The Effects of the Fraction Isolated from Iranian *Buthotus shach* Scorpion Venom on Synaptic Plasticity, Learning, Memory, and Seizure Susceptibility

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Abstract

Epilepsy, as a neurological disease, can be defined as frequent seizure attacks. Further, it affects many other aspects of patients’ mental activities, such as learning and memory. Scorpion venoms have gained notice as compounds with potential antiepileptic properties. Among them, *Buthotus schach* (BS) is one of the Iranian scorpions studied by Aboutorabi et al., who fractionated, characterized, and tested this compound using electrophysiological techniques in brain slices (patch-clamp recording). In the present study, the fraction obtained from gel electrophoresis was investigated through behavioral and electrophysiological assays. At first, ventricular cannulation was performed in rats, and then the active fraction (i.e., F3), carbamazepine, and the vehicle were microinjected into the brain before seizure induction by the subcutaneous (SC) injection of pentylenetetrazol (PTZ). Seizure behaviors were scaled according to Racine stages. Memory and learning were evaluated using the Y-maze and passive avoidance tests. Other groups entered evoked field potential recording after microinjection and seizure induction. Population spike (PS) and field excitatory postsynaptic potential (fEPSP) were measured. The F3 fraction could prevent the fifth stage and postpone the third stage of seizure compared to the control (carbamazepine) group. There was no significant improvement in memory and learning in the group treated with the F3 fraction. Also, PS amplitude increased significantly and long-term potentiation was successfully formed after the high-frequency stimulation of the performant pathway. Our results support the antiepileptic effects of the F3 fraction of BS venom, evidenced by behavioral and electrophysiological studies. However, the effects of this fraction on memory and learning were not in the same direction, suggesting the involvement of two different pathways.

Keywords: Epilepsy, Scorpion, Memory, Learning, Synaptic, Plasticity, Field, Recording, Acute, Seizure.

1. Background

Epilepsy is one of the most common neurological impairments around the world. Statistics show an incidence rate of 10% in the general population (1). Frequent seizure attacks aside, epilepsy can have profound negative effects on cognition, consciousness, and motor function (2). Current treatments for epilepsy are mainly divided into two groups: pharmaceutical therapy and surgery. Both of these therapeutic approaches may cause serious side effects, and they may not be able to completely control seizure attacks. Also, current medications merely alleviate epileptic symptoms but have no impact on epileptogenesis (3). Another remarkable point is that epilepsy medicines have a wide range of interference with other medications and even foods.

Long-term potentiation (LTP) is defined as the purposeful reorganization or regeneration of neurons in response to persistent stimuli in order to change neuronal function and structure (or even both) to strengthen neuronal reaction to the stimulus (4). The hippocampus
region has an enormous role in LTP formation in the brain, and the Dentate gyrus region of the hippocampus is one of the most important players in this event (5). Long-term potentiation is known to have a substantial role in regulating brain functions, such as memory and learning, and also in the development of diseases like epilepsy and depression (4, 6). The fact that epilepsy can cause memory and learning problems has been widely studied (7-10), and both of these conditions are believed to be rooted in the same origin and follow similar mechanisms. Therefore, it is crucial to more deeply explore this potential link.

Natural compounds are gaining attention as potential treatments for seizures and epilepsy. Researchers have proved the anti-seizure potency of compounds like flavonoids, terpenoids, and alkaloids (11). A large number of polypeptides with anti-seizure properties have been extracted and purified from animals, including wasps, bees, spiders, and scorpions (12, 13). Scorpion envenomation is a lethal situation that can lead to death in a significant number of victims. There are controversial reports on the clinical outcomes and applications of scorpion envenomation. One of the symptoms in people sting by scorpions is acute seizure. On the other hand, there is growing interest in purifying the fractions of scorpion venom with anti-seizure properties, leading to promising achievements (14). Scorpion venoms are mixtures of different peptides, including long-chain peptides with modulating effects on sodium channels and short-chain peptides with blocking effects on potassium channels. Buthotus schach (BS) is a scorpion belonging to the Buthidae family with a limited distribution in some parts of Iran. The most recognized symptoms following BS envenomation are arrhythmia, respiratory depression, convulsion, and cardiac arrest (17).

