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Mas Receptor Agonism Attenuates Seizures in a Pentylenetetrazole Kindling Model of Experimental Epilepsy in Mice

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Abstract

To investigate the effects of the Mas receptor (MasR) agonist AVE 0991 on seizure parameters, anxiety-like behavior, and learning-memory functions in a pentylenetetrazole (PTZ)-induced epilepsy model in mice, as well as its impact on MasR, NR2B, and BDNF mRNA expression levels. Seventy-one male Swiss albino mice were used. A chronic kindling model was induced with PTZ (37 mg/kg, i.p.). Following the kindling procedure, animals were assigned to four groups: naïve control, PTZ control (saline), and AVE 0991 at 3 mg/kg or 10 mg/kg. ECoG electrodes were implanted, and AVE 0991 or saline was administered either as a single dose (30 min before PTZ) or daily for 14 days. Seizure severity, first myoclonic jerk latency, generalized seizure latency and duration, spike count during generalized seizures, and total spike number were assessed. After long-term administrations, behavioral evaluations were conducted using the open field test, elevated plus maze, and Y-maze test. Cortical and hippocampal tissues were collected for qPCR analysis of MasR, NR2B, and BDNF mRNA expression. While acute single-dose administration of AVE 0991 failed to alter seizure parameters, long-term AVE 0991 (10 mg/kg) administration reduced seizure severity and spike activity, and prolonged seizure latencies. It also attenuated PTZ-induced anxiety-like behavior and cognitive impairment. Molecular analyses showed that AVE 0991 increased MasR mRNA expression in the cortex and hippocampus and suppressed the PTZ-induced pathological upregulation of NR2B and BDNF mRNA expression. It is suggested that MasR-targeted therapies may hold therapeutic potential in epilepsy, warranting further investigation.

Highlights

• Long-term MasR agonism reduced seizure severity and spike activity while delaying the onset of myoclonic jerk and generalized seizure.

• Long-term MasR agonism reduced anxiety-like behavior and improved learning and memory.

• Long-term MasR agonism increased Mas mRNA expression in the cortex and hippocampus.

• Long-term MasR agonism decreased the PTZ-induced increase in NR2B mRNA expression in the cortex and hippocampus.

• Long-term MasR agonism mitigated the PTZ-induced increase in BDNF mRNA expression in the cortex and hippocampus.

Introduction

Although clinically effective antiepileptic drugs are widely used today, approximately one-third of epilepsy patients are resistant to drugs. This situation highlights the importance of identifying new therapeutic targets for seizure control [1]. The kindling model is a chronic experimental epilepsy model characterized by generalized seizures, in which pentylenetetrazole (PTZ) is commonly used for induction [2]. PTZ competitively binds to the picrotoxin-binding site of the GABA-A receptor, thereby reducing chloride influx across the membrane and inducing generalized tonic-clonic seizure formation [3].

The renin-angiotensin system (RAS) consists of two arms: the classical and the alternative arm. The alternative arm is formed by the ACE2/Angiotensin-(1–7) [(Ang-(1–7)]/MasR axis [4]. Ang-(1–7) is an endogenous ligand for the Mas receptor (MasR), which belongs to the G-protein coupled receptor (GPCR) family [5]. It is known that Ang-(1–7) acts via MasR, which is expressed primarily in the hippocampus, amygdala, piriform cortex, thalamus, striatum, astrocytes, and microglia [6, 7]. A growing body of evidence indicates that the ACE2/Ang-(1–7)/MasR axis is involved in various

conditions, including Alzheimer’s disease [8], Parkinson’s disease [9], neuropathic pain [10], and neuropsychiatric disorders [11].

Research on the involvement of MasR in epilepsy remains limited. A single acute PTZ administration resulted in a short-term (2–6 h) upregulation of hippocampal Mas mRNA levels, followed by a return to baseline at 24 h post-seizure [12]. On the other hand, in a pilocarpine-induced epilepsy model, intracerebroventricular (i.c.v.) infusion of Ang-(1–7) for 28 days led to a decrease in hippocampal MasR protein levels [13]. Due to short half-life and rapid proteolytic degradation of Ang-(1–7) [14, 15], the longer-acting non-peptide MasR agonist AVE 0991 was used in this study [16]. AVE 0991 has been shown to exert neuroprotective effects in neurodegenerative diseases such as Alzheimer's disease [7, 16] and Parkinson's disease [17]. To our knowledge, this is the first study to investigate the effect of systemic administration of AVE 0991 in an epileptic seizure model.

