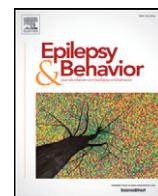


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## Experiencing neonatal maternal separation increased the seizure threshold in adult male mice: Involvement of the opioid system



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### ABSTRACT

Experiencing early-life stress has been considered as a potent risk factor for the development of many of brain disorders, including seizures. Intervening mechanisms through which neonatal maternal separation (MS) alters the seizure susceptibility in adulthood have not been well studied. In the current study, by applying 180 min of MS stress (PND 2–14), we determined the seizure susceptibility and considered the role of the opioid system. Maternal separation increased the seizure threshold, and administration of anticonvulsant/proconvulsant doses of morphine (1 and 30 mg/kg, respectively) reversed the impact of MS. Using tail flick and hot plate tests, we exposed animals to 30 min Restraint stress (RS) and found that MS decreased the pain threshold, suggesting the hyporesponsiveness of the opioid system. These results supported the abnormal seizure activity observed in the MS mice and suggested that abnormalities in the opioid system following MS alter seizure susceptibility in later life.

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### 1. Introduction

The social environment plays a pivotal role in shaping the brain and behaviors during development [1]. Emerging lines of research indicate that exposure to stress during one's lifespan has negative effects on seizure activity. Specifically, experiencing chronic stress during development of the brain has been reported to alter seizure susceptibility in adulthood [2,3]. In this regard, there are pieces of evidence suggesting that early-life stress such as maternal separation (MS) adversely affects brain development and increases the risk of occurrence of behavioral difficulties [4,5]. Clinical and preclinical studies have demonstrated that several mechanisms account for the deleterious consequences of MS in adulthood such as alterations in the neurotransmission in different areas of the brain [6,7].

On the other hand, the endogenous opioid system and their receptors are widely expressed in various regions of the CNS [8]. Under stressful conditions, the opioid system undergoes considerable changes that contribute to the regulation of several behaviors such as

nociception, addiction, and seizure susceptibility [9–11]. In this regard, recent studies reported that experiencing adversity during childhood such as MS induced long-lasting alterations in the regulation of the endogenous opioid system in both humans and animals [12,13]. In this respect, previous research reported that MS stress reduced the pain threshold in animals and that this effect was correlated with hypofunction of the opioid system in the CNS [14–16]. It has been reported that morphine dose dependently has both anticonvulsant and proconvulsant properties in the experimental models of seizure [17, 18]. Considering that the opioid system is involved in the pathophysiology of seizure disorders, its role in the MS-induced changes in the epileptogenesis is of interest but has not been clarified.

In this study, using pentylenetetrazole (PTZ)-induced seizures, we aimed to investigate the effects of morphine on the seizure threshold in adult male mice that had experienced neonatal MS.

### 2. Materials and methods

#### 2.1. Animals

Pregnant NMRI mice were obtained from the Pasteur Institute of Iran (Tehran) and were maintained under standard laboratory conditions (in the Department of Pharmacology, Tehran University of Medical Sciences), and the day of birth was considered as postnatal day 0 (PND 0). The litters

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were afterward assigned to a maternal separation paradigm. For this purpose, pups were briefly handled and separated from their dams for 180 min daily during PND 2 to PND 14, beginning at 09:00 a.m. At the end of the separation period, pups were returned to the nest cage. At PND 21, male offspring were housed in groups until the experiment day (PND 50).

All procedures in this study were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication # 80-23) and institutional guidelines for animal care and use (Department of Pharmacology, School of Medicine, TUMS). Also, each experimental group contained 6 to 8 animals.

## 2.2. Study design

Maternally separated and control mice were randomly divided into 7 experimental groups as follows: 1: saline, 2: naltrexone (3 mg/kg), 3: morphine (1 mg/kg), 4: morphine (30 mg/kg), 5: 30-min restraint stress, 6: 30-min restraint stress plus morphine 1 mg/kg, and 7: 30-min restraint stress plus naltrexone (3 mg/kg). All behavioral assessments were performed in the early adulthood (PND 50) of both maternally separated (MS) and control mice. In addition, mice were treated intraperitoneally (i.p.) with a 5-ml/kg (animal weight) dose of morphine (60 min prior to seizure test), naltrexone (45 min prior to seizure test), and saline. In order to test the responsiveness of the opioid system, acute stress (30-min restraint stress) was applied to both experimental groups before evaluation of behavioral measures. Dosage and time of drug administration were chosen according to our previous studies [18].

