df=1$, $P<.001$). Moreover, most females (82.5%) displayed RB clonus, while only 47.5% of the males presented such behavior ($\chi^2=10.8$, $df=1$, $P<.001$). Only one female exhibited THE, dying immediately after the seizure. No significant difference in mean latencies was found between males and females.

3.2. Second experiment

A significant association between scores and dose was found: increasing the amount of PTZ that was injected resulted in significantly higher scores ($\tau=0.571$, $P<.01$). Scores were also affected by sex: females displayed more severe seizures more often than males ($\tau=0.155$, $P<.05$).

When doses were analyzed separately, the absence of abnormal behavior in most animals (Fig. 1) was the main finding in the 40-mg/kg dose group (females: 60%, males: 70%). The distribution of scores was similar for both sexes (Wilc; $Z=0.66$, with correction for ties; $P>.10$, two-tailed), and no significant difference was found between sexes for the average score.

As in the first experiment, the K-S test revealed a significant difference between the distribution of scores of males and females ($P=.013$, two-tailed) when the 50-mg/kg dose was used. This dose elicited clonic seizures on 85% of the females. In contrast, just 35% of males showed such behavior ($\chi^2=7.8$, $df=1$, $P<.01$). On the other hand, males (50%) presented the less severe myoclonus seizure as their most intense one significantly more often then females (10%) ($\chi^2=7.6$, $df=1$, $P<.01$). Furthermore, the average score of males (1.15 ± 0.16) and females (1.80 ± 0.12) were significantly different from each other (M-W; $Z=3.99$, $P=.002$). When compared to the 40-mg/kg dose group, the percentage of animals, in both sexes, that presented either RB clonus or THE was higher (Fig. 1).

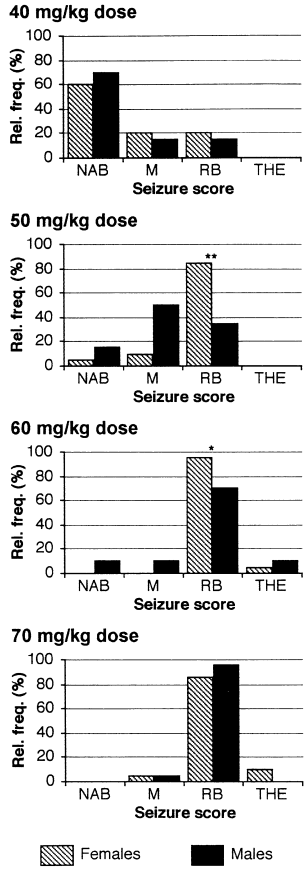

Fig. 1. Distribution of scores according to dose group. Note that the percentage of females presenting RB clonus is significantly higher for the 50- and 60-mg/kg doses. No significant differences were found for the 40- and 70-mg/kg doses. These results indicate that female mice are more susceptible to PTZ (intraperitoneally)-induced seizures than male mice, and this sex difference is present in a limited range of doses. NAB: no abnormal behavior; M: myoclonus; RB: running bouncing clonus; THE: tonic hind limb extension. $**P<.01$; $*P<.05$.

The Wilc test failed to reveal a significant difference ($Z=1.17$, with correction for ties; $P>.10$, two-tailed) between the distribution of scores of males and females for the 60-mg/kg dose (Fig. 1). However, we observed a significant difference between males and females in the percentage of animals that presented RB clonus ($\chi^2=4.3$, $df=1$, $P<.05$). Notwithstanding with the previous result, no significant difference between sexes was found for the average scores. For both sexes, but mainly for males, the percentage of animals that presented either RB clonus or THE was higher than when the 50-mg/kg dose was used. One female and two males died during the testing session immediately after a THE seizure.

Administration of 70 mg/kg resulted in similar distributions of scores for both sexes (Wilc; $Z=0$, with correction for ties; $P>.10$, two-tailed) (Fig. 1), and no significant difference between sexes was found for the average scores. This dose resulted in an increased percentage of males that displayed RB clonus or THE, as compared to the 60-mg/kg dose. On the other hand, the percentage of females in which

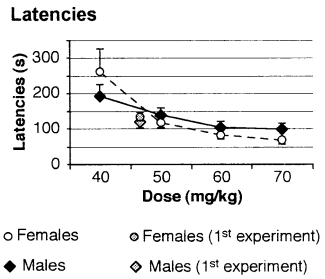

Fig. 2. Dose–response curve showing the latencies for the onset of the first observed seizure (regardless of severity). Note that latency decreases with increasing dosage. No sex differences regarding latencies were found. Each point represents the mean ± S.E.M.

either RB clonus or THE was induced was quite similar to that found for the 60-mg/kg dose. Two females died during the testing session immediately after a THE seizure.

Regarding latencies (Fig. 2), the ANOVA revealed a significant effect of the dose ($P < .001$). For instance, males presented a mean latency of 190.8 ± 32.5 s when injected with 40 mg/kg of PTZ. The mean latency decreased to 97.6 ± 13.7 s when males were injected with the 70-mg/kg dose. No significant effect was found for sex ($P = .96$). By way of illustration, males and females presented mean latencies of 138.7 ± 17.5 and 117.6 ± 10.8 s, respectively, for the 50-mg/kg dose.

4. Discussion

Our results indicate that female Swiss mice are more susceptible than males to seizures elicited by intraperitoneally injected PTZ. However, this sex difference is dose-dependent, since it was demonstrated specifically for the 50- and 60-mg/kg doses. The 40-mg/kg dose is probably too low to elicit seizures in a number of animals that would make the sex effect distinguishable. On the other hand, the 70-mg/kg dose is high enough to elicit RB clonus in most animals, either males or females. In addition, we demonstrated that latencies decrease with increasing dosage for both sexes, but we found no significant difference between males and females.

