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The milk-derived opioid peptide β-casomorphin-7 delays progression to the tonic–clonic phase and limits the increase in gamma band activity in acute pentylenetetrazole seizures in rats

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Abstract

Objective

This study aimed to evaluate the effects of β-casomorphin-7 (BCM-7) on behavioural seizure responses and electrocorticographic (ECoG) activity in a pentylenetetrazole (PTZ)-induced acute seizure model, together with the possible contribution of the opioid receptor system to these effects.

Methods

Fifty-six male Wistar albino rats were randomly allocated to seven groups, designated SAL + SAL, VEH + SAL, BCM-7 + SAL, VEH + PTZ, BCM-7 + PTZ, NLX + BCM-7 + PTZ and NLX + VEH+PTZ, where SAL denotes saline, VEH the BCM-7 vehicle and NLX naloxone. BCM-7 (1 mg/kg), naloxone (2 mg/kg) and PTZ (70 mg/kg) were administered intraperitoneally. In the four PTZ-treated groups, the latencies to myoclonic jerk and to tonic–clonic seizure, the progression interval between these two events, seizure severity scored blind on coded video recordings according to the revised Racine scale, mortality and time of death were assessed, and survival was analysed using the Kaplan–Meier method and the log-rank test. ECoG recordings were examined by power spectral density analysis, and the absolute and relative powers of the frequency bands, total absolute power, median frequency and band ratios were calculated.

Results

In the planned comparison for myoclonic jerk latency, the latency was longer in the BCM-7 + PTZ group than in the VEH + PTZ group ($p = .034$). BCM-7 prolonged the tonic–clonic seizure latency approximately 1.5-fold and the myoclonic jerk–tonic–clonic seizure progression interval approximately 2.7-fold (both $p < .001$), and these effects were preserved in the presence of naloxone. PTZ markedly increased absolute power across all frequency bands in the groups in which naloxone was not administered alone. In the post-treatment measurements, gamma ($p = .024$) and HFA ($p = .003$) absolute powers were lower in the BCM-7 + PTZ group than in the VEH + PTZ group. The difference in total absolute power remained at the limit of significance ($p = .053$); however, this difference and those in the delta and HFA bands were also present before treatment, and the PTZ-induced increase in total power was comparable in the two groups. No significant difference was detected in theta, alpha or beta absolute power. Administration of naloxone alone limited the PTZ-induced increase in all absolute power measures. No overall difference between groups was found in Racine scores ($p = .103$), mortality rates ($p = .182$) or survival (log-rank $p = .226$). Nevertheless, death occurred later in the BCM-7 + PTZ group than in the VEH + PTZ group (median 17.0 vs. 10.0 min), whereas seizure severity ($p = .020$) and the risk of death ($p = .043$) were lower in the NLX + BCM-7 + PTZ group than in the BCM-7 + PTZ group.

Conclusion

In the acute PTZ model, BCM-7 displays a profile that delays progression to the tonic–clonic phase and limits the increase in gamma activity without markedly altering the PTZ-induced increase in total cortical power, rather than a classical anticonvulsant effect that fully prevents seizures. The preservation of the behavioural effects in the presence of naloxone, together with the independent ECoG effects of naloxone, suggests that the effects of BCM-7 may not be explained solely by naloxone-sensitive opioid mechanisms. The reduction in seizure severity and in the risk of death under co-administration with naloxone further suggests that a naloxone-sensitive component of BCM-7 may contribute to seizure severity and mortality.

Introduction

Epilepsy is a chronic brain disorder characterised by recurrent seizures that usually occur without an overt external trigger and that may lead to substantial neurobiological, cognitive, psychological and social consequences. Epileptic seizures are characterised by transient motor, sensory, autonomic or behavioural signs arising from abnormal, excessive and synchronous electrical activity in neuronal networks (Fisher et al., 2005; Sarmast et al., 2020). In their pathophysiology, neuronal hyperexcitability and excessive synchronisation produced by a disturbance of the balance between glutamatergic excitation and GABAergic inhibition, by changes in voltage-gated ion channels and by disturbances in ion distribution play a central role (Anwar et al., 2020; Bromfield et al., 2006; Vera-González, 2022). Depending on the networks involved, seizures may be focal or generalised (Boleti et al., 2024; Kanner & Melo Bicchi, 2022), and the clinical findings may range from mild sensory changes to tonic–clonic seizures (Sarmast et al., 2020). Although the available antiseizure medications target different ion channels and neurotransmitter systems, adequate seizure control cannot be achieved in approximately one third of patients, which makes the investigation of new molecular mechanisms and neuromodulatory agents necessary (Mesraoua et al., 2023).

Pentylenetetrazole (PTZ) is a convulsant that is widely used in experimental seizure research and that produces cortical hyperexcitability by disrupting the inhibitory function of the GABA-A receptor complex. PTZ has been reported to interact with the picrotoxin-sensitive site of the GABA-A receptor complex (Ramanjaneyulu & Ticku, 1984), to act at a domain that overlaps with but is distinct from that of picrotoxin and to reduce the GABA-activated chloride current in a concentration-dependent manner (Huang et al., 2001), whereas acute administration lowers GABA-A receptor subunit mRNA levels (Walsh et al., 1999). The PTZ model is therefore used to evaluate seizure onset and generalisation as well as the effects of pharmacological interventions on seizure development; in the PTZ kindling model, the anticonvulsant and antiepileptogenic effects of GABA-A receptor ligands have been demonstrated with this approach (Hansen et al., 2004). Because the model allows behavioural and electrophysiological measures to be considered together, both sets of measures could be evaluated jointly in the present study. In this context, whether the excitation–inhibition balance can be modulated by food-derived opioid peptides is of importance for investigating the effects of β-casomorphin-7 in the PTZ model. β-

casomorphin-7 (BCM-7) is an opioid-like heptapeptide with the sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile that is released during the proteolytic digestion of bovine β-casein (Bolat et al., 2024). BCM-7 has been reported to bind to μ-opioid receptors and to influence endocrine and neurological processes (Bolat et al., 2024; Giribaldi et al., 2022) and, at the gastrointestinal level, to alter the mucosal immune response (Miyagawa et al., 2024), while a role for serotonergic mechanisms in its central effects has been proposed on the basis of the demonstration that BCM-7 interacts with 5-HT2 receptors (Sokolov et al., 2005). The literature on the effects of casomorphin derivatives on seizures and cortical oscillations is limited: β-casomorphin-(1–5) derivatives have been reported to exert derivative-dependent proconvulsant or anticonvulsant effects in PTZ-, picrotoxin- and electrically induced seizures (Becker et al., 1991a); the des-Tyr derivative BCH-325 has been reported not to protect adult rats against PTZ-induced convulsions while affording protection in immature animals (Becker et al., 1991b); and the same peptide has been reported to increase the power of the alpha-2 and beta-1 bands while decreasing delta and theta power in the cortical EEG (Diedrich et al., 1994). However, no study on the effects of BCM-7 in the acute PTZ model was found in the literature. The aim of the present study was therefore to evaluate the effects of BCM-7 on myoclonic and tonic–clonic seizure latencies, seizure severity, mortality and the absolute powers of the ECoG frequency bands in a PTZ-induced acute seizure model, and to investigate the possible contribution of the opioid receptor system to these effects by means of naloxone.