Therefore, we aimed to investigate the effects of the Mas receptor agonist AVE 0991 on seizures in an experimental epilepsy model induced by PTZ, both behaviorally and electrophysiologically. In addition, the potential effects of MasR stimulation on anxiety-like behaviors and learning-memory processes have been evaluated. Furthermore, the influence of MasR activation on seizure-induced changes in the brain expression of MasR, NR2B, and BDNF, which play crucial roles in epileptic seizures, has been examined. Through this approach, the study aims to provide a comprehensive evaluation of the effects of MasR activation on the electrophysiological, behavioral, and neurobiological alterations induced by epileptic seizures.

Material and Methods

Animals

This study was approved by the Ondokuz Mayıs University Local Ethics Committee for Animal Experiments with decision number 2023/16 and then supported by the Ondokuz Mayıs University Scientific Research Project Management Office (Project no: BAP04-B-2024-4961).

Seventy-one male Swiss Albino, 5-month-old mice, weighing 32 ± 4 g, were obtained from the Ondokuz Mayıs University Experimental Animal Research Center. The mice were housed under standard laboratory conditions with a controlled ventilation and heating-cooling system, an automatically adjusted diurnal rhythm (12 hours light/12 hours dark cycle), a humidity level of 55 ± 3%, and a room temperature of 23 ± 1°C. Food and water were provided ad libitum. Animals were randomly assigned to the experimental groups using a computer-based randomization sequence. All procedures were conducted in accordance with the ARRIVE guidelines and the Guide for the Care and Use of Laboratory Animals as per the US National Institutes of Health (8th edition, National Academies Press, 2011).

Drugs

AVE 0991, PTZ, and sterile physiological saline were used in the experiments. AVE 0991 (MedChem Express) and PTZ (Merck) were dissolved in sterile saline and administered intraperitoneally (i.p.) at a constant volume of 10 mL/kg. The doses of AVE 0991 (3 and 10 mg/kg) were determined based on previously reported i.p. administration regimens in mice, selecting those that are effective in central nervous system studies without inducing hypotensive side effects [7, 16, 18].

Experimental Design

PTZ Kindling Process

Before the experiments, the mice were randomly divided into two groups as: a naïve control group (n = 11) and a kindling group (n = 60).

Initially, the animals were placed in transparent plexiglas cages (30x30x30 cm), allowing them to move freely for 7 consecutive days (2 hours per day) to acclimatize them to the laboratory environment. After the acclimatization period, experiments began on the 8th day (the first day of the experiment). PTZ injections (37 mg/kg) were administered up to 15 times at 48-hour intervals [19]. Each mouse was placed in an observation cage and monitored for 30 minutes after PTZ injection (Fig. 1), and seizure behaviors were recorded using a video camera. Seizure behaviors were evaluated off-line according to the Modified Racine Score as: Stage − 1: normal activity; stage 0: muscle twitching; stage 1: sudden behavioral stiffness; stage 2: facial jerk (sudden movement); stage 3: sudden jerks in the neck; stage 4: clonic seizures (sitting); stage 5: clonic, tonic-clonic seizures (lying face down); stage 6: clonic, tonic-clonic seizures (lying on the side) & wild jumping; stage 7: tonic extension, respiratory arrest and death [20, 21].