Considering previous reports, the presence of sex differences in sensitivity to GABA-related convulsants seems to depend on the drug that is being used and on the methodology used to inject it. The former case can be illustrated by the fact that, while acting on the GRC, bicuculline and DMCM presented opposite results when intravenously injected in rats (Finn and Gee, 1994). Even when two convulsants share the same binding site of the GRC, the observed sex difference may not systematically favor one sex. For instance, Pericic et al. (1986) demonstrated that intraperitoneally injected picrotoxin affects male mice more than females. In the present study, we report that PTZ, which is known to act on the picrotoxin-binding site of the GRC, affects females more than males. It has been suggested that the sex difference demonstrated by the use of picrotoxin intraperitoneally reflects differences in pharmacokinetics rather than in pharmacodynamics (Pericic and Bujas, 1997). In order to explain the lower average scores of males in our study as a difference in pharmacokinetics, the amount of PTZ in the male brain would have to be lower than in the female one. However, it was demonstrated that PTZ accumulates rather rapidly in the brain of male mice. Approximately 5 min after injection, the concentration of PTZ in the brain was found to be equivalent to that originally injected in the peritoneal space (Yonekawa et al., 1980). Moreover, our results concerning the lack of sex differences in latencies also support the possibility that there are no striking differences in pharmacokinetics between sexes for intraperitoneally injected PTZ.

The pertinence of the method of injection for the study of sex differences in sensitivity to chemoconvulsants has been demonstrated by Pericic and Bujas (1997). These authors verified that, using bicuculline as the convulsant, the intravenous method presented opposite results when compared to the intraperitoneal method. It was also suggested that the intravenous method was more reliable than the intraperitoneal method. Interestingly, however, the intravenous method does not seem to present consistent results when different reports are considered. For instance, Finn and Gee (1994) showed, in rats, that bicuculline affects females more intensely than males. On the other hand, Pericic and Bujas (1997) and Guillet and Dunham (1995) reported the opposite in their study. Finally, Devaud et al. (1995) did not find a significant difference between sexes. Reports concerning intravenously injected PTZ also presented contrasting results. Kokka et al. (1992) demonstrated that male rats were more susceptible than females, while Finn and Gee (1994) reported no significant differences in seizure threshold between sexes. In addition, Finn and Gee (1994) reported a seizure threshold that was more than twice of that found by Kokka et al. (1992). Another relevant consideration to be made, regarding the route of injection, stems from the fact that intraperitoneally injected PTZ has been frequently used in routine assessments of the anticonvulsant efficacy of several drugs (Löscher et al., 1991). Some of these studies have drawn conclusions based on the pooled results of males and females (Kubová and Mares, 1993; Kubová et al., 1993). These assessments frequently use high doses of PTZ (85 mg/kg, CD97). Our results (specifically with the 70-mg/kg dose) would seem to indicate that no relevant sex differences should be discernible at such higher doses. However, the anticonvulsant acts by reducing the potency of the PTZ. In some cases, it is conceivable that the effectiveness of the PTZ in inducing seizures might have been decreased to a point in which sex differences are present. In this sense, our findings suggest that the analysis of the results in such tests might profit from separately studying the data obtained from each sex.

The existence of a sex difference in the sensitivity to PTZ could be explained by the modulation of the GRC by sexual hormones and their metabolites. Seyle (1942) first reported the anticonvulsant effect of progesterone in rodents. Furthermore, it has been demonstrated that the progesterone metabolite 3α-hydroxy-5α-pregnan-20-one (3α,5α-P) also presents anticonvulsant properties (Belelli et al., 1989). Specifically, this metabolite increases the threshold dose for convulsions elicited by PTZ (Finn and Gee, 1994). Testosterone also seems to have a protective effect regarding seizures (Woodbury, 1969). However, sexual hormones are not restricted to the protective role. In fact, estrogen increases seizure susceptibility (Woodbury, 1969).

Sex differences in seizure susceptibility are not restricted to hormonal modulation of receptor activity. Alterations in the concentration of neurotransmitters, especially glutamate and GABA, may account for the variations in the susceptibility to seizures (Zilles et al., 1999). For instance, it has been reported that an epilepsy-prone mice strain has low concentration of GABA in the cortex (Dolina et al., 1993). In addition, it has been shown, in rats, that the GABAergic activity in the male hypothalamus (ventromedial and preoptic nucleus) is about two-fold greater than in females (Grattan and Selmanoff, 1997). Interestingly, the ventromedial nucleus projects to the central gray of the brain stem (Sakuma, 1984; Sakuma and Tada, 1984), which is a probable generating center of the RB clonus (Browning and Nelson, 1986). This functional dimorphism may account for the higher susceptibility of females to PTZ, which, in our case, was associated with a higher incidence of RB clonus in animals of this sex. In fact, it was demonstrated that injection of γ-vinyl-GABA (an inhibitor of GABA degradation) in the hypothalamus is capable of blocking PTZ seizures (Miller et al., 1987).

In summary, our findings indicate that female mice are more sensitive to the convulsive activity of the PTZ than males for a specific range of doses. Our report demonstrates that it is possible to use a simple, well-known and reliable method (intraperitoneally injected PTZ) to observe sex differences in susceptibility to seizures. In this sense, this method could be used as a tool to investigate the role played by sexual hormones in regulating the sensitivity of the GRC.

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