Materials and Methods

Ethical approval

and experimental animals

The study was conducted in accordance with approval no. 77.637.435/317 of the Local Ethics Committee for Animal Experiments of Manisa Celal Bayar University. A total of 56 male Wistar albino rats (10–12 weeks old, 300–400 g) in seven groups were used. The animals were housed at 22 ± 1°C and approximately 50% humidity under a 12-h light/12-h dark cycle, with free access to standard chow and water. The four PTZ-treated groups initially comprised 32 rats; owing to a problem with the behavioural recordings of two rats, the behavioural analyses were performed on the data of 30 rats. These analyses covered the latencies to myoclonic jerk and to tonic–clonic seizure, Racine scores and mortality rates. Because of artefacts and recording irregularities observed in the ECoG traces, the recordings of three rats were not included in the ECoG analyses, which were therefore completed on the data of 53 rats.

Epidural electrode implantation and the postoperative period

Stereotaxic surgery for epidural electrode implantation was performed under anaesthesia induced by an intraperitoneal combination of ketamine (80 mg/kg) and xylazine (20 mg/kg). The scalp was first opened through an incision along the anteroposterior (AP) axis. With bregma as the reference, the active (+) electrode was placed at approximately AP + 4 mm and mediolateral (ML) + 3 mm (right), the reference

(−) electrode at AP − 4 mm and ML + 3 mm (right) and the ground electrode at AP − 4 mm and ML − 3 mm (left), all positioned epidurally with the integrity of the dura mater preserved. Screw electrodes were used for electrode placement. The electrodes and the connector socket were fixed in place using insulating material and dental composite. To allow postoperative recovery and to reduce possible surgery-related inflammatory effects, the animals were given a 7-day recovery period and were observed daily during this period for any adverse condition.

Experimental groups and pharmacological treatments

After surgery, the animals that continued in the experimental protocol were randomly allocated by lot to seven groups. The group design and the treatments are shown in Table 1. In the first stage of the study, baseline ECoG recordings were obtained from the groups. The primary pharmacological treatment was then administered and a 10-min interval was allowed. At the end of this interval, the second treatment was administered: saline to the groups not receiving PTZ and pentylenetetrazole to the PTZ groups, both intraperitoneally. In the PTZ-treated acute hyperexcitability groups, video and ECoG recordings were obtained simultaneously with the treatment. The BCM-7 dose was selected as 1 mg/kg on the basis of systemic administrations in previous rodent studies; naloxone was used at 2 mg/kg and PTZ at 70 mg/kg. The BCM-7 dose was determined on the basis of previous studies reporting that systemic administration of 1 mg/kg produces biological and behavioural effects in rodents (Dubynin et al., 2000; Maslennikova et al., 2008; Zozulia et al., 2009). In those studies, this dose produced cellular and behavioural effects in newborn and infant rats (Dubynin et al., 2000; Maslennikova et al., 2008) and a change in a serotonergically mediated behavioural response in mice (Zozulia et al., 2009). No study directly demonstrating the efficacy of the same dose in adult rats was found in the literature. The 2 mg/kg intraperitoneal naloxone dose used to evaluate the contribution of opioid receptors was determined with reference to the study of Havemann and Kuschinsky (1981), which reported that the muscular rigidity induced by β-casomorphin-4 in rats was reversed by the same dose of naloxone.

Table 1
Experimental groups and the scheme of pharmacological treatments

Group	Behavioural seizure analysis (n)	ECoG analysis (n)	First treatment	Second treatment (10 min later)	Abbreviation
1	–	8	Saline, 1 mL/kg	Saline, 1 mL/kg	SAL + SAL
2	–	8	BCM-7 vehicle, 1 mL/kg	Saline, 1 mL/kg	VEH + SAL
3	–	8	BCM-7, 1 mg/kg	Saline, 1 mL/kg	BCM-7 + SAL
4	8	7	BCM-7 vehicle, 1 mL/kg	PTZ, 70 mg/kg	VEH + PTZ
5	7	8	BCM-7, 1 mg/kg	PTZ, 70 mg/kg	BCM-7 + PTZ
6	7	7	Naloxone, 2 mg/kg + BCM-7, 1 mg/kg	PTZ, 70 mg/kg	NLX + BCM-7 + PTZ
7	8	7	Naloxone (2 mg/kg) + BCM-7 vehicle	PTZ, 70 mg/kg	NLX + VEH+PTZ

*– = Behavioural seizure assessment was not performed in the groups not receiving PTZ.

Saline and vehicle were administered at 1 mL/kg in the respective groups. The BCM-7 solution was prepared by dissolving 1 mg BCM-7 in 1 mL of a mixture containing 10% DMSO (dimethyl sulfoxide), 40% PEG300 (polyethylene glycol), 5% Tween-80 (polysorbate) and 45% saline, and was dosed according to the body weight of the animals. The same solvent was administered in the same volume to the vehicle groups. The bovine BCM-7, pentylenetetrazole and naloxone used in the study were obtained from MedChemExpress (Monmouth Junction, NJ, USA).

ECoG recordings, power spectral density and spectral analysis

Recordings were obtained from freely moving animals at a sampling rate of 4 kHz using a PowerLab/SP8 system (ADInstruments, Australia) with the LabChart interface. In the PTZ groups, recording was started before the PTZ injection and continued for 60 min. In the groups not receiving PTZ, the recording period was set at 10 min. For the ECoG analyses, the mean of three separate 30-s epochs free of marked artefact was used for the baseline and final measurements of the groups not receiving PTZ and for the baseline measurements of the groups to receive PTZ. In the PTZ-treated groups, the final measurement was obtained from a 30-s epoch beginning at the moment at which the first myoclonic jerk was identified on the video and ECoG recordings. A 30-s epoch duration is one of the epoch durations used in the spectral evaluation of rodent EEG recordings (Yan et al., 2011). Power spectral density (PSD) was computed using Welch's averaged periodogram method with an 8192-point

FFT, 87.5% overlap and a 50-Hz notch filter (Welch, 1967). The frequency bands were defined as delta (1–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), beta (12–30 Hz), gamma (30–80 Hz) and high-frequency activity (HFA; 80–500 Hz). The recording band was limited to 1–1000 Hz at the amplifier level. Absolute band powers were calculated in mV^2; relative band powers, total absolute power, median frequency and band ratios were also evaluated in the study.

Behavioural seizure assessment

In the behavioural seizure assessments, the time of the PTZ injection was taken as the zero point; the time to the first myoclonic jerk was calculated as the myoclonic jerk latency, the time to the first tonic–clonic seizure lasting at least three seconds as the tonic–clonic seizure latency, and the interval between these two events as the progression interval. Behavioural scores of seizure severity were evaluated according to the revised form of the classical Racine approach for PTZ seizures (Racine, 1972; Van Erum et al., 2019). Scoring used stages from − 1 to 7, ranging from the stage without convulsive behaviour to tonic extension and death. Seizure stages and time of death were assessed on video recordings labelled with codes containing no group information, by an investigator blind to the treatment groups. Mortality was assessed within the 60-min recording period after PTZ and reported as a group-level proportion; time of death was defined as the moment at which respiration and movement ceased completely. All deaths occurred within the recording period, and surviving animals were sacrificed 24 h later.