2. Objectives

Regarding the fact that scorpion venoms affect ion channels and interfere with cellular functions, we investigated if BS venom could modulate the function of ion channels in Wistar rats.

3. Methods

3.1. Experiment 1

3.1.1. Effects of F3 Fraction on Susceptibility to Seizure, Learning, and Memory

Buthotus schach crude venom was provided by the Department of Poisonous Animals, Razi Vaccine and Serum Research Institute, Karaj, Iran. The venom was obtained by electrically stimulating the scorpion telson and then was lyophilized using a freeze dryer (18). Venom fractions were separated by gel filtration as described in a previous study. According to Aboutorabi et al., the F3 fraction, compared to other fractions, could decrease sodium flow in patch clamp recording. It has been confirmed that sodium flow has a significant role in the occurrence and progression of seizures, so its blocking may promote anti-seizure effects. Regarding the above-mentioned, we decided to divulge the potential anti-seizure effects of this fraction more deeply (17).

3.1.2. Animals

Eighteen male Wistar rats in the weight range of 250 - 300 gr were randomly divided into three groups (n = 6 per group). The animals were obtained from the Pasteur Institute (Tehran, Iran), had free access to water and pellet food, and were kept in standard condition (12/12 h light-dark cycle, temperature of 22 ± 2ºC, and 40% humidity). All experiments were carried out according to the guidelines of the Ethical Committee of Shahid Beheshti University of Medical Sciences, and the code of ethics was IR.SBMU.PHARMACY.REC.1402.038.

The animals were anesthetized by ketamine (100 mg/kg) and xylazine (10 mg/kg) and fixed on the stereotaxic apparatus. An incision was made on the skin, and extra tissue was carefully removed from the target area. After the spots and midline became visible, the left ventricle of the brain was marked and drilled. A stainless-steel guide cannula was placed in the whole, according to Paxinos and Watson’s atlas. Dentistry cement was used to fix the cannula and close the wound. After a 5- to 7-day recovery period, the rats were injected by the target fraction through the intra-cerebroventricular (i.c.v) route. Control animals received normal saline; carbamazepine-injected rats were regarded as positive controls (400 µg/rat). The last group received the active fraction of BS venom (F3, 10 µg/kg). Thirty minutes after the i.c.v injection, 60 mg/kg of PTZ was subcutaneously injected into the back in all groups. Seizure behaviors were observed for 30 minutes and scaled in reference to
the Racine seizure grading system. The Y-maze and passive avoidance tests were performed after seizure induction to assess the effects of seizure on learning and memory.

In the Y-maze test, animals were placed in the central part of a 3-compartment maze, and the number and order of entrance in each arm were recorded for 10 minutes. The alternation percentage was calculated and compared between the groups (19).

The passive avoidance test was performed immediately after the Y-maze test. Animals were placed in the bright compartment and allowed to move freely to become familiar with the environment. Then, the gate between the two compartments was opened, and the animals entered the dark compartment out of curiosity. When the animal walked into the dark compartment, a low-frequency unpleasant stimulus (current = 0.2 mA, frequency= 50 Hz, duration = 5s) was applied to the animal's feet to create a memory to prevent further entrance. On the second day of the experiment, the animals were placed in the bright compartment, and the gate was opened. The cut-off time was set as 120 seconds, and not entering the dark compartment after 120 seconds was considered as passing the test; otherwise, the animal failed this test. The experiment was repeated three times after the first adaptation test (20).

