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Protective effects of M8-B, a TRPM8 antagonist, on febrile- and pentylenetetrazol-induced seizures

Nazanin Zandi[1], Nosaibeh Riahi Zaniani[2], Ali Moghimi[2] and Ali Roohbakhsh[3]*

[1] Department of Pharmacodynamics and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran,
[2] Rayan Center for Neuroscience and Behavior, Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran,
[3] Pharmaceutical Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran,
** Email: roohbakhsha@mums.ac.ir*

Epilepsy is a life-threatening disorder that is marked by recurrent seizures. Febrile seizure is a common neurological disorder observed in neonates. In many cases, reducing body temperature can prevent febrile seizure. Transient receptor potential cation channel subfamily M member 8 (TRPM8) is a cation channel that is involved in body thermoregulation. It was reported that M8-B, a TRPM8 antagonist, can reduce body temperature. Thus, we aimed to investigate the effect of M8-B on different experimental seizure models. Eight-day-old male Wistar rat pups were used for induction of febrile seizure. M8-B and diazepam were injected intraperitoneally. The rat pups were then transferred to a heated plexiglas chamber and the latency to the first febrile seizure was measured. In addition, different groups of mice were pretreated with M8-B and received a convulsant dose of pentylenetetrazol (PTZ). Latencies to stages 2 and 4 and duration of stage 5 seizure episodes were measured. Furthermore, the effect of M8-B on electroshock-induced seizures was also investigated and hindlimb extension time was measured. The results showed that M8-B decreased the body temperature of rat pups and increased the latency to the first febrile seizure, implying a significant protective effect. It also induced a significant anticonvulsant effect in PTZ - but not electroshock-induced convulsions. M8-B showed anticonvulsant effects in both febrile- and PTZ-induced seizures. M8-B had a hypothermic effect with significant protective effects on febrile- and PTZ-induced seizures; however, it did not produce similarly protective effects on seizures induced by electroshock.

Key words: febrile seizure, TRPM8, hypothermia, hyperthermia, rat pups

INTRODUCTION

Epilepsy is a chronic condition that has beset humans for centuries. Currently, it is estimated that 50 million people suffer from epilepsy and more than 40 types of epilepsy have been identified. This life-threatening disorder is associated with recurrent and unpredictable seizures with sensory, motor, and autonomic attacks, with or without disturbances of consciousness (Falco-Walter et al., 2018). Epileptic seizures are exhibited following synchronized electrical discharges in the brain. Dysfunction in inhibitory synaptic transmission mediated by gamma-aminobutyric acid (GABA), enhanced glutamatergic excitatory synaptic mechanisms, increases in calcium flow, and the intensification of spontaneous neuronal firing are the primary mechanisms responsible for seizure episodes.

Fever has been implicated as the main cause of seizures in infants and young children with fever leading to febrile seizure in one out of every 20–50 children (Dube et al., 2007). Generally, febrile seizure does not induce epilepsy. However, some studies show that prolonged febrile seizures increase the risk of developing epilepsy (Raspall-Chaure et al., 2006; Provenzale et al., 2008).

A variety of medications have been used to treat different types of epilepsy. However, their therapeutic efficacy is hampered by numerous drug interactions and side effects. The transient receptor potential (TRP) family of ion channels has been divided into seven subfamilies. TRP