Statistical analysis

Analyses were performed using GraphPad Prism 11 and SPSS 31. Normality of distribution for continuous variables was assessed with the Shapiro–Wilk test and homogeneity of variances with the Brown–Forsythe test. Because the absolute and relative band powers, total absolute power, median frequency and band ratios obtained from the ECoG recordings took only positive values and showed a right-skewed distribution whose variance increased with the mean, they were subjected to log10 transformation before analysis. The transformed data were analysed by two-way repeated-measures ANOVA (general linear model, GLM) with the factors group (seven levels; between subjects) and time (pre- vs. post-treatment; within subjects). For the measures in which the group × time interaction was significant, post hoc comparisons were performed with Fisher's LSD test, as the groups to be compared had been specified in advance; pre- and post-treatment values were compared, and the groups were compared with one another at each measurement time. In the graphs, untransformed (absolute) values are displayed on a logarithmic axis. Because the myoclonic jerk and tonic–clonic seizure latencies and the progression interval showed differences in variance between groups, they were analysed by one-way ANOVA after log10 transformation and compared with Fisher's LSD test; these measures are reported as geometric means. Because of the ordinal nature of the Racine scores, the groups were evaluated with the Kruskal–Wallis test and the planned group pairs were compared with Dunn's test. Mortality distributions were analysed with the two-sided Fisher–Freeman–Halton exact test and pairwise comparisons with Fisher's exact test. Survival was evaluated by the Kaplan–Meier method; the groups

were compared overall and for the planned group pairs with the log-rank test, and hazard ratios with 95% confidence intervals were calculated from the log-rank statistic. Animals alive at the end of the recording period were censored. In the animals that died, the time of death was compared with the Mann–Whitney U test for exploratory purposes. Effect sizes are reported for the parametric analyses.

Results

Behavioural seizure results

Within the study, the myoclonic jerk and tonic–clonic seizure latencies were calculated by evaluating the video and ECoG recordings of the animals in the VEH + PTZ (n = 8), BCM-7 + PTZ (n = 7), NLX + BCM-7 + PTZ (n = 7) and NLX + VEH+PTZ (n = 8) groups in the PTZ-induced acute seizure model. A myoclonic jerk and a tonic–clonic seizure stage were observed in all PTZ-treated animals. In all of these calculations, the time of the intraperitoneal injection was taken as the reference time point.

In the analyses, the overall group effect on myoclonic jerk latency was not significant ($F(3,26) = 2.518$; $p = .080$; $\eta p^2 = .225$). Nevertheless, a significant difference was detected between the VEH + PTZ and BCM-7 + PTZ groups, with a longer latency in the BCM-7 + PTZ group (geometric mean 92.6 s vs. 72.9 s; $t(26) = 2.236$; $p = .034$). A significant difference between groups was found for tonic–clonic seizure latency ($F(3,26) = 9.704$; $p < .001$; $\eta p^2 = .528$). In the comparisons, the BCM-7 + PTZ group had a longer tonic–clonic seizure latency than the VEH + PTZ group (128.3 s vs. 85.8 s; $t(26) = 3.897$; $p < .001$), and the NLX + BCM-7 + PTZ group had a longer latency than the NLX + VEH+PTZ group (129.4 s vs. 88.2 s; $t(26) = 3.723$; $p = .001$). The group effect was also significant for the progression interval between the myoclonic jerk and the tonic–clonic seizure ($F(3,26) = 22.409$; $p < .001$; $\eta p^2 = .721$). The progression interval was approximately 2.7-fold longer in the BCM-7 + PTZ group than in the VEH + PTZ group (34.2 s vs. 12.6 s; $t(26) = 5.594$; $p < .001$) and approximately 2.9-fold longer in the NLX + BCM-7 + PTZ group than in the NLX + VEH+PTZ group (37.5 s vs. 12.9 s; $t(26) = 5.978$; $p < .001$).

The PTZ-treated groups reached seizure severities between stages 5 and 7 on the revised Racine scale. No significant difference between groups was detected for the Racine scores (Kruskal–Wallis $H = 6.187$; $p = .103$). In the comparisons, seizure severity was lower in the NLX + BCM-7 + PTZ group than in the BCM-7 + PTZ group (Dunn's test, $p = .020$). The proportion of rats reaching stage 7 was 1/7 in the NLX + BCM-7 + PTZ group and 5/7 in the BCM-7 + PTZ group. Mortality rates were 62.50%, 71.43%, 14.29% and 50.00% in the VEH + PTZ, BCM-7 + PTZ, NLX + BCM-7 + PTZ and NLX + VEH+PTZ groups, respectively, and did not differ significantly between groups (Fisher–Freeman–Halton exact test, $p = .182$). All deaths occurred within the 60-min recording period after PTZ. In the Kaplan–Meier analysis, no difference in survival was found between the groups (log-rank $\chi^2(3) = 4.351$; $p = .226$). In the comparisons, the risk of death was significantly lower in the NLX + BCM-7 + PTZ group than in the BCM-7 + PTZ group (log-rank $p = .043$; hazard ratio 0.19; 95% CI 0.04–0.95). Although the mortality rate was similar in the BCM-7 + PTZ and VEH + PTZ groups, among the animals that died, death occurred later in the BCM-7 + PTZ group than in the VEH + PTZ group (median [minimum–maximum] 17.0 [13.8–40.3] min vs. 10.0 [8.0–14.5] min;

Mann–Whitney U test, p = .016). Mortality occurred in all rats reaching stage 7, whereas all rats remaining at lower stages survived (Fig. 1).

Pre- and post-treatment comparisons of ECoG absolute power between groups

The absolute power values of the frequency bands obtained from the pre- and post-treatment ECoG recordings of the study groups were evaluated by PSD analysis. To examine the pre- to post-treatment change together with the differences between groups, two-way repeated-measures ANOVA was applied to the log10-transformed data. The absolute band powers, geometric means and significance levels of the principal comparisons are presented in Fig. 2.

In the analyses, the group × time interaction was significant for delta, theta, alpha, beta, gamma, HFA and total absolute power [delta: $F(6,46) = 5.968$; $p < .001$; $\eta p^2 = .438$; theta: $F(6,46) = 11.821$; $p < .001$; $\eta p^2 = .607$; alpha: $F(6,46) = 13.336$; $p < .001$; $\eta p^2 = .635$; beta: $F(6,46) = 10.410$; $p < .001$; $\eta p^2 = .576$; gamma: $F(6,46) = 8.029$; $p < .001$; $\eta p^2 = .512$; HFA: $F(6,46) = 12.382$; $p < .001$; $\eta p^2 = .623$; total power: $F(6,46) = 12.375$; $p < .001$; $\eta p^2 = .617$]. In the groups not receiving PTZ (SAL + SAL, VEH + SAL and BCM-7 + SAL), no significant change from baseline was detected in any band (all $p > .05$). In contrast, after PTZ administration, a significant increase was found in all bands and in total absolute power in the VEH + PTZ, BCM-7 + PTZ and NLX + BCM-7 + PTZ groups (all $p < .01$). This increase ranged from 4.2-fold (delta) to 20.2-fold (beta) in the VEH + PTZ group, from 5.1-fold (gamma) to 15.2-fold (alpha) in the BCM-7 + PTZ group and from 3.6-fold (delta) to 8.4-fold (beta) in the NLX + BCM-7 + PTZ group. In the NLX + VEH+PTZ group the increase remained more limited (1.8–3.7-fold) and was significant for theta, alpha, beta, HFA and total absolute power ($p = .004–.031$) but not for the delta ($p = .052$) or gamma ($p = .085$) bands.