## 2.3. Assessment of seizure threshold

To evaluate the MS-induced changes in seizure susceptibility, we assessed seizure threshold using the method that was previously described [19]. Briefly, to determine the clonic seizure threshold, we inserted a 30-gauge dental needle into the lateral tail vein of the mice. The needle was attached to the tail by a narrow piece of adhesive tape. With the mouse moving freely, the PTZ solution (0.5%) was slowly infused into the tail vein at a constant rate of 1 ml/min using an infusion pump (NE 1000, New Era Pump System, Inc.), which was connected to the dental needle by polyethylene tubing. Infusion was stopped when general clonus (forelimb clonus followed by full clonus of the body) was observed. The minimal dose of PTZ (mg/kg of mice weight) needed to induce general clonus was recorded as an index of clonic seizure threshold. In this regard, the seizure threshold is dependent on the PTZ dose administered and is time-related.

## 2.4. Restraint stress procedure

Restraint stress was carried out according to the criteria described by Bonneau et al. [20]. Falcon tubes (50 ml) with suitable ventilation holes (0.4 cm in diameter) were used. The mice (PND 50) were restrained for 30 min in the tube without any food and water. The control mice were left in cages at the same time without food and water supply.

## 2.5. Assessment of nociception

In order to evaluate the effect of maternal separation on analgesia, hot-plate and tail-flick tests were performed. For the hot-plate test, mice were placed on a  $52 \pm 0.2$  °C-heated plate (Socrel Hot-plate Model DS37; Ugo Basile, Comerio, VA, Italy), and the latency to lick the fore paw, hind paw, or jump was measured [21]. We considered a deadline time of 60 s to avoid tissue damage. The tail-flick test was done according to the procedure described by D'Amour and Smith [22]. Radiant heat was applied to the tail 5–8 cm from the tip using a tail-flick apparatus (type 812, Hugo Sachs Electronics, Germany). Tail-flick latency was considered as the time interval between the application of a standardized beam focused on the tail and the sudden

removal of the tail from the nociceptive stimulus. The cutoff time was 10 s. Each mouse was only used for one test.

## 2.6. Statistical analysis

Comparisons between experimental groups were performed by one-way ANOVA followed by Tukey's post hoc test when appropriate or by *t*-test. A value of  $P < 0.05$  was considered to be significant.

## 3. Results

### 3.1. Anticonvulsant effect of maternal separation is partly modulated via the opioid system

Seizure thresholds were determined for morphine (1 and 30 mg/kg) and naltrexone (3 mg/kg). As shown in Fig. 1a, while administration of morphine (1 mg/kg, 60 min prior to the test, i.p.) significantly increased seizure threshold ( $*P < 0.05$ ), treating mice with morphine (30 mg/kg, 60 min prior to the test, i.p.) considerably decreased the seizure threshold in comparison with control animals ( $**P < 0.01$ ). Fig. 1b shows that MS significantly increased the seizure threshold in comparison with control animals ( $***P < 0.001$ ). Moreover, administration of morphine (1 and 30 mg/kg, 60 min prior to the test, i.p.) and naltrexone (3 mg/kg) in MS mice significantly increased the seizure threshold in comparison with the control group ( $*P < 0.05$ ,  $***P < 0.001$ , and