Animals exhibiting Stage ≥ 4 seizures in three consecutive injections were considered kindled (chronic epilepsy), and PTZ administration was subsequently discontinued [19, 22]. Animals that did not have consecutive seizures of Stage 4 or higher received PTZ injections every other day, up to a maximum of 15 times. Animals that failed to exhibit three consecutive Stage ≥ 4 seizures within 15 injections were classified as non-kindled (n = 19), while animals that died due to severe seizures (n = 3) were likewise excluded from the study. One week after the completion of the kindling process, 38 kindled mice were administered PTZ (37 mg/kg) to assess seizure susceptibility. Animals exhibiting seizures below Stage 4 during seizure assessment were classified as non-kindled (n = 5) and excluded from the study. The fully kindled animals (n = 33) were allocated to 3 groups (n = 11 per group) according to their PTZ injection counts (animals with similar injection counts were distributed evenly across the groups). Non-kindled and excluded mice were not used for further analyses. Three days later, together with the naïve control group, the four experimental groups (total n = 44) underwent electrode implantation for electrocorticogram (ECoG) recordings.

Electrode Implantation and Electrocorticography (ECoG) Recordings

Animals were anesthetized with ketamine/xylazine (100/20 mg/kg i.p.). The absence of corneal and hindpaw withdrawal reflexes verified the depth of anesthesia. The scalps of the anesthetized mice were shaved, and the heads were fixed to a stereotaxic frame (Stoelting Company, USA). Next, a 1.5 cm incision was made along the rostracaudal axis of the scalp, the skin was retracted, and the underlying skull was exposed. After cleaning the periosteum, the bregma was identified. Using the bregma as the reference point, three holes were drilled at the following coordinates (positive: anterior; 2 mm, left lateral; 1.5 mm; negative: posterior; 2 mm, left lateral; 1.5 mm; ground: posterior; 2 mm, right lateral; 1.5 mm) using an automatic drill with a 0.6 mm wide drill bit. Three stainless steel screws were placed in these holes, in contact with the dura mater, for ECoG recording. The tripolar electrode assembly was connected to the screws and was fixed to the skull with dental acrylic (Fig. 1). Mice were monitored until they regained mobility after surgery, and ketoprofen (5 mg/kg/day) was administered subcutaneously (s.c.) twice daily every 12 hours for 48 hours to prevent post-operative pain [23]. Subsequently, animals were observed for 3 days postoperatively for signs of pain or surgical complications. Following electrode implantation, the animals were allowed a one-week recovery period.

Determination of Single-dose and Long-term Effects of AVE 0991

To determine the single-dose effect, 7 days after electrode implantation, fully kindled mice (n = 33) were administered saline, 3 mg/kg, or 10 mg/kg AVE 0991 30 minutes prior to the PTZ (37 mg/kg) injection. To assess the immediate

pharmacological response under this acute condition, electrocorticography (ECoG) recordings were obtained following the PTZ injection. Naïve control mice (n = 11) were administered saline 30 minutes prior to the saline (10 mL/kg) injection.

For the determination of the long-term effect, 24 hours after the single-dose drug injections, the drugs were administered again, and this process was continued for 13 consecutive days (a total of 14 injections/day). To evaluate the long-term impact of the treatment, PTZ was administered 24 hours after the final drug injection on the 14th day, and subsequent ECoG recordings were obtained to analyze the alterations in seizure activity.

Behavioral Tests (Open field test, Elevated plus maze, Y-maze)

Twenty-four hours after PTZ administration, mice underwent a series of behavioral tests conducted in a dedicated, quiet behavioral testing room between 09:00 and 16:00 h. To minimize handling stress, the tests were performed sequentially from the least to the most stressful, with a 1-h rest interval between tests, in the following order: open field test (OFT), elevated plus maze test (EPM), and Y-maze test. All behavioral sessions were recorded via an overhead digital camera and analyzed off-line by an investigator blinded to the experimental groups.

Open Field Test

The open field test is a behavioral test based on rodents' tendency to explore their environment by overcoming their fear in unsafe and open areas. The test was conducted in a 40×40 cm square glass cage to assess the animals' anxiety-like behaviors. The apparatus was surrounded by walls 38 cm in height to prevent the animal from escaping [24]. The anxiety level of the mice was assessed by allowing them to freely explore the open area for 5 minutes. Each mouse was placed in the center of the arena, and their movements were recorded by camera throughout the test. After each mouse, the test arena was cleaned with 70% ethanol and dried. The anxiety levels of the mice were evaluated by observing parameters such as the time spent in the center, the number and duration of their rearings, and the number of fecal boli.