In the comparisons made after the pharmacological treatments, the absolute power values of the VEH + PTZ group were significantly higher than those of the BCM-7 + PTZ group for gamma ($t(92) = 2.290$; $p = .024$) and HFA ($t(92) = 3.043$; $p = .003$). The difference in the delta band remained at the limit of significance ($t(92) = 1.988$; $p = .050$). No significant difference between these two groups was found in the theta ($p = .141$), alpha ($p = .215$) or beta ($p = .096$) bands. After treatment, total absolute power was approximately 2.9-fold higher in the VEH + PTZ group than in the BCM-7 + PTZ group, but this difference remained at the limit of significance ($t(92) = 1.961$; $p = .053$). The pre-treatment delta ($t(92) = 2.549$; $p = .012$), HFA ($t(92) = 2.397$; $p = .019$) and total absolute power ($t(92) = 2.030$; $p = .045$) values of the VEH + PTZ group were, however, also higher than those of the BCM-7 + PTZ group. The baseline difference in total absolute power (approximately 3.0-fold) was of similar magnitude to the post-treatment difference, and the PTZ-induced increase was comparable in the VEH + PTZ (approximately 9.4-fold) and BCM-7 + PTZ (approximately 9.7-fold) groups. When the VEH + PTZ and NLX + VEH+PTZ groups were compared in order to evaluate the effect of naloxone alone in the PTZ model, significant differences were detected in all bands and in total absolute power: delta ($t(92) = 2.472$; $p = .015$), theta ($t(92) = 2.845$; $p = .005$),

alpha ($t(92) = 3.029$; $p = .003$), beta ($t(92) = 2.853$; $p = .005$), gamma ($t(92) = 2.993$; $p = .004$), HFA ($t(92) = 3.259$; $p = .002$) and total absolute power ($t(92) = 3.117$; $p = .002$) were all 4.2- to 7.1-fold higher in the VEH + PTZ group. In addition, the VEH + PTZ group had higher post-treatment absolute power values than the NLX + BCM-7 + PTZ group in all bands ($p = .006–.031$). No significant difference was found between the BCM-7 + PTZ and NLX + BCM-7 + PTZ groups in any band or in total absolute power (all $p > .05$).

Pre- and post-treatment comparisons of ECoG relative power between groups

The relative power values obtained from the ECoG recordings were examined for the group effect and the group × time interaction in the delta, theta, alpha, beta, gamma and HFA bands. In addition, the differences between the VEH + PTZ and BCM-7 + PTZ, BCM-7 + PTZ and NLX + BCM-7 + PTZ, NLX + BCM-7 + PTZ and NLX + VEH+PTZ, and VEH + PTZ and NLX + VEH+PTZ groups, which constituted the principal experimental comparisons of the study, were evaluated in the post-treatment measurements (Fig. 3).

In the analyses, the group × time interaction was significant only for delta ($F(6,46) = 2.813$; $p = .020$; $\eta p^2 = .268$) and gamma ($F(6,46) = 4.111$; $p = .002$; $\eta p^2 = .349$) relative power. The group × time interaction was not significant for theta, alpha, beta or HFA relative power (all $p > .05$); among these bands, only the group effect was significant for theta, alpha and beta relative power ($p \leq .002$). Delta relative power decreased after PTZ administration in the VEH + PTZ ($p < .001$) and BCM-7 + PTZ ($p = .006$) groups, but no significant difference was detected in any of the planned post-treatment comparisons (all $p > .05$). Gamma relative power decreased after treatment in the BCM-7 + PTZ ($p < .001$) and NLX + VEH+PTZ ($p = .024$) groups and was unchanged in the VEH + PTZ ($p = .954$) and NLX + BCM-7 + PTZ ($p = .672$) groups. Among the groups not receiving PTZ, gamma relative power increased after treatment in the VEH + SAL group ($p = .044$) and was higher than in the SAL + SAL group ($t(92) = 2.116$; $p = .037$). In this band, the only significant difference in the planned post-treatment comparisons was between the BCM-7 + PTZ and NLX + BCM-7 + PTZ groups ($t(92) = 2.099$; $p = .039$), with lower gamma relative power in the BCM-7 + PTZ group.

Findings on the other pre- and post-treatment ECoG measures

Median frequency and the beta/theta, delta/beta and (delta+theta)/(alpha+beta) absolute power ratios were also calculated in the study (Fig. 4). The group × time interaction was not significant for median frequency ($F(6,46) = 1.160$; $p = .344$; $\eta p^2 = .131$), whereas the group ($F(6,46) = 7.937$; $p < .001$; $\eta p^2 = .509$) and time ($F(1,46) = 11.301$; $p = .002$; $\eta p^2 = .197$) effects were significant. For the beta/theta ratio, neither the group × time interaction ($F(6,46) = 1.195$; $p = .326$; $\eta p^2 = .135$) nor the group effect ($p = .847$) was significant.

The group × time interaction was significant for the delta/beta ($F(6,46) = 2.811$; $p = .021$; $\eta p^2 = .268$) and (delta+theta)/(alpha+beta) ($F(6,46) = 2.484$; $p = .036$; $\eta p^2 = .245$) ratios. Both ratios decreased after PTZ administration in the VEH + PTZ (both $p < .001$), BCM-7 + PTZ ($p = .017$ and $p = .025$) and NLX + BCM-7 + PTZ ($p = .035$ and $p = .041$) groups, whereas they showed no significant change in the NLX + VEH+PTZ group ($p = .143$ and $p = .204$). No significant difference was detected in any of the planned post-treatment comparisons between the principal PTZ groups (all $p > .05$).

Discussion

When the behavioural and electrocorticographic findings of the present study are considered together, BCM-7 appears to modulate in particular the timing of tonic–clonic seizure onset and seizure progression in the PTZ-induced acute seizure model. In the BCM-7 + PTZ group, the tonic–clonic seizure latency was prolonged approximately 1.5-fold and the myoclonic jerk–tonic–clonic seizure progression interval approximately 2.7-fold relative to the VEH + PTZ group. The prolongation of the myoclonic jerk latency was significant only in the planned comparison specified before the study, and the overall group effect across the four PTZ groups did not reach significance; the effect of BCM-7 on the emergence of the first myoclonic sign therefore appears more limited than its effect on progression. Taken as a whole, the behavioural findings suggest that BCM-7 can delay the transition to the more advanced motor phases in particular. Given that PTZ interacts with the picrotoxin binding site of the GABA-A receptor complex (Ramanjaneyulu & Ticku, 1984), reduces the GABA-activated Cl^- current in a concentration-dependent manner in recombinant GABA-A receptors (Huang et al., 2001) and that acute administration can reduce GABA-A receptor subunit mRNA levels and the coupling between GABA and benzodiazepine binding (Walsh et al., 1999), the prolongation of the latencies suggests that the temporal progression of the PTZ-induced excitation–inhibition imbalance may be modulated by BCM-7. Although the mortality rate was unchanged in the BCM-7 + PTZ group, the later occurrence of death relative to the VEH + PTZ group is also consistent with this delaying effect. Nevertheless, the development of seizures in all PTZ-treated animals and the absence of an overall group difference in Racine scores, mortality distribution and survival support the evaluation of BCM-7 as a neuropeptide that modulates seizure onset and progression rather than as an anticonvulsant that fully prevents seizures.

The literature on the effects of casomorphins on seizure activity does not present a unidirectional profile. Different β-casomorphin derivatives have been reported to exhibit proconvulsant or anticonvulsant properties in experimental seizure models, and these effects have been reported to vary with peptide structure, age and seizure model (Becker et al., 1991a; Becker et al., 1991b). Although the μ-opioid receptor agonist properties of BCM-7 are supported, the variability of the evidence concerning its neurological and systemic effects (Ali et al., 2026) calls for caution in mechanistic inferences. In the present study, the post-treatment gamma and HFA absolute powers of the BCM-7 + PTZ group were lower than those of the VEH + PTZ group and the difference in the delta band remained at the limit of significance, whereas no significant difference was detected between the two groups in theta, alpha or beta absolute power. After treatment, total absolute power was approximately 2.9-fold higher in the VEH + PTZ group than in the BCM-7 + PTZ group, but this difference remained at the limit of significance ($p =$

.053). The total absolute power of the VEH + PTZ group was, however, also higher than that of the BCM-7 + PTZ group before treatment by a similar factor (approximately 3.0-fold), and the PTZ-induced increase in total power was of almost identical magnitude in the two groups (approximately 9.4- and 9.7-fold). In this sense, BCM-7 did not markedly alter the magnitude of the overall increase in cortical activity produced by PTZ. The presence of similar baseline differences in the delta and HFA bands, together with the comparable magnitude of the PTZ-induced increase in the delta band in the two groups, indicates that the difference in the delta band in particular may reflect the baseline difference rather than an effect of BCM-7. In the gamma band, where no baseline difference was present, the more limited PTZ-induced increase in the BCM-7 + PTZ group (approximately 5.1-fold) compared with the VEH + PTZ group (approximately 9.3-fold) suggests that the effect of BCM-7 emerges most consistently in gamma activity. This points to BCM-7 modulating particular frequency components rather than suppressing the entire spectrum. Diedrich et al. (1994) reported that a different casomorphin derivative decreased delta and theta power while increasing alpha-2 and beta-1 activity in the cortical EEG. However, because of the experimental differences between the two studies, such as the type, chemical structure and sequence of the peptide used, receptor activity, dose, recording conditions and acute PTZ hyperexcitability, generalising those results to the present study is not considered appropriate.