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cation channel subfamily M member 8 (TRPM8), which acts as a cold temperature sensor, belongs to the TRPM subfamily. It is a nonselective ion channel with low calcium permeability. Its endogenous ligands are phosphatidylinositol biphosphate and lysophospholipids (Gavva et al., 2012). TRPM8 expression has been reported in brain regions such as hypothalamus, hippocampus, and amygdala (Voronova et al., 2015; Ivask et al., 2018; Kozyreva et al., 2018; Sutton et al., 2018). It is activated at 22–28°C and is a voltage-, temperature-, and ligand-gated ion channel (Voets et al., 2007). Menthol is a TRPM8 agonist with well-known cooling attribute. Intravenous administration of this substance makes animals prefer warmer places, indicating its important role in the modulation of temperature. Previous studies have shown that TRPM8 knock-out (KO) mice exhibit a disturbed perception of coldness (Bautista et al., 2007) with an absence of normal avoidance response to harmful coldness (Dhaka et al., 2007). Activation of TRPM8 increases core body temperature while its blockade decreases core body temperature. Its selective antagonists have been studied for the treatment of increased sensitivity to coldness in neuropathic patients, pain management, and overactive bladder (Lashinger et al., 2008; Winchester et al., 2014). In accordance, intraperitoneal (i.p.) injection of M8-B, as a TRPM8 selective antagonist, reduced core temperature as much as 0.9°C (Almeida et al., 2012). To the best of our knowledge, the role of the TRPM8 channel in epileptogenesis has not yet been evaluated. Considering the physiological and pharmacological features of the TRPM8 channel, we aimed to evaluate the effect of a selective TRPM8 antagonist in different experimental models of seizure.

METHODS

Eight-day-old male Wistar rat pups (13–15 g) were used for induction of febrile seizures. Male albino mice (25–30 g) were used for PTZ- and electroshock-induced convulsions. The animals were housed under standard conditions (22–25°C and 12 h light/dark cycle). They had free access to food and water at all times except when being tested. Each experimental group included eight animals. The present study was approved by the Ethics Committee of Mashhad University of Medical Sciences (no. 941081).

Eight-day-old rat pups were used for febrile seizure induction. At this age, rat pups' brains are developmentally similar to a six-month old human infant (Bender et al., 2007). To evaluate the effects of M8-B (a selective TRPM8 antagonist, Sigma, Germany) on body temperature, it was dissolved in sterile saline 0.9% and two groups of rat pups received i.p. injections of M8-B at a dose of 6 or 9 mg/kg. The doses were selected according to a previous study (Almeida et al., 2012). Then, their rectal temperature was measured every 5 min for 1 hour using a sensitive digital thermometer. After that, three groups of rat pups received M8-B (3, 6, and 9 mg/kg; i.p.) 30 min prior to the test, and two groups, as a control, received sterile saline 0.9% (10 ml/kg, i.p.) or diazepam (3 mg/kg, i.p.). Then, they were transferred to a heated plexiglas chamber. The temperature of the chamber was constantly measured by two thermometers. The pups were kept in the chamber at 40°C for 20 min and their body temperatures were measured every 2 min (Koyama, 2017). They were removed from the chamber if they showed symptoms of febrile seizure. The body temperatures after removing pups were between 40.8 and 41.8°C. Then, pups were transferred to a water container to control for hyperthermia. All experiments were videotaped and the latency to the first sign of febrile seizure, including increased frequency of urination and tonic-clonic contraction, was recorded.

For induction of PTZ-induced seizure, PTZ was administrated at a dose of 80 mg/kg, 30 min after administration of normal saline (10 ml/kg), diazepam (3 mg/kg), or M8-B (3, 6, and 9 mg/kg). The experiments were videotaped and the following parameters were recorded: stage 2 (S2L) and stage 4 (S4L) latencies, and stage 5 duration (S5D) (Kordi Jaz et al., 2017).

For induction of electroshock-induced seizure, the ears of each mouse subject were treated with normal saline 0.9% prior to placing the electrodes in order to enhance conductivity. A stimulus (60 Hz, 50 mA, 0.2 s) was applied via ear-clips connected to the ears. Five groups of mice received normal saline (10 ml/kg), diazepam (3 mg/kg), or M8-B (3, 6, and 9 mg/kg) 30 min before the test. Then, hindlimb extension time was measured (Hosseinzadeh and Parvardeh, 2004).

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) and repeated measures ANOVA, which were followed by Tukey's as a post-test if necessary. $P<0.05$ was set as the significance level.