3.2. Experiment 2

3.2.1. Electrophysiological Studies

Evoked-field potential (e-LFP) animals were divided into 4 groups (6 rats in each group); stereotaxic surgery and the i.c.v injection of the active fraction were performed as described in the previous section (i.e., experiment 1, behavioral studies). After 30 min, PTZ was subcutaneously injected into the animals (60 mg/kg), which were anesthetized by the intraperitoneal injection of Urethane (1.5 g/kg). Thirty minutes after seizure induction, animals' heads were fixed on the apparatus using ear bars. Covering cement was removed; the skull was cleaned, and the two target points (dentate gyrus and perforant pathway) were marked, where holes were drilled into the skull. Electrode coordinates were applied for the performant pathway (anteroposterior = -8.1 mm; mediolateral = ±4.3 mm; dorsoventral = 3 - 3.3 mm) and dentate gyrus in the left hemisphere (anteroposterior = -3.8 mm; mediolateral = ±2.4 mm; dorsoventral = 2.7 - 3.2 mm). Two electrodes made of double-strand stainless steel wire were placed above the marked regions and lowered slowly to reach the accurate point under the skull surface according to the spike shape, and this process was closely monitored on a screen. A stability period of 10 - 20 min was given to the recording cells. After that, the I/O curve was plotted, and the test stimulus current was obtained from 60% of the maximum response. Signals were amplified (100 ×) and filtered (1 to 3 kHz bandpass) using a differential amplifier. Baseline recording was performed for 15 minutes (one stimulus with a frequency of 100 mHz was induced every minute, and the spike was recorded). Then, LTP was induced according to the high-frequency stimulation protocol (10 trains of 20 pulses at 400 Hz with 80% of maximum intensity every 10 seconds). In the final step, the dentate gyrus area was monitored for 60 minutes. It was expected to see a 25% acceleration in two measuring parameters, including population spike (PS) amplitude and excitatory postsynaptic potential (EPSP) (i.e., the slope of the line connecting the first and second peaks of the record). Rats with incorrect electrode positioning were excluded. Recording and data analysis were performed by e-probe and e-lab setup (21, 22).

4. Results

4.1. Experiment 1

In behavioral studies, statistical analysis showed that the administration of the F3 fraction of BS venom increased latency to the stage 3 seizure according to the Racine seizure scale. Remarkably, the effectiveness of the F3 fraction was considerably close to that of carbamazepine (an approved epilepsy medicine), both of which could postpone the occurrence of stage 3 PTZ-induced seizure. Figure 1 depicts the impact of the F3 fraction on latency to stage 3 seizure in comparison with other groups.

The effects of the F3 fraction on latency to stage 5 (tonic-clonic) seizure have been shown in Figure 2. The F3 fraction could completely prevent the occurrence of tonic-clonic seizures.

4.2. Y-maze and Passive Avoidance Tests

In the Y-maze test, we calculated the alternation percentage. The number and order of animals’ entries to the arms were recorded. A consecutive rotation between the two arms was considered an alternation. The number and percentage of alternations were calculated using the equation shown in Figure 3A. The results showed that there was no significant difference between the study groups, meaning that the F3 fraction could not improve memory and learning in animals (Figure 3B).

The passive avoidance test was repeated three times for each animal at a cut-off time of 120 seconds. No entry to
Figure 1. The effect of F3 fraction on latency to stage 3 PTZ-induced acute seizure in rats. Six animals were used in each group. One-way ANOVA was used for multiple comparisons between groups, followed by Tukey's post-hoc test. Mean ± SD was reported.

Figure 2. The effect of F3 fraction on latency to PTZ-induced acute stage 5 seizure in rats. Six animals were used in each group. One-way ANOVA was used for multiple comparisons between groups, followed by Tukey's post-hoc test. Mean ± SD was reported.

the dark compartment after 120 seconds was considered passed; otherwise, the test result was regarded as failed. In this test, no significant difference was observed in the number of animals entering the dark compartment between the rats treated with the F3 fraction and other groups. Figure 3 shows the results of these two tests. The results of the passive avoidance test have been shown in Figure 3C.

4.3. Experiment 2

In electrophysiological studies, recording was performed in the dentate gyrus area, and data were obtained before and after LTP induction according to the high-frequency stimulation (HFS) protocol. Between- and within-group analyses (Figure 4A) indicated some traces in field potential recording before and after HFS induction in the study groups. Also, the Input/Output curve (I/O curve) was provided (part B in the very same figure).