Considering the absolute and relative power results together shows that the effect of PTZ emerges in particular on the absolute magnitude of cortical electrical activity. While absolute powers increased markedly in all bands in the PTZ-treated groups, a significant group × time interaction was observed in relative power only in the delta and gamma bands. The decrease in delta relative power occurred similarly in the VEH + PTZ and BCM-7 + PTZ groups and no difference between groups was detected in the planned comparisons; this change therefore reflects the PTZ-induced spectral shift rather than being specific to BCM-7. The decreases in the delta/beta and (delta+theta)/(alpha+beta) ratios were likewise seen in the PTZ-treated groups but did not differ between groups in the planned comparisons. In contrast, the decrease in gamma relative power in the BCM-7 + PTZ group and its lower value in this group than in the NLX + BCM-7 + PTZ group suggest that BCM-7 may also affect the share of the gamma band within the total spectrum. Nevertheless, the fact that naloxone alone also reduced gamma relative power requires this finding to be interpreted with caution. BCM-7 is therefore considered to affect particular components, principally the gamma band, to a limited extent rather than to reorganise the frequency distribution within the total spectrum as a whole. Gamma oscillations are reported in the literature to be related to the interaction between excitatory pyramidal cells and fast-spiking parvalbumin-positive inhibitory interneurons (Buzsáki & Wang, 2012; Sohal et al., 2009). The more limited increase in gamma absolute power in the BCM-7 + PTZ group is therefore consistent with the possibility that the cortical network-level reflection of the excitation–inhibition balance disrupted by PTZ may be modulated by BCM-7. The serotonergic system has also been reported to modulate the activity of these networks (Athilingam et al., 2017; Marek & Aghajanian, 1999). Activation of 5-HT2A receptors has been shown to increase excitatory postsynaptic currents in prefrontal cortex pyramidal cells (Marek & Aghajanian, 1999) and excitability in fast-spiking interneurons (Athilingam et al., 2017). Enhancement of NMDA receptor-mediated glutamatergic responses through Src kinase has been reported in rat jaw-

closing motoneurons (Dantsuji et al., 2019). Human BCM-7 has been shown to bind to 5-HT2 receptors in the rat frontal cortex, while both human and bovine BCM-7 were found to act in an antagonist-like manner by suppressing serotonin-induced aggregation in human platelets (Sokolov et al., 2005). Taken together, these findings suggest that a possible antagonistic effect of BCM-7 on 5-HT2 receptors may contribute to the more limited PTZ-induced increase in gamma activity. However, as this antagonistic action was demonstrated under in vitro conditions and at micromolar concentrations, and as serotonergic receptor activity, serotonin levels and the underlying mechanistic process were not evaluated in the present study, the change in gamma power cannot be attributed to a specific mechanism; this possibility is regarded as one that should be tested in further studies.

The naloxone-treated groups occupy an important place in the study with respect to the evaluation of the mechanism of action of BCM-7. The absence of a significant difference between the BCM-7 + PTZ and NLX + BCM-7 + PTZ groups in tonic–clonic seizure latency and progression interval, together with the longer latency and progression interval of the NLX + BCM-7 + PTZ group relative to the NLX + VEH+PTZ group and the failure of naloxone alone to alter any latency measure significantly, suggests that the behavioural effects of BCM-7 are preserved in the presence of naloxone. In line with these findings, the effect of BCM-7 on progression is considered to possibly include a component that cannot be blocked by naloxone. However, it cannot be inferred from these findings that this component is serotonergic. Zozulia et al. (2009) found that both human and bovine BCM-7 suppressed the serotonergic response induced by 5-hydroxytryptophan in mice and that this suppressive effect was abolished completely by 10 mg/kg naloxone. This indicates that the effect of BCM-7 on the serotonergic system may also be naloxone-sensitive. Another possibility is that the 2 mg/kg dose used in the present study did not fully antagonise the opioid receptors. Nevertheless, as these mechanisms could not be verified directly with the present findings, they should be regarded as hypotheses to be tested in further research. This suggests that the effects of BCM-7 may not be explained solely by naloxone-sensitive opioid mechanisms, although it does not prove that they are entirely independent of the opioid system. The interaction of BCM-7 with μ-opioid receptors is nonetheless stated in the literature (Ali et al., 2026; Bolat et al., 2024; Giribaldi et al., 2022). In addition, the finding in the comparisons that both seizure severity (proportion of rats reaching stage 7, 1/7 vs. 5/7) and the risk of death were lower in the NLX + BCM-7 + PTZ group than in the BCM-7 + PTZ group indicates that naloxone may affect seizure severity and survival when administered together with BCM-7. The absence of similar protection in the NLX + VEH+PTZ group, in which naloxone was given alone (4/8 deaths), suggests that this effect may be related to co-administration with BCM-7 rather than to an independent effect of naloxone. This pattern raises the possibility that, while the progression-delaying effect of BCM-7 is preserved in the presence of naloxone, a component contributing to seizure severity and mortality may be naloxone-sensitive. However, because the overall group effect was not significant for Racine scores or survival and the number of animals per group was limited, this finding should be interpreted with caution. The electrophysiological consequences of naloxone show a more complex pattern. The differentiation of all of the delta, theta, alpha, beta, gamma, HFA and total absolute powers between the VEH + PTZ and NLX + VEH+PTZ groups, and the fact that the PTZ-induced increase in absolute power remained most limited in the NLX + VEH+PTZ group, show that naloxone can by itself

alter cortical spectral activity under acute PTZ conditions. The absence of a difference between BCM-7 + PTZ and NLX + BCM-7 + PTZ cannot therefore be regarded as evidence that the effect of BCM-7 alone is non-opioid. Previous studies have shown that naloxone can affect cognitive or neuronal processes in PTZ kindling models without changing seizure outcome (Becker et al., 1994; Erdtmann-Vourliotis et al., 1998; Omrani et al., 2007). Similarly, naloxone alone has been reported not to alter seizure expression in fully kindled rats (Palizvan & Ghaznavi-Rad, 2014). Morphine studies have shown that morphine can exert a proconvulsant effect on PTZ seizures (Foote & Gale, 1984) and that its effect is biphasic, being inhibitory at a low dose and facilitatory at higher doses (Rashan et al., 2021). The findings that PTZ seizures are more severe and their latencies shorter in mice with low endogenous opioid system activity (Ruszczak et al., 2024), whereas naltrexone, an opioid antagonist different from naloxone, can reduce seizure-like activity in certain genetic and chemically induced seizure models (Sturgeon et al., 2021), show that the effect of the opioid system on seizure susceptibility can vary with endogenous opioid tone, type of antagonist, dose and experimental model. Explaining the BCM-7–naloxone relationship with a simple agonist–antagonist model is therefore not considered appropriate. The finding that gamma and HFA absolute powers were lower than those of the VEH + PTZ group in both the BCM-7 + PTZ and the NLX + VEH+PTZ groups also limits the interpretation of the decrease in these bands as a BCM-7-specific opioid receptor effect. The absence of a significant change in basal electrocorticographic band powers with BCM-7 in the groups not receiving PTZ shows that it does not produce a marked cortical electrophysiological effect under physiological conditions. Although this raises the possibility that the passage of BCM-7 across the blood–brain barrier may be limited, this finding does not constitute direct evidence of insufficient central access. Indeed, PTZ-induced acute generalised seizures have been reported to increase blood–brain barrier permeability in mice (Danjo et al., 2013). In this context, the regulatory effects of BCM-7 on the gamma band, particularly in the PTZ-treated groups, together with the observed changes in seizure latency and progression interval, suggest that seizure-related pathophysiological processes may alter the central effects of BCM-7. Nevertheless, as the brain and plasma levels of BCM-7 and blood–brain barrier permeability were not evaluated in the present study, it cannot be asserted with certainty that the observed electrophysiological and behavioural effects arise from increased central access.