Elevated Plus Maze

This test is based on the instinctive behavior of rodents to avoid risky environments (open arms) and to prefer safe environments (closed arms). The aim was to determine the animals' anxiety-like activity by observing their behavior in the elevated plus maze. Mice were allowed to explore the plus-shaped maze, consisting of two open arms (15x5.5) and two closed arms (15x5.5, enclosed by 15-cm high walls), approximately 45 cm above the ground, for 5 minutes, and their movements were recorded on video and subsequently evaluated and analyzed [24]. After each mouse, the apparatus was cleaned with 70% ethanol and left to dry.

Y-Maze Test

Rodents exhibit a natural tendency to explore novel and previously unvisited arms of a maze, driven by their innate curiosity and reliance on spatial memory to distinguish between familiar and unfamiliar environments [25]. The Y-maze test was conducted to assess the spatial working (navigation) memory of rodents by observing their exploratory behavior. The experiment was performed in a Y-shaped maze consisting of three arms (40x8x15 cm) positioned adjacent to one another at 120-degree angles [24]. Mice were placed in the center of the apparatus, facing one of the three arms, and allowed to explore the three arms freely for 5 minutes. After each mouse, the apparatus was cleaned with 70% ethanol and left to dry.

In the Y-maze test, an arm entry was recorded when all four paws of the mouse entered the arm zone, and the sequence of arm entries was manually scored off-line by the blinded observer. The alternating behavior of mice entering the arms sequentially was used to assess spatial working memory in rodents. Alternation can occur in various combinations (such as ABC, BCA, CAB, BAC, CBA, ACB, …). Entries into each arm were recorded on video for 5 minutes, and the percentage of spontaneous alternations was calculated by determining the number of alternations and the total number of arm entries as [24]:

Spontaneous Alternation (%)=[Number of Alternations/(Total Arm Entries − 2)]×100.

Tissue Replacement and qPCR Protocol

Following behavioral testing, mice were intraperitoneally anesthetized with ketamine/xylazine (100/20 mg/kg, i.p.), and the absence of corneal and paw withdrawal reflexes confirmed the depth of anesthesia. Brains were then rapidly excised following transcardial perfusion with 0.9% saline, and the right somatosensory cortex (S1 barrel field; +0.5 to − 1.5 mm posterior, + 2.4 to + 3.4 mm lateral) and right hippocampus were microdissected. Tissue samples were immediately immersed in 1.5 mL tubes containing 1 mL TRIzol and cryopreserved at − 80°C until analysis.

RNA Isolation from Tissue Samples

To prevent RNA degradation, a key concern for neural tissue analysis, RNA isolated from cryopreserved somatosensory cortex and hippocampus samples under strict RNase-free conditions with minimized processing times. Tissue samples stored at − 80°C in 1.5 mL tubes with 1 mL TRIzol reagent (per 50–100 mg tissue) were allowed to equilibrate to room temperature before homogenization. Then, it was homogenized with a homogenizer probe for 3–5 cycles of 90 seconds each to achieve complete lysis. Homogenates were incubated at room temperature for 5 min and centrifuged at 12,000×g for 10 min at 4°C.

The upper aqueous phase containing RNA was carefully transferred to a new RNase-free tube, mixed gently with an equal volume of chloroform by inversion, incubated for 2–3 min at room temperature, and centrifuged at 12,000×g for 15 min at 4°C. The aqueous phase was recovered, combined with 500 µL of isopropanol, and incubated at room temperature for 10 minutes to precipitate RNA. The pellet was collected by centrifugation at 12,000×g for 10 minutes at 4°C. The supernatant was discarded, and the RNA pellet was washed with cold 75% ethanol and centrifuged at 7,500×g for 5 minutes at 4°C. After removing the ethanol, the pellet was air-dried and resuspended in 20 µL of distilled water, completing RNA isolation.