Postsynaptic activities were recorded by the extracellular field potential recording technique. Baseline activities and post-LTP behaviors were compared at different times (Figure 5). Statistical analysis (two-way ANOVA) demonstrated a significant impact on F3 fraction [F (74, 1036) = 6.747, P < 0.0001]. There was also a significant difference between the groups at different time points [F (3, 1036) = 121.1, P < 0.0001]. Administration of the F3 fraction significantly increased PS amplitude in comparison with the control group (vehicle) in almost all time points. Rats in other groups (CBZ and PTZ) showed no significant changes in PS-LTP amplitude. So, it can be concluded that the F3 fraction of BS could affect the action potential pathway and neuronal firing by modulating the activity of ion channels.

In another experiment, the fEPSP factor was recorded and compared between the study groups (Figure 6). Two-way ANOVA analysis revealed the significant impact of time on the fEPSP factor [interaction F (222, 1040) = 1.201, P=0.0353; time F (3, 1040) = 241.8, P < 0.0001]. Further analysis by Bonferroni's post-hoc test revealed that there was a significant rise in slope in the control group vs. the PTZ-received group after LTP induction.

As shown in Figure 7, two-way ANOVA showed that the PS amplitude was significantly higher in the F3-treated group than other groups 0 to 15 minutes after HFS induction and also in the last 15 minutes of LTP baseline recording (45 - 60 mins) [interaction F (6, 843) = 24.96, P < 0.0001; treatment F (2, 843) = 304.4, P < 0.0001; time F (3, 843) = 91.74, P < 0.0001].

Comparing the fEPSP slope factor between the study groups at different times revealed a significant difference between the PTZ group and other groups after LTP induction (Figure 8) [interaction F (6, 843) = 5.628, P < 0.0001; time F (3, 843) = 22.33, P < 0.0001].

5. Discussion

Our results showed that the active fraction of BS venom (i.e., F3) could affect the severity of PTZ-induced seizure and the latency of each stage. As it has been proven in previous research performed by Aboutorabi et al., this fraction exerts different impacts on ion channels in comparison with the whole venom and its other fractions, evidenced...
by the patch clamp technique, indicating its potential antiepileptic properties (17). However, our data contradict the results of some previous studies.

We conducted two routine tests to assess the impact of the F3 fraction on the memory and learning impairments caused by PTZ-induced seizure (23, 24). It is known that epilepsy and seizure can shift from the LTP pattern to epileptogenesis, disturbing the memory formation pathway (25, 26). The Y-maze and passive avoidance tests were performed to discover the effects of the active fraction of BS scorpion venom on the functional patterns of short-term and long-term memory after seizure, respectively (27). In fact, our results did not support this hypothesis that the F3 fraction could improve seizure-induced memory dysfunction, evidenced by observing no significant difference between the F3-received group and other groups. It could be a hint that F3 may interact with different pathways and act through other mechanisms to modulate memory and learning patterns.

Long-term potentiation is one of the most important markers of synaptic plasticity during both physiological and pathological events in the brain. Actually, LTP happens in response to repetitive stimuli and potentiates neuronal connections and functions in a specific way (4). As it has been pointed out previously, the hippocampus, and specifically the dentate gyrus region, is mostly responsible for LTP formation (28). Field potential recording is one of the electrophysiological tools widely used to study cerebral functions. Also, some studies have used field potential recording to track LTP, most commonly in the dentate gyrus of the hippocampus (21, 22, 29, 30). In this study, the field potential technique was used to complement the results of behavioral studies by recording fEPSP and PS amplitudes. The PS amplitude is an indicator of neuronal activation following ion movements and the generation of action potentials (31). According to our results, the F3 fraction of BS enhanced the PS amplitude. A previous experiment pointed out three major functional peptides existing in scorpion venom (32), including peptides affecting sodium currents by interacting with open/closed gates (33), peptides that modulate potassium ion currents by blocking pores, and finally, those modulating calcium currents through Ryanodine channels (34, 35). It seems that the F3 fraction has an effect on neuronal firing following high-frequency
Figure 4. (A) Traces show the difference in the field activity of neurons before and after HFS induction in different groups. (B) The method to determine the intensity. Input stimuli were given to neurons, the PS amplitude was recorded, and when it reached a steady state, 60% of the maximum was considered as the target stimulus.

stimulation, which can originate from sodium-oriented interactions. The PS amplitude raised following treatment with the F3 fraction, which might be the first step of memory and learning formation in a convulsive brain; however, we could not prove this notion in our behavioral assessments.