RESULTS

The effect of M8-B on body temperature and febrile seizure

Fig. 1 shows that administration of M8-B reduced the body temperature of rat pups at doses of 6 and 9 mg/kg ($F_{12,72}=12.51$, $P<0.001$). M8-B, at a dose of 9 mg/kg, increased the latency of the first febrile seizure compared to the normal saline-treated group ($F_{4,35}=93.95$, $P<0.05$). Howev-

er, it did not induce a significant protective effect against febrile seizure at doses of 3 or 6 mg/kg (Fig. 2). Diazepam, administered as a control drug, significantly increased the latency of the first febrile seizure ($F_{4,35}$=93.95, P<0.001).

The effect of M8-B on PTZ-induced seizure

M8-B at a dose of 9 mg/kg, but not 3 or 6 mg/kg, increased the latency of S2L and S4L ($F_{4,35}$=37.3, P<0.001; $F_{4,35}$=209, P<0.001, Figs 3, 4) and decreased the S5D ($F_{4,35}$=278.57, P<0.001, Fig. 3C). Diazepam increased S2L ($F_{4,35}$=37.3, P<0.001) and S4L ($F_{4,35}$=209, P<0.001) and decreased S5D ($F_{4,35}$=278.57, P<0.001).

The effect of M8-B on seizures induced by electroshock

Neither doses of M8-B had a significant effect on hindlimb extension time induced by electroshock (Fig. 4). Diazepam (3 mg/kg) reduced the hindlimb extension time significantly ($F_{4,35}$=12.33, P<0.001).

DISCUSSION

This study was designed to evaluate the effect of a TRPM8 receptor antagonist (M8-B) on febrile-, PTZ-, and electroshock-induced seizures in related animal models. The results showed that M8-B reduced the body temperature of rat pups, confirming that the TRPM8 channel has an active role in thermoregulation in the body. In recent years, TRPM8 antagonists have been introduced as efficient pharmacological tools for reduction of core body temperature. Almeida et al. (2012) reported that i.p. injection of M8-B decreased core body temperature as much as 0.9°C in 20 min. They showed that intracerebroventricular and intraspinal administration of M8-B did not decrease body temperature. As a result, they concluded that the effects of M8-B were mainly due to its peripheral action (Almeida et al., 2012). Another study revealed that a single dose of AMG-9678, as a selective TRPM8 antagonist, resulted in a reduced body temperature that lasted for 12 h with peak effect occurring one hour after injection (Gavva et al., 2012). Repeated administration of the drug reduced body temperature by 0.62°C. The researchers also showed that AMG-2850, as another selective TRPM8 antagonist, attenuated core body temperature by 1°C after 2 hours (Gavva et al., 2012).

Considering its precise control of body temperature through various mechanisms, TRPM8 has been introduced as a target that can change body temperature set