Taken together, the findings show that BCM-7 can delay progression to the tonic–clonic phase in particular in the acute PTZ model, and that this behavioural change is accompanied by a more limited PTZ-induced increase in gamma and HFA absolute powers. The difference in total absolute power that remained at the limit of significance ($p = .053$) and the difference in delta absolute power are considered to be largely related to the pre-treatment difference between the groups, and BCM-7 is considered not to have markedly altered the PTZ-induced increase in total cortical power. The findings suggest that BCM-7 produces a profile that modulates seizure progression and cortical network activity rather than acting as a classical anticonvulsant that fully prevents seizures. The later occurrence of death in the BCM-7 + PTZ group is also consistent with this delaying profile. The preservation of the progression-delaying effect in the presence of naloxone, together with the reduction in seizure severity and risk of death under co-administration with naloxone, suggests that the contribution of the opioid system may differ according

to the type of effect; however, the presence of independent ECoG effects of naloxone precludes a definitive mechanistic conclusion on this point. Studies in which the serotonergic system, opioid receptor subtypes, the central access of BCM-7 and cardiorespiratory responses are directly evaluated are considered necessary to clarify the mechanism of this effect.

Limitations

Some limitations of the study should be taken into account when interpreting the findings. First, a single BCM-7 dose and a single PTZ dose were used, and the dose–response relationship was not evaluated. As the BCM-7 dose was selected on the basis of studies conducted in newborn and infant rats and in mice, a different effect profile may emerge at different doses in adult rats. Second, naloxone was administered at a single dose. The report that the effect of BCM-7 on the serotonergic system is blocked by 10 mg/kg naloxone in mice (Zozulia et al., 2009) suggests that the 2 mg/kg dose used in the present study may have been insufficient to antagonise the opioid-mediated effects of BCM-7 completely. As the design did not include a group in which naloxone was administered alone without PTZ, the effect of naloxone on basal cortical activity could not be separated from its interaction with PTZ. In addition, selective antagonists for δ and κ opioid receptors and pharmacological tools directed at the 5-HT2A receptor were not used, and receptor activation was not measured directly.

Third, the plasma and cerebrospinal fluid (CSF) levels of BCM-7 were not measured. Given that the peptide can be degraded by dipeptidyl peptidase IV (Bolat et al., 2024), the extent to which it reaches the central nervous system after intraperitoneal administration is unknown. The presence of differences between groups in absolute power values before treatment makes it difficult to exclude a contribution of inter-individual variability related to electrode placement and impedance. The respiratory and cardiac changes underlying mortality were also not recorded. Finally, the number of animals per group is limited for detecting medium-sized effects in secondary outcomes such as mortality and Racine scores. The findings regarding these outcomes should be regarded as exploratory. The study was conducted only in male rats and in an acute seizure model, and the generalisability of the findings to female animals and to chronic epilepsy models is unknown.

Conclusion

In the PTZ-induced acute seizure model, BCM-7 prolonged the onset of tonic–clonic seizures and the myoclonic jerk–tonic–clonic seizure progression interval compared with the VEH + PTZ group. The prolongation of the myoclonic jerk latency was significant only in the planned comparison specified before the study, and the overall group effect across the four PTZ groups did not reach significance. In the electrocorticographic evaluations, PTZ markedly increased absolute power in all frequency bands, and post-treatment gamma and HFA absolute powers were lower in the BCM-7 + PTZ group than in the VEH + PTZ group. The difference in total absolute power remained at the limit of significance ($p = .053$) and, together with the differences in the delta and HFA bands, was already present before treatment, whereas no significant difference was found in theta, alpha or beta absolute power. BCM-7 is therefore

considered to affect high-frequency components such as gamma and HFA more selectively rather than the PTZ-induced increase in total cortical power. The fact that the changes in relative power and band ratios were largely confined to the effect of PTZ suggests that BCM-7 does not reorganise the spectral distribution as a whole and that its effect emerges most clearly in the gamma band. The preservation of the effects of BCM-7 on tonic–clonic seizure latency and progression interval in the presence of naloxone indicates that this behavioural effect may not be explained solely by naloxone-sensitive opioid receptor mechanisms. In contrast, the limited PTZ-induced increase in absolute power across all measures in the group receiving naloxone alone shows that the electrophysiological results cannot be interpreted within the framework of a simple agonist–antagonist relationship. No overall difference between groups could be demonstrated in Racine scores, mortality distribution or survival; nevertheless, death occurred later in the BCM-7 + PTZ group, and seizure severity and the risk of death were lower in the NLX + BCM-7 + PTZ group than in the BCM-7 + PTZ group. Taken together, these findings suggest that, in the acute PTZ model, BCM-7 may present the profile of a neuropeptide that modulates the timing of seizure onset, progression to the tonic–clonic phase and cortical network activity rather than that of an agent that fully prevents seizures in the classical sense.

Declarations

Ethical Statement

The animal experiments were carried out with approval no. 77.637.435/317 of the Local Ethics Committee for Animal Experiments of Manisa Celal Bayar University.

Conflict of Interest

The authors declare that there is no commercial, financial or personal conflict of interest related to the research, authorship and/or publication of this study.

Clinical Trial Number

Clinical trial number: not applicable.

Declarations

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Author Contribution

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Data Availability

The electrophysiological recordings of this study, the absolute values derived from these recordings and the behavioural seizure scores are available from the corresponding author upon reasonable request.