RNA Purity Measurement

Following resuspension of the RNA pellet in 20–30 µL of nuclease-free water, the purity and concentration of the RNA samples were quantified using a Thermo NanoDrop spectrophotometer [26]. Spectrophotometric Measurements were recorded and archived offline via the instrument's integrated software.

cDNA Synthesis

Primers specific to MasR, NR2B, and BDNF were designed by the researchers using the NCBI database (Table 1). Following RNA isolation and quantification, complementary DNA (cDNA) was synthesized from purified RNA according to the manufacturer's instructions for the cDNA synthesis kit. In accordance with the manufacturer's instructions for the cDNA synthesis kit. Reaction parameters were optimized based on NanoDrop spectrophotometry data, followed by thermal cycling executed via the standardized program on a PCR instrument.

Primer Specificity and Efficiency

The specificity of PCR amplification for each primer set was confirmed by a single peak in melting curve analyses. Preliminary evaluation confirmed that the candidate reference gene was expressed in mouse brain tissues, including the right somatosensory cortex and hippocampus. Primer efficiency (E) was calculated from the slope of the standard curve generated using fluorescence values obtained from a 5-fold serial dilution of a calibrator cDNA sample.

Quantification of MasR, NR2B, and BDNF Genes by qRT-PCR

Following cDNA synthesis, quantitative real-time reverse transcription PCR (qRT-PCR) assays were assembled in accordance with the manufacturer's specifications for the iScript qRT-PCR kit, employing the recommended volumes and reagent concentrations. Primer mixes targeting genes of interest (MasR, NR2B, and BDNF) and reference gene were aliquoted into reaction mixtures arrayed on a 96-well PCR plate. The plate was subsequently loaded into the qPCR instrument, and the manufacturer-recommended thermal cycling protocol was used.

Amplification was monitored in real time, enabling accurate quantification of gene expression from Ct values. Reliability of the results was ensured through careful pipetting, strict contamination control, and consistency across technical replicates.

Table 1
Primer design for MasR, NR2B, BDNF, and the target gene.

Gene (symbol)	Target	Species	Forward Primer (5'→3')	Reverse Primer (5'→3')
Mas1 (MasR)	Mas receptor	Mouse	CTGACAGCCATCAGTGTGGAGA	GTGGTCACCAAGCACGAAAGTG
BDNF	Brain-derived neurotrophic factor	Mouse	GGCTGACACTTTTGAGCACGTC	CTCCAAAGGCACTTGACTGCTG
GRIN2B (NR2B)	NMDA receptor subunit NR2B (GluN2B)	Mouse	CTGGTGACCAATGGCAAGCATG	GGCACAGAGAAGTCAACCACCT
HBB-Betaglobulin (Housekeeping)	β-globin gene (hemoglobin beta chain)	Mouse	GCTTCTGACACAACTGTGTTCA	CCACTGACAGCCTCACTTAA

Statistical Analysis

All data were analyzed using SPSS v21 (SPSS Inc., Chicago, IL) Software. The normality of the data distribution was assessed using the Shapiro-Wilk test. For the seizure stage, first myoclonic jerk latency, generalized seizure latency and duration, number of spikes in a generalized seizure, total number of spikes, behavioral tests, and qPCR analyses, one-way ANOVA followed by post-hoc Tukey-Kramer test was applied to data showing normal distribution, while Kruskal-Wallis followed by post-hoc Dunn test was applied to non-normally distributed data. Data other than qPCR were presented as mean ± standard error (SEM), while qPCR data were presented as mean ± standard deviation. $p < 0.05$ was considered statistically significant.

Results

No statistically significant differences were observed among groups in the initial body weight measurements obtained after completion of the kindling procedure and in the final weight following completion of drug administration

(Supplemental Fig. 1).

Effects of Single-Dose and Long-Term AVE 0991 Administration in Kindled Rats

The distribution of seizure stages evaluated according to the Modified Racine Scale is presented in Supp. Figure 2.

Representative ECoG recordings obtained from the study groups are presented in Fig. 2.

Single doses of saline or AVE 0991 (3 or 10 mg/kg) did not affect any seizure parameter (Fig. 3a-f).