According to previous studies, NMDA receptors and their ligands have a great role in the formation of LTP and LTD in the hippocampus. For instance, in the study of Migaud et al., a mutation lowering the level of PSD-95, an NMDA receptor binding protein, caused an imbalance between the LTP and LTD patterns and shifted the activity toward LTP. Behavioral studies show that learning and memory form upon a balanced state between LTP and LTD, so stimuli-induced overexpression of LTP can impair LTD formation, adversely affecting memory and learning (36). In another study by Gu et al., the Fmr2 gene was knocked out, which reproduced the same results (37). In our study, the F3 fraction was observed to exert the same effect on the LTP-LTD balance; however, the enhancement of LTP in recordings was not associated with positive impacts on memory and learning.

Repetitive firing can nurture a saturated environment in the synaptic space, hindering LTP formation. Seizure enforces neuronal networks to function at an intense level that saturates synapses and prevents robust LTP
Figure 5. F3 fraction of BS venom affected firing in the synaptic space. According to two-way ANOVA analysis, the F3 fraction significantly accelerated the PS factor in almost all time points in comparison with the control group (n = 8, *P < 0.05).

Figure 6. PTZ-received rats showed a significantly lower slope after high-frequency stimulation in comparison with F3-treated animals, suggesting the opposite actions of F3 and PTZ in LTP formation in the dentate gyrus region (n = 8, ****P < 0.0001).
formation in the DG following high-frequency stimulation of the perforant pathway. Our results indicated that F3 administration before seizure induction showed higher synaptic flexibility mirrored by stronger LTP expression in comparison with epileptic rats treated with the vehicle. Seemingly, the F3 fraction regulates the tone and frequency of neuronal activity and keeps them in a balanced state, providing free spaces for synaptic potentiation and LTP formation.

Field excitatory postsynaptic potential reflects the excitatory drive toward neurons (38). We found that PTZ treatment at the dose of 60 mg/kg decreased this parameter to a significantly lower level than F3 treatment. This observation suggests that PTZ and F3 act in two
completely different directions. The known mechanism of action of PTZ is through antagonizing the GABA pathway, leading to repetitive firing and seizure induction (39). On the contrary, our data suggested that F3 could suppress PTZ-induced seizure; however, F3 also facilitated LTP formation after HFS induction. It seems that F3 exerts its seizure-preventing effects through a mechanism other than LTP formation or by targeting a different region in the brain. As has been described in the previous paragraph, F3 modulates synaptic activity and prevents the unbridled firing of neurons. As we showed in this study, this activity of F3 could explain fEPSP elevation after HFS induction. On the other hand, we witnessed a great decrease in the relevant slope in the vehicle-treated group before seizure induction, which might be the result of synaptic hyper-activation and gradual neuronal debilitation. However, a number of neurons remain active.

**Figure 8.** Two-way ANOVA followed by Tukey’s post-hoc test for comparing the fEPSP factor between the study groups in different time ranges, indicating a significant difference between the PTZ group vs. all other groups after LTP induction [*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001].**
in this situation and fail to reach the highest level of activation. Also, this phenomenon could be related to the fall of the driving force due to the influx of sodium ions into cells, as well as because of the depolarization of the resting membrane potential after HFS induction and the disability of the membrane to reach the previous resting potential and sodium homeostasis.

5.1. Conclusions

In conclusion, our results showed that the F3 fraction isolated from BS scorpion venom could enhance neuronal connections and plasticity by inducing LTP and preventing PTZ-induced seizure in rats. However, the F3 fraction could not significantly improve memory and learning functions following seizure induction.

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Footnotes


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