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Figures

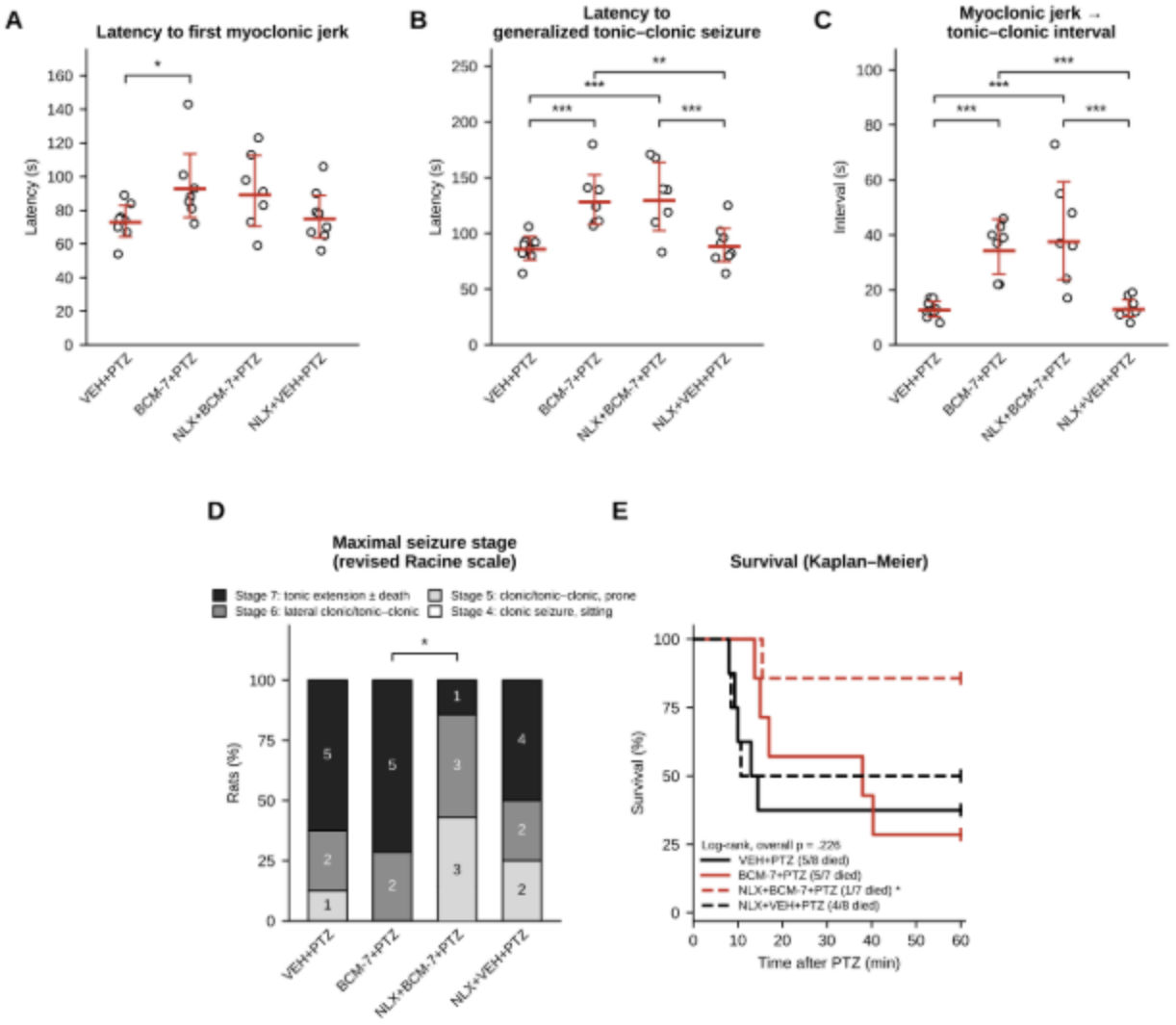


Figure 1

Behavioural seizure results in the PTZ groups. (A) Myoclonic jerk latency, (B) tonic–clonic seizure latency, (C) progression interval between the myoclonic jerk and the tonic–clonic seizure, (D) distribution of the highest seizure stage reached according to the revised Racine scale (Van Erum et al., 2019) and (E) Kaplan–Meier survival curves. (A–C) Circles show individual values and red lines the geometric mean with the 95% confidence interval (one-way ANOVA after log10 transformation and Fisher's LSD test). (D) The numbers within the bars indicate the number of rats at the corresponding stage (Kruskal–Wallis and Dunn's test). (E) Vertical ticks indicate animals alive at the end of the recording period (censored) and the values in the legend the number of rats that died out of the total (log-rank test; *p ≤ .05 vs. the BCM-7+PTZ group). *p ≤ .05, **p ≤ .01, ***p ≤ .001.