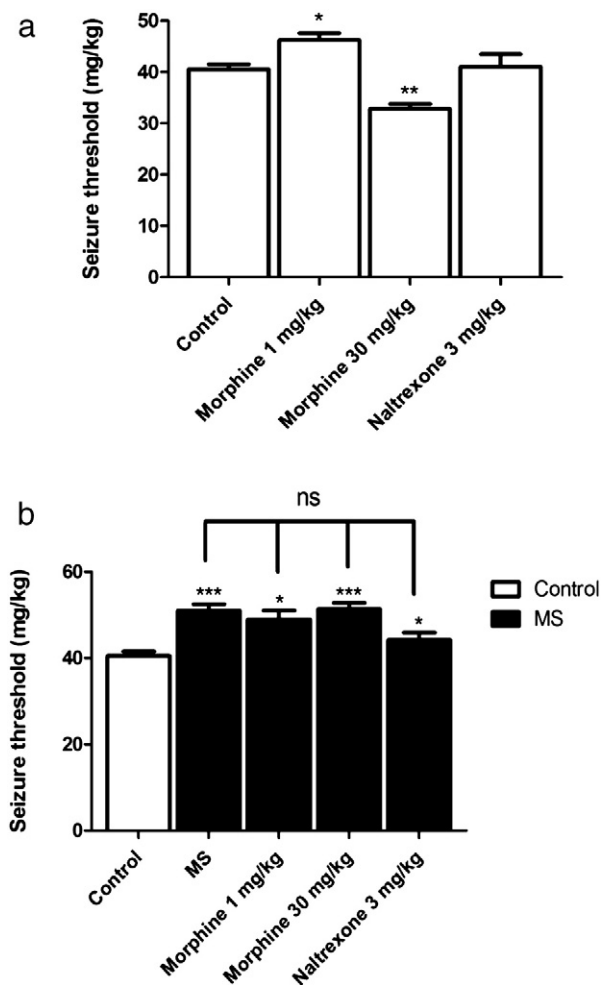
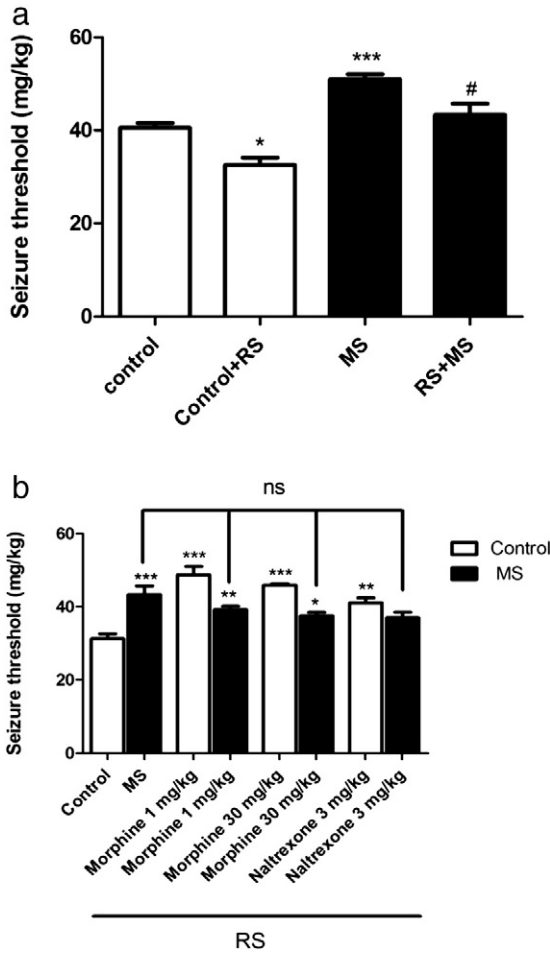


Fig. 1. Effects of morphine and naltrexone treatments on the seizure threshold in male mice; data are expressed as the mean  $\pm$  S.E.M. ( $n = 7$ ) and were analyzed using one-way ANOVA followed by Tukey's post hoc test.  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$  compared with the control groups, and  $P > 0.05$  is defined as nonsignificant (ns).

\* $P < 0.05$ , respectively). However, there was no significant difference in the seizure threshold between saline-treated MS mice and morphine-treated or naltrexone-treated MS mice ( $P > 0.05$ ).