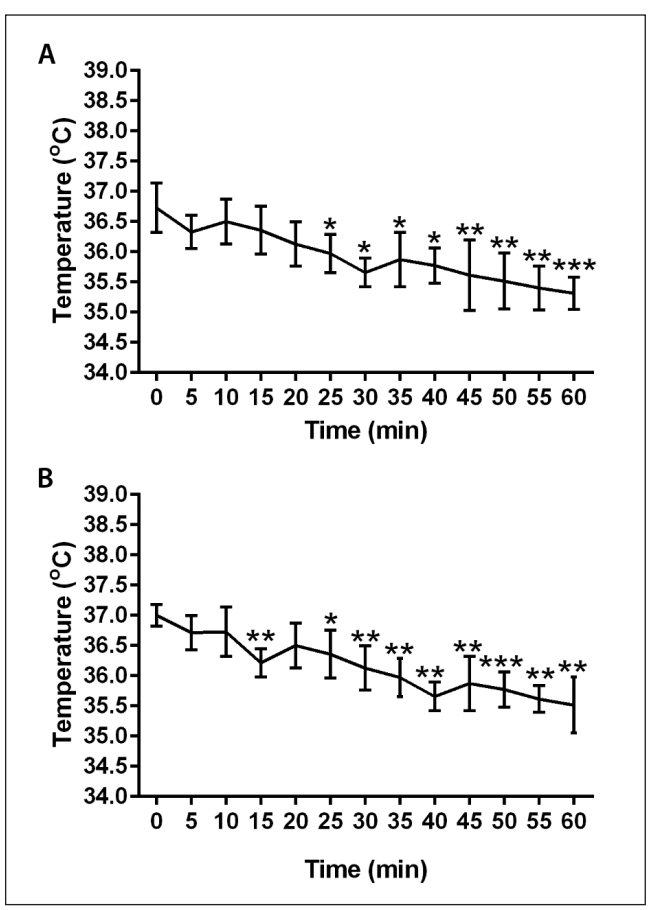

Fig. 1. The effect of M8-B on the body temperature of rat pups at doses of 6 mg/kg (A) and 9 mg/kg (B) Data are presented as mean ± SEM. n=8. ***: P<0.001, **: P<0.01, and *: P<0.005 compared to their body temperature before injection.

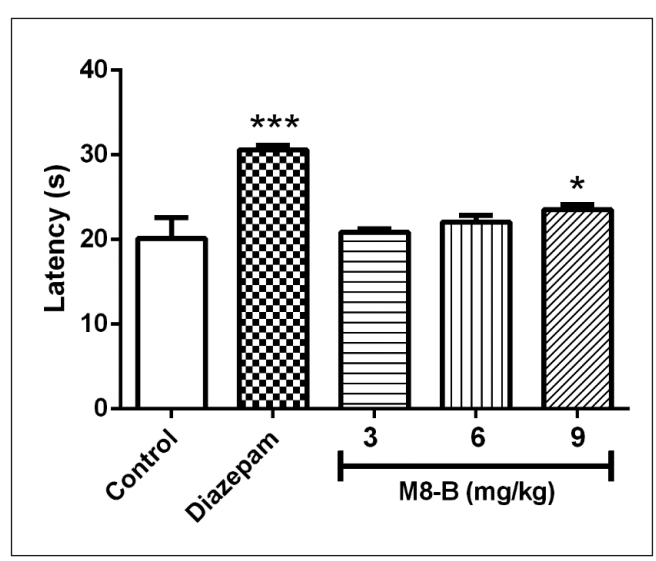

Fig. 2. The effect of M8-B on the latency of first febrile seizure of rat pups. Data are presented as mean ± SEM. n=8. ***: P<0.001 and *: P<0.05 compared to control group.