Effect of AVE 0991 on Seizure Stage

Long-term administration of the 10 mg/kg dose significantly reduced the seizure stage compared to the saline control ($F_{2,30}$=4.750; p = 0.014). The respective seizure stages for the saline, 3 mg/kg, and 10 mg/kg groups were 4.72 ± 0.27, 4.36 ± 0.24, and 3.54 ± 0.31, respectively (Fig. 3a).

Effect of AVE 0991 on the Latency of First Myoclonic Jerk and Generalized Seizure

Long-term administration of the 10 mg/kg dose significantly delayed the myoclonic jerk latency compared to the saline control and the 3 mg/kg group ($F_{2,30}$=8.832; p < 0.001 and p = 0.042, respectively). The latencies to the first myoclonic jerk for the long-term administered saline, 3 mg/kg, and 10 mg/kg AVE 0991 groups were 91.64 ± 4.90, 107.60 ± 5.79, and 132.80 ± 9.42 sec, respectively (Fig. 3b).

The maximum seizure latency observed in mice that exhibited generalized seizures was 373 seconds. Therefore, animals that did not develop generalized seizures were assigned a latency of 400 seconds for statistical analysis.

After long-term drug administration, the 10 mg/kg AVE 0991 group had delayed generalized seizure latency compared to the saline and 3 mg/kg AVE 0991 groups (p = 0.006 and p = 0.037, respectively). The generalized seizure latencies of the long-term administered saline, 3 mg/kg AVE 0991, and 10 mg/kg AVE 0991 groups were calculated as 208.50 ± 25.22, 224.50 ± 21.55, and 331.10 ± 23.80 sec, respectively (Fig. 3c).

Effect of AVE 0991 on The Duration of Generalized Seizure

The seizure durations of animals that did not develop generalized seizures were considered to be zero.

With the long-term drug administration, the 10 mg/kg AVE 0991 group showed a reduced seizure duration compared with the saline-treated group (p = 0.030). Following long-term administration, the generalized seizure durations of the PTZ control (saline), 3 mg/kg AVE 0991, and 10 mg/kg AVE 0991 groups were calculated as 21.27 ± 2.60, 20.18 ± 2.27, and 10.09 ± 3.06 sec, respectively (Fig. 3d).

Effect of AVE 0991 on The Number of Spikes During Generalized Seizures and on Total Spike Count

The number of spikes during seizures was considered to be zero in animals that did not develop generalized seizures.

After long-term administrations, the 10 mg/kg AVE 0991 group showed a reduction in the number of spikes during generalized seizures compared with the saline and 3 mg/kg AVE 0991 groups (p = 0.042 and p = 0.038, respectively). Following long-term administration, the number of spikes during generalized seizures in the PTZ control (saline), 3

mg/kg AVE 0991, and 10 mg/kg AVE 0991 groups was calculated as 182.50 ± 22.36, 184.90 ± 22.28, and 87.27 ± 26.32 spike, respectively (Fig. 3e).

With the long-term administrations, the 10 mg/kg AVE 0991 group showed a reduction in total spike count compared with the saline-treated group ($F_{2,30}$=4.449; p = 0.023). Following long-term administration, the total spike counts of the PTZ control (saline), 3 mg/kg AVE 0991, and 10 mg/kg AVE 0991 groups were calculated as 400.50 ± 28.77, 373.90 ± 35.61, and 264.60 ± 37.39 spike, respectively (Fig. 3f).

Behavioral Test Evaluation

Behavioral tests were performed 24 hours after the last PTZ injection. Animals were subjected to the open field test, elevated plus maze test, and Y-maze test.

Effect of AVE 0991 on Open Field Test Parameters

Long-term administrations of 3 and 10 mg/kg AVE 0991 did not significantly alter the number of fecal boli compared with the saline-treated group ($F_{3,40}$=1.602) (Fig. 4a).

In kindled animals treated with saline, the time spent in the center during the open field test was reduced compared with the naïve control group (p < 0.001). In contrast, the group treated with 10 mg/kg AVE 0991 showed an increased time spent in the center compared with the PTZ control (saline) group ($F_{3,40}$=7.961; p = 0.029). The time spent in the center for the naïve control, PTZ control (saline), 3 mg/kg AVE 0991, and 10 mg/kg AVE 0991 groups was calculated as 49.73 ± 3.00, 24.64 ± 3.47, 36.64 ± 4.81, and 39.73 ± 3.08 sec, respectively (Fig. 4b).