3.2. Effect of restraint stress on seizure threshold

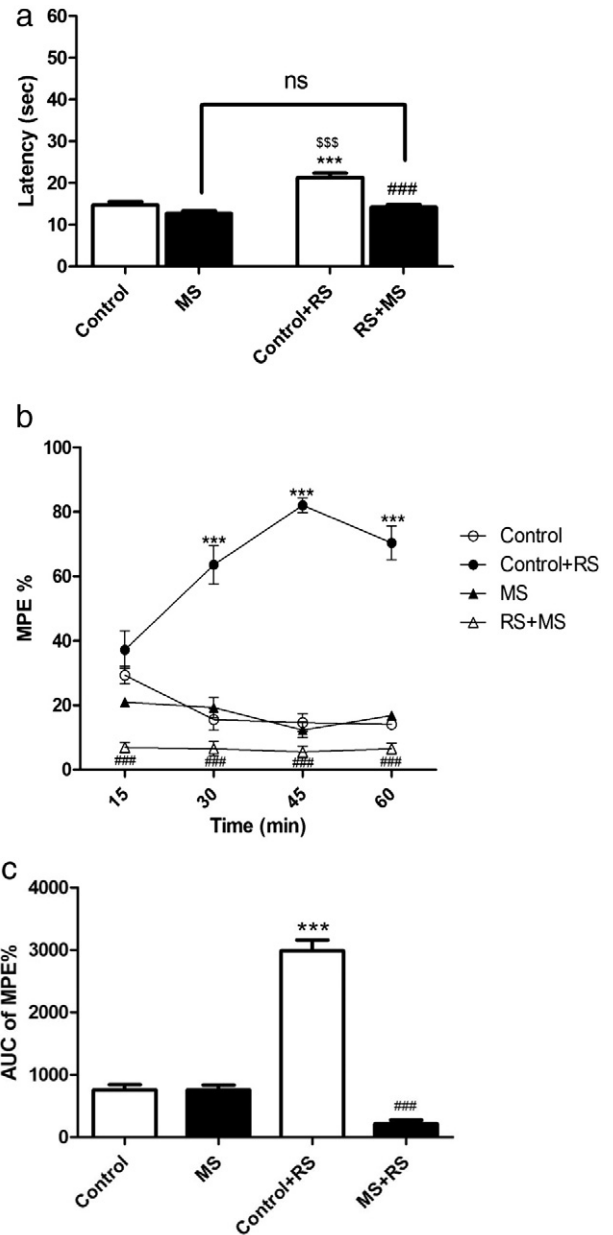
As shown in Fig. 2a, RS (30 min prior to the test) significantly decreased seizure threshold in both control mice (\* $P < 0.05$ ) and MS mice ( $^{\#}P < 0.05$ ) in comparison to their control counterparts. In respect to the effects of morphine and naltrexone, Fig. 2b reveals that administration of morphine (1 and 30 mg/kg, 60 min before the test, i.p.) or naltrexone (3 mg/kg, 60 min before the test, i.p.) to control mice significantly abolished the proconvulsant effect of restraint stress (RS) (30 min before the test) when compared to saline-treated controls ( $^{***}P < 0.001$  for both morphine 1 and 30 mg/kg and,  $^{***}P < 0.01$  for naltrexone 3 mg/kg). However, there was no significant difference between saline-treated and morphine/naltrexone-treated MS mice when animals underwent coadministration of morphine/naltrexone and RS ( $P > 0.05$ ). In comparison with controls, MS mice had a considerable increase in seizure threshold when coadministered morphine (1 and 30 mg/kg) and RS ( $^{**}P < 0.01$  and  $^{*}P < 0.05$  for RS + morphine 1 mg/kg and RS + morphine 30 mg/kg, respectively).



**Fig. 2.** a: Effects of RS (30 min before the test), MS and RS + MS on seizure threshold in male mice; values are expressed as the mean  $\pm$  S.E.M. ( $n = 7$ ) and were analyzed by  $t$ -test. \* $P < 0.05$  and  $^{***}P < 0.001$  compared with the control group,  $^{\#}P < 0.05$  compared with the MS group. b: Effects of morphine and naltrexone on seizure threshold in both experimental groups (controls and MS) that experienced RS for 30 min. Data are shown as the mean  $\pm$  S.E.M. ( $n = 7$ ) and were analyzed by one-way ANOVA followed by Tukey's post hoc test. \* $P < 0.05$ ,  $^{**}P < 0.01$ , and  $^{***}P < 0.001$  compared with the RS control groups.  $P > 0.05$  is defined as nonsignificant (ns).