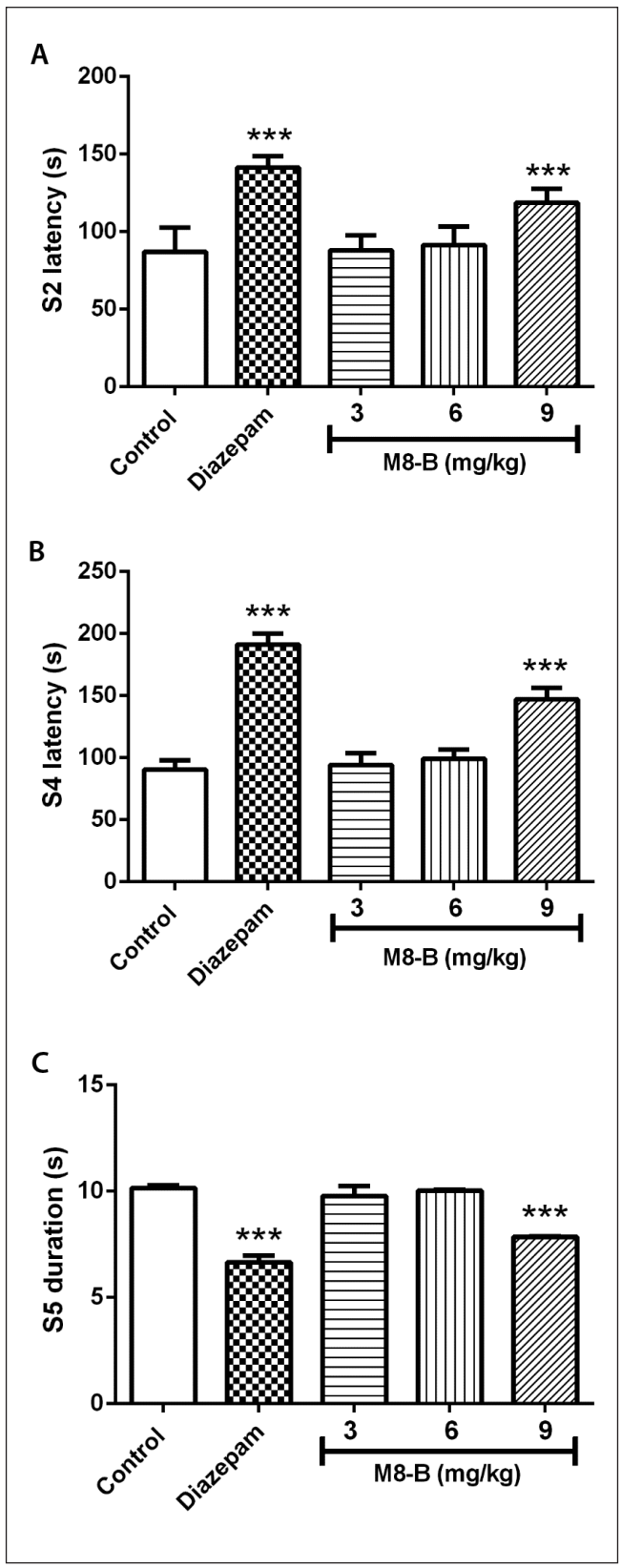

Fig. 3. The effect of M8-B on latency of stage 2 (A), latency of stage 4 (B), and duration of stage 5 (C) of PTZ-treated mice. Data are presented as mean ± SEM. n=8. ***: *P*<0.001 compared to control group.

point efficiently. Therefore, we hypothesized that its antagonist, M8-B, may induce protective effects in febrile seizure either by reduction of core body temperature and/or blockade of TRPM8, as an ion channel. Our results showed that M8-B, similar to diazepam, could decrease latency to the first febrile seizure compared to the control group. It is noteworthy that lowering body temperature has been introduced as an effective way of controlling seizure. Previous studies show that changes as little as 2° to 3°C in brain temperature affect neuronal properties and brain functions (Ritchie et al., 1956; Schiff et al., 1985). Hypothermia has been demonstrated to protect against seizure in both in vitro and in vivo models within seconds, without causing acute or delayed injury to the cooled brain (Inoue et al., 2017). Cooling affects field excitatory postsynaptic potentials and population spikes, in parallel with its effect on spontaneous epileptiform activity. It has inhibitory effects on neuronal transmission that are induced mainly via the GABAergic system (Motamedi et al., 2013). However, a study showed that both glutamate and GABA concentrations are decreased following cooling in extracellular fluid of patients with intractable epilepsy (Nomura et al., 2017). The researchers concluded that glutamate has a more important role than GABA in reduction of epileptic discharges during cooling. According to these studies, heat-sensitive molecules and receptors, including TRPM8, have a crucial role in maintaining brain functions as normal. The present results showed that M8-B reduced febrile seizures only at a dose of 9 mg/kg. Considering the hypothermic effect of M8-B at a dose of 6 mg/kg, we hypothesized that mechanisms other than hypothermic effect may also be involved in the beneficial effects of M8-B on febrile seizure.

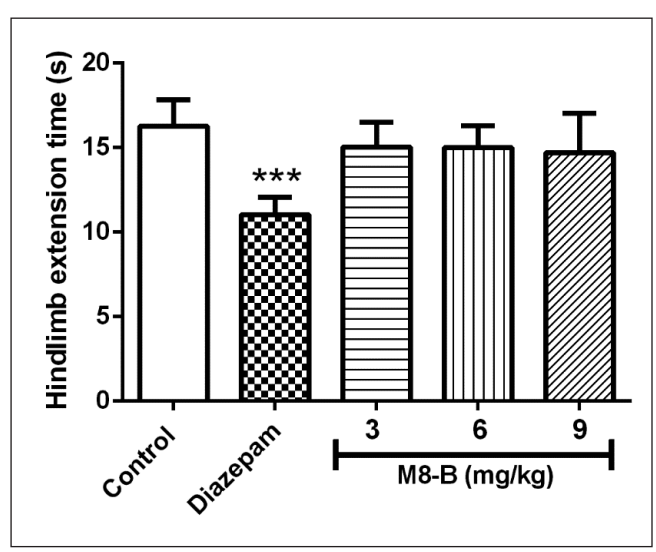

Fig. 4. The effect of M8-B and diazepam on hindlimb extension time induced by electroshock in mice. Data are presented as mean ± SEM. n=8. ***: *P*<0.001 compared to control group.