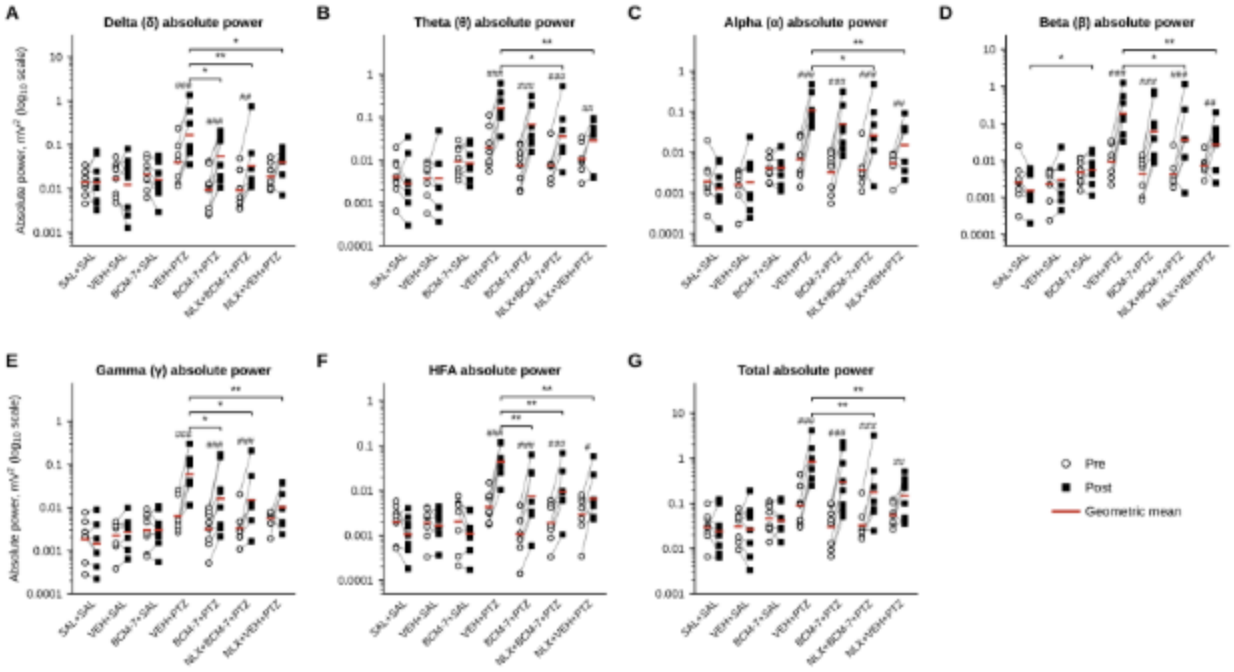


Figure 2

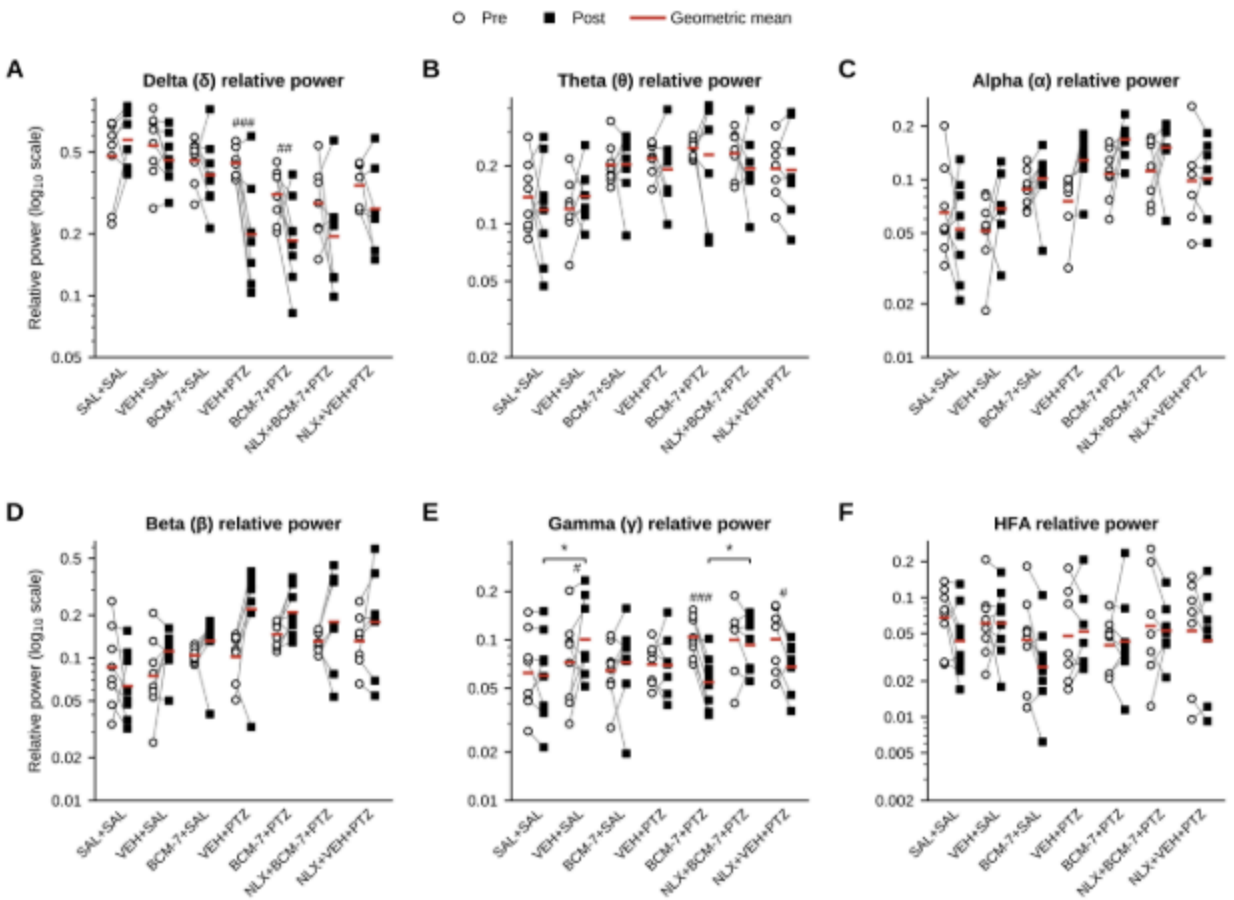


Figure 3

Pre- and post-treatment ECoG relative band powers of the groups. (A) Delta, (B) theta, (C) alpha, (D) beta, (E) gamma and (F) HFA. Circles show individual pre-treatment values and squares individual post-treatment values; thin lines connect measurements from the same rat and red lines indicate the geometric mean. The y-axis is logarithmic; analyses were performed on log10-transformed data by two-way repeated-measures ANOVA and Fisher's LSD test. #p ≤ .05, ##p ≤ .01, ###p ≤ .001 vs. baseline within the same group; *p ≤ .05, **p ≤ .01, ***p ≤ .001 between the indicated groups after treatment. Post hoc results are shown only for the measures in which the group × time interaction was significant.

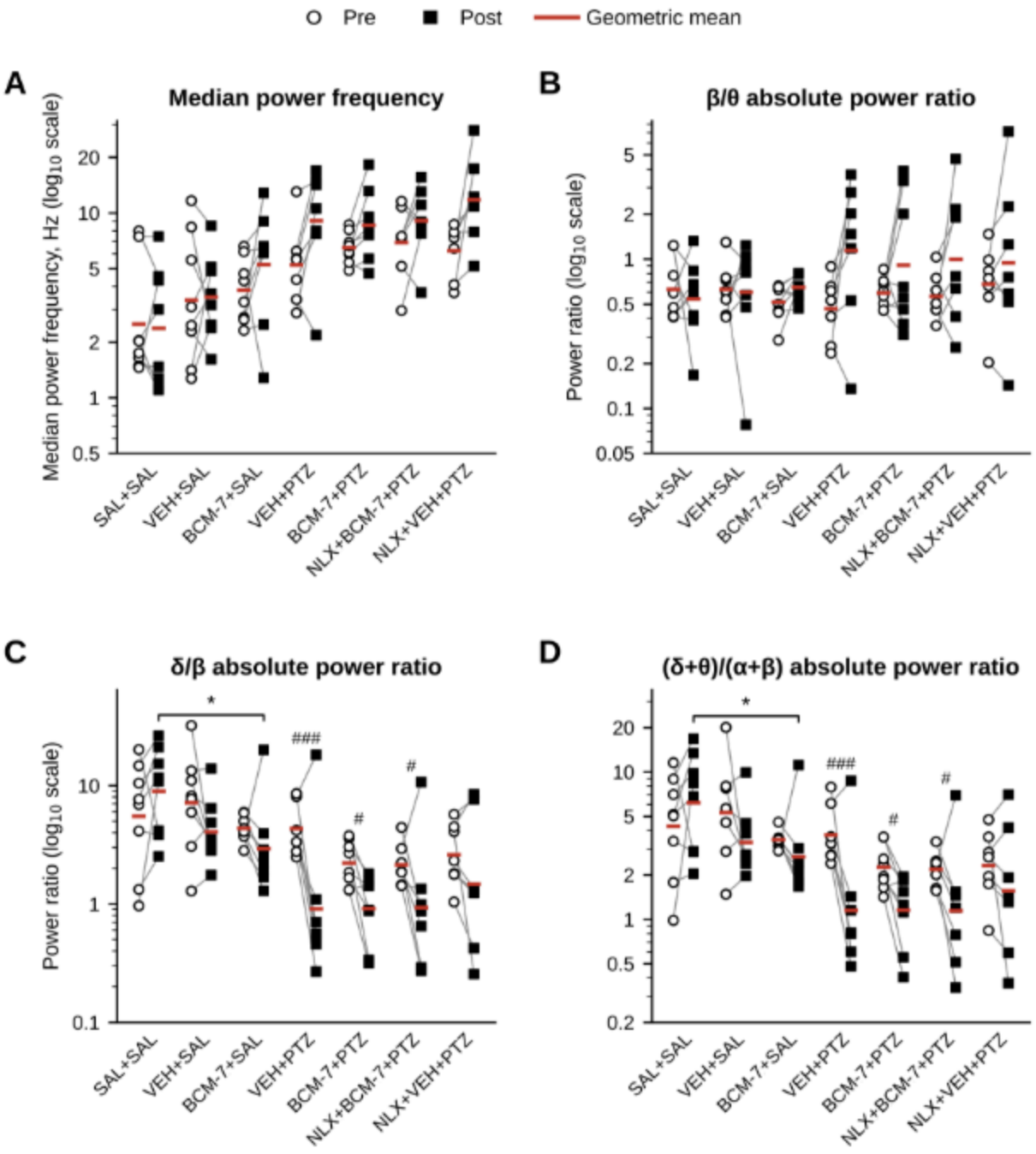


Figure 4

Pre- and post-treatment (A) median frequency, (B) beta/theta, (C) delta/beta and (D) (delta+theta)/(alpha+beta) absolute power ratio measurements of the groups. Circles show individual pre-treatment values and squares individual post-treatment values; thin lines connect measurements from the same rat and red lines indicate the geometric mean. The y-axis is logarithmic; analyses were performed on log10-transformed data by two-way repeated-measures ANOVA and Fisher's LSD test. #p ≤ .05, ##p ≤ .01, ###p ≤ .001 vs. baseline within the same group; *p ≤ .05, **p ≤ .01, ***p ≤ .001 between the indicated groups after treatment. Post hoc results are shown only for the measures in which the group × time interaction was significant.