3.3. Effect of MS and RS on nociceptive behaviors in animals

Hot-plate and tail-flick tests were carried out to evaluate the effects of RS, MS, and their combination (RS + MS) on the pain responses in both MS and control groups. Fig. 3a shows that RS (30 min before the test) significantly increased hot-plate latency in control mice in comparison with nonstressed controls ( $^{***}P < 0.001$ ). However, the same response was not observed in MS mice exposed to RS (30 min before the test) when compared to nonstressed MS mice ( $P > 0.05$ ). Furthermore, while there was no significant difference between nonstressed MS and control mice in the hot-plate latency ( $P > 0.05$ ),



**Fig. 3.** Nociceptive responses of RS, MS, and RS + MS groups in the hot-plate test; data are shown as the mean  $\pm$  S.E.M. ( $n = 7$ ). One-way ANOVA followed by Tukey's post hoc test was used for data analysis.  $^{***}P < 0.001$  compared with the control group,  $^{###}P < 0.001$  compared with the control + RS group (a). Nociceptive responses of RS, MS, and RS + MS groups in the tail-flick test; data are shown as the percentage of maximal possible effect (MPE%). Values are represented as the mean  $\pm$  S.E.M. for 8 mice (b). Area under the curve (AUC) for MPE% in 60 min. Values are shown as the mean  $\pm$  S.E.M. for 8 mice (c).  $^{***}P < 0.001$  compared with the control group,  $^{###}P < 0.001$  compared with the MS group.

control + RS mice showed a sizable increase in the hot-plate latency when compared to MS + RS mice ( $###P < 0.001$ ).

As shown in Fig. 3b, one-way ANOVA for maximum possible effect (MPE %) revealed that exposure to RS (30 min before the test) significantly increased the MPE % of the tail-flick latency in control mice, reflecting stress-induced analgesia ( $***P < 0.001$ ). In contrast, exposure to RS (30 min before the test) significantly decreased the MPE % of the tail-flick latency in MS mice when compared to nonstressed MS mice ( $###P < 0.001$ ). In addition, one-way ANOVA for the normalized area under the curve (AUC) values in Fig. 3c shows that RS significantly increased the AUC of MPE % in control mice in comparison with non-stressed mice in the tail-flick test ( $***P < 0.001$ ). In contrast, RS significantly decreased the AUC of MPE % in MS mice when compared to their nonstressed MS counterparts ( $###P < 0.001$ ).

#### 4. Discussion

Data of the present study revealed that maternal separation, as a cogent animal model of early-life stress, decreased seizure susceptibility to PTZ in the adult male mice. These alterations in the seizure threshold were associated with the hyporesponsiveness of the opioid system, as reflected by the observations that MS mice showed no response to the analgesic effects of 30-min RS in both tail-flick and hot-plate tests. Also, applying 30-min RS or different doses of morphine and naltrexone to MS mice confirmed the finding that perturbations in the opioid system may play a role in altered seizure susceptibility to PTZ.

Emerging lines of evidence suggest that experiencing early-life stress has profound and long-lasting effects on the brain and behavior in later life [1]. In the current study, our results revealed that MS mice were resistant to the convulsive effects of PTZ in their adulthood state. In this regard, there are pieces of evidence that indicate that maternal separation is able to prime seizures in rodents. The discrepancy between our result and those of previous studies may be associated with the differences in the type of manipulation (number of days separated), age and strain of animals used in the test, gender, or the mode of seizure induction. To describe this, it is important to note that effects of stress on seizure susceptibility depend on the time, intensity, and type of stress exposure [23]. There are limited studies focusing on the effects of repeated neonatal maternal separation on seizure susceptibility. Most of these investigations reported that MS increases the risk of limbic epileptogenesis and stated that such effect is sex-specific [2,3]. These observations are in agreement with the fact that the prevalence of some mental disorders such as depression and mesial temporal lobe epilepsy (MTLE) is higher in women [24]. Using PTZ to evaluate the seizure susceptibility in adult rats exposed to MS, Lai et al. showed that MS rats treated with pilocarpine in early life had a lower seizure threshold during adulthood [25]. In our study, we did not apply any pharmacological or behavioral manipulation to MS mice, and seizure activity was determined in adulthood. Also, studies on the effects of MS on seizure vulnerability poorly addressed the underlying mechanisms responsible for changes in seizure activity. Involvement of HPA axis components, neurochemical dysregulation, and structural abnormalities are some of the reported intervening mechanisms [23].