The total number of rearings was significantly reduced compared with the naïve control group (p < 0.001). In contrast, administration of 10 mg/kg AVE 0991 significantly increased the total number of rearings compared with the PTZ control (saline) group ($F_{3,40}$=7.980; p = 0.043). The total rearing counts in the naïve control, PTZ control (saline), 3 mg/kg AVE 0991, and 10 mg/kg AVE 0991 groups were 49.91 ± 2.56, 32.91 ± 3.01, 34.36 ± 3.05, and 44.00 ± 2.79, respectively (Fig. 4c). The total rearing duration was significantly reduced in kindled mice compared with the naïve control group (p = 0.006). Administration of 10 mg/kg AVE 0991 increased the total rearing duration compared with the PTZ control (saline) group ($F_{3,40}$=5.715; p = 0.048). The total rearing duration for the naïve control, PTZ control (saline), 3 mg/kg, and 10 mg/kg AVE 0991 groups was calculated as 100.90 ± 6.90, 69.27 ± 6.28, 73.91 ± 6.89, and 93.55 ± 5.24 sec, respectively (Fig. 4d).

Effect of AVE 0991 on Elevated Plus Maze Test Parameters

Open-arm entries in kindled mice were reduced compared with the naïve control group (p < 0.001). In the 3 mg/kg and 10 mg/kg AVE 0991 groups, the number of entries into the open arms increased compared with the PTZ control (saline) group ($F_{3,40}$=9.543; p = 0.031 and p = 0.003, respectively). The number of open-arm entries for the naïve control, PTZ control (saline), 3 mg/kg, and 10 mg/kg AVE 0991 groups was 12.27 ± 0.95, 5.54 ± 0.80, 9.27 ± 0.89, and 10.36 ± 1.00, respectively (Fig. 5a).

With long-term drug administration, no significant differences were observed among the groups in the number of entries into the closed arms of the elevated plus maze ($F_{3,40}$=2.909) (Fig. 5b).

The saline-treated kindled mice spent significantly less time in the open arms and significantly more time in the closed arms compared with the naïve control group (p = 0.010). Administration of AVE 0991 at a dose of 10 mg/kg significantly increased the time spent in the open arms while decreasing the time spent in the closed arms compared with the PTZ control (saline) group ($F_{3,40}$=4.829, p = 0.023). The time spent in the open arms by the naïve control, PTZ

control (saline), 3 mg/kg AVE 0991, and 10 mg/kg AVE 0991 groups was 216.40 ± 10.91, 155.30 ± 10.36, 178.00 ± 17.90, and 210.60 ± 11.64 s, respectively (Fig. 5c). Corresponding values for time spent in the closed arms were 83.64 ± 10.91, 144.70 ± 10.36, 122.00 ± 17.90, and 89.36 ± 11.64 s, respectively (Fig. 5d).

Evaluation of the Effect of AVE 0991 on Y-Maze Test Parameters

Regarding the percentage of alternation, the PTZ control (saline) group showed a reduction compared with the healthy naïve control group ($p = 0.004$). Compared with the PTZ control (saline) group, the 10 mg/kg AVE 0991 group exhibited an increased percentage of alternation ($F_{3,40}=6.329$; $p = 0.036$). The percentage of alternation for the naïve control, PTZ control (saline), 3 mg/kg, and 10 mg/kg AVE 0991 groups was 65.46 ± 2.91, 46.63 ± 4.10, 49.27 ± 3.66, and 61.03 ± 3.65, respectively (Fig. 6).