In another part of the study, we aimed to evaluate the effect of M8-B on PTZ- and electroshock-induced seizures. The results showed that pretreatment with M8-B (9 mg/kg) increased the stage 2 and stage 4 latencies and attenuated stage 5 duration compared to the control group. This finding implies that M8-B exhibited a significant anticonvulsant effect in the PTZ-induced convulsion model. However, it did not produce such protective effects in the electroshock model. In agreement with our results, Rauca et al. (2000) showed that hypothermia prevented PTZ-induced kindling and its associated memory deficits. Moreover, hypothermia decreased blood-brain barrier permeability following PTZ treatment (Oztas et al., 1994). PTZ blocks GABAA receptors, Ca^{2+}, Na^{+}, and K^{+} channels and induces hyperexcitability in the neuronal system (Papp et al., 1987; Hansen et al., 2004). On the other hand, following TRPM8 activation, sodium and calcium ions enter the cells, thereby leading to cellular depolarization and generation of action potentials. It is a possibility that M8-B via its hypothermic effect and/or inhibition of either calcium or sodium entry exhibited anticonvulsant effects in the PTZ model. Recent evidence shows that TRPM8 and inositol 1,4,5-trisphosphate (InsP3) receptor are co-localized (Melanaphy et al., 2016). InsP3 receptor acts as an important calcium channel. Hence, it may be suggested that blockade of TRPM8 and eventually inhibition of calcium entry via the channel and/or by modulation of InsP3 receptor prevented seizure. As mentioned, M8-B did not prevent seizures induced by electroshock. The difference in effectiveness of M8-B in two experimental models of epilepsy may be due to the underlying mechanism(s) of action. Similarly, some antiepileptic drugs such as ethosuximide and tiagabine were able to inhibit PTZ but not electroshock seizures (White et al., 1995; Dalby et al., 1997). Generally, drugs that prevent electroshock-induced seizures are suitable for the treatment of generalized tonic-clonic seizures, whereas drugs that suppress clonic seizures induced by PTZ are useful for the management of generalized absence seizures (White et al., 1995). To our knowledge, the role of the TRPM8 channel in an experimental model of epilepsy has not been evaluated in previous studies. Therefore, we cannot provide evidence to support our findings. However, reports related to other TRP channels show that their antagonists may be beneficial in the treatment of seizures. For example, it was reported that TRPM7 antagonists such as carvacrol or waxiencin reduced neuronal excitability in both in vitro and in vivo models (Khalil, 2016). Likewise, a TRPV1 antagonist reduced seizures in PTZ-induced chemical kindling (Shirazi et al., 2014). In addition, ablation of TRPC1, TRPC4, and TRPC5 channels reduced seizures and concomitant neuronal cell death in mice (Phelan et al., 2012; 2013). Based on the present findings, we suggest that TRPM8 may be a potential target for finding novel antiepileptic therapeutics. However, many additional studies are needed to elucidate the role of TRPM8 in the pathophysiology of different types of seizures. In line with these findings, topical administration of TRPM8 agonists has been used to resolve chronic itching and dry eye in two recent clinical studies (Stander et al., 2017; Yang et al., 2017). Therefore, the future pharmacotherapy based on TRPM8 ligands appears promising.

CONCLUSION

The results of the present study showed that M8-B, as a TRPM8 antagonist, exerted a hypothermic effect with significant protective effects on febrile- and PTZ-induced seizures. However, it did not produce similar protective effects on seizures induced by electroshock.

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