It has been well documented that the opioid system strongly contributes to mother–infant attachment in that opioids not only mitigate the stress response in offsprings but are also involved in the development of the CNS [14,26]. Several lines of research postulated that the opioid system plays a modulatory role in seizure susceptibility in that administration of morphine at low doses induces anticonvulsant effects while high doses of morphine have proconvulsant properties [18, 27]. Consistent with findings of previous studies, our results showed that administration of morphine at a low dose increased the seizure threshold in control mice while treating animals with high-dose morphine induced a proconvulsant effect. The same treatments failed to decrease the elevated seizure threshold in MS mice but not in control mice [18,27]. These results suggest that the opioid system in MS mice

could be impaired. To support our finding, we applied tail-flick and hot-plate tests which are well-known classic paradigms in the evaluation of the pain response [21,22]. It has been known that applying 30-min RS to rodents induced a strong analgesic effect through enhancement of the endogenous opioid system [28]. Interestingly, unlike control mice, MS mice showed no increase in the pain threshold following 30 min of RS either at the spinal (tail-flick test) or supraspinal level (hot-plate test). These results were in line with those of previously published studies showing that MS markedly disrupts opioid system development and decreases the analgesic effects of morphine [29,30]. In addition, other studies showing the field confirmed that maturation of the endogenous opioid system is highly relevant to critical periods of brain development, in that exposure to stress during postnatal period results in hypofunction of the opioid system [14–16].

In this work, we also showed that applying 30 min of RS (versus 120 min) facilitates seizure occurrence. This result was in agreement with the recent study that acute stress, depending on the duration of exposure, has bimodal effects on seizure activity [31]. We applied RS (30 min) to mice for 2 reasons. An acute episode of RS is known to increase the endogenous opioid tone, thus, we first applied RS to see the response of MS mice to the analgesic effects of RS as an endogenous source of opioids. Second, we wanted to see the effects of acute stress exposure on the MS mice that underwent developmental challenge. Apart from the analgesic effects of RS, this stress is able to increase excitatory neurotransmission in several regions of the brain [32]. Although the majority of animal studies have reported that acute stress has anticonvulsant properties, human studies conversely revealed that people with seizure disorders are vulnerable to seizure exacerbation from acute stress [11,33]. Albeit the proconvulsant effect of acute RS is beyond the scope of the current study, we found that a steep increase in the activity of excitatory neurotransmission (such as glutamate) plays a role in the effects of RS on seizure susceptibility (data not shown). In respect to the effects of RS, while treating control animals with a subeffective dose of naltrexone decreased the proconvulsant effects of RS, the same treatment induced no alteration in the seizure susceptibility to PTZ in MS mice. Interestingly, while morphine pretreatment effectively blocked the proconvulsant effect of RS in control mice, the same effect was not observed in MS mice. These results suggest that pretreatment with morphine/naltrexone effectively inhibits the proconvulsant effect of RS in control mice but not in MS mice. In this respect, there are pieces of evidence that indicate that pretreatment of subjects with 1 or 30 mg of morphine potently inhibits the activation of the hypothalamus–pituitary–adrenal (HPA) axis through blocking the CRH in the hypothalamus [34,35]. Failure of the morphine pretreatment in antagonizing the effects of RS may be associated with developmental abnormalities observed in these animals.

The results of the current work showed that the type of stress (chronic or acute) and timing of exposure to stress (early life or adulthood) differently change seizure susceptibility to PTZ. Also, we showed that MS mice were resistant to the convulsive effect of PTZ suggesting the abnormal development of neurotransmission systems, mostly the opioid and GABAergic systems.

Supporting the differential effects of the opioid system on seizure susceptibility, we recently showed that juvenile social isolation stress has a proconvulsant effect in male mice, and administration of low-dose morphine (anticonvulsant dose) not only did not have an anticonvulsant effect but also produced proconvulsant effects in adult mice [19, 36]. Thus, the potential for stress exposure to have an effect on seizure susceptibility depends on the temporal relationship of the stress to brain development including, maturation of neurotransmission systems.

In conclusion, the results of our study provided preliminary data which indicate that experiencing MS alters seizure susceptibility to PTZ in adult male mice. Also, these changes were partly associated with the significant decrease in the activity of the opioid system of MS mice. Further research dedicated to finding the underlying mechanisms

involved in the effects of MS on the seizure activity on later life is warranted.

### Conflict of interest

The authors have no conflicts of interest to declare regarding the study described in this article and preparation of the article.

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