Effect of AVE 0991 on MasR mRNA Expression in the Cortex and Hippocampus

No significant difference between the naïve control group and the PTZ control (saline) group in either the cortex or the hippocampus regarding MasR mRNA levels. Long-term administrations of AVE 0991 at doses of 3 and 10 mg/kg significantly increased MasR mRNA expression compared with the PTZ control (saline) group in both brain regions (cortex: $F_{3,12}=32.74$, $p = 0.040$ and $p < 0.001$; hippocampus: $F_{3,12}=44.41$, $p = 0.015$ and $p < 0.001$ for 3 and 10 mg/kg, respectively). Additionally, the 10 mg/kg AVE 0991 group exhibited higher MasR mRNA levels than the 3 mg/kg group in both the cortex ($p = 0.003$) and hippocampus ($p = 0.002$). MasR mRNA levels for the naïve control, PTZ control (saline), 3 mg/kg, and 10 mg/kg AVE 0991 groups were 0.98 ± 0.17, 1.54 ± 0.45, 2.65 ± 0.44, and 4.28 ± 0.77 in the cortex, and 0.99 ± 0.16, 2.00 ± 0.35, 3.55 ± 0.62, and 5.60 ± 0.95 in the hippocampus, respectively (Fig. 7a,b).

Effect of AVE 0991 on NR2B mRNA Expression in the Cortex and Hippocampus

NR2B mRNA levels were significantly higher in the PTZ control (saline) group than in the naïve control group in both the cortex and hippocampus ($p < 0.001$; $p < 0.001$, respectively). Administration of 10 mg/kg AVE 0991 significantly reduced NR2B mRNA expression compared with the PTZ control (saline) group in both brain regions (cortex: $F_{3,12}=28.32$, $p = 0.028$; hippocampus: $F_{3,12}=21.86$, $p = 0.015$). NR2B mRNA levels in the cortex for the naïve control, PTZ control (saline), 3 mg/kg, and 10 mg/kg AVE 0991 groups were 0.98 ± 0.13, 2.56 ± 0.33, 2.36 ± 0.27, and 1.95 ± 0.26, respectively (Fig. 7c). In the hippocampus, NR2B mRNA levels were 1.00 ± 0.18, 3.67 ± 0.55, 3.24 ± 0.64, and 2.37 ± 0.50 for the naïve control, PTZ control (saline), 3 mg/kg, and 10 mg/kg AVE 0991 groups, respectively (Fig. 7d).

Effect of AVE 0991 on BDNF mRNA Expression in the Cortex and Hippocampus

BDNF mRNA levels revealed a significant increase in the PTZ control (saline) group compared with the naïve control group in both the cortex and hippocampus (cortex: $p < 0.001$; hippocampus: $p < 0.001$). Administration of 10 mg/kg AVE 0991 decreased BDNF mRNA levels in the cortex compared with both the PTZ control (saline) and 3 mg/kg AVE 0991 groups ($F_{3,12}=36.69$; $p = 0.002$ and $p = 0.044$, respectively). In the hippocampus, both 3 mg/kg and 10 mg/kg AVE 0991 treatments decreased BDNF mRNA levels compared with the PTZ control (saline) group ($F_{3,12}=32.99$; $p = 0.012$ and $p < 0.001$, respectively). BDNF mRNA levels in the cortex for the naïve control, PTZ control (saline), 3 mg/kg, and 10 mg/kg AVE 0991 groups were 0.97 ± 0.06, 2.83 ± 0.33, 2.48 ± 0.37, and 1.90 ± 0.18, respectively (Fig. 7e). In the

hippocampus, BDNF mRNA levels were 0.98 ± 0.14, 4.04 ± 0.52, 2.86 ± 0.56, and 2.33 ± 0.39 for the naïve control, PTZ control (saline), 3 mg/kg, and 10 mg/kg AVE 0991 groups, respectively (Fig. 7f).

Discussion

The renin–angiotensin system (RAS), a primary therapeutic target in cardiovascular disease, also functions within the brain [27]. Pharmacological inhibition of angiotensin II (Ang-II) via angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) shifts the RAS equilibrium from the classic pressor arm toward the protective cascade, thereby increasing Ang-(1–7) levels [28]. Activation of the protective RAS axis promotes neuroprotection by reducing anxiety [29], improving cognitive performance, and mitigating pathological alterations observed in disorders such as Alzheimer's [30] and Parkinson's diseases [31].

AVE 0991, a non-peptide analog of Ang-(1–7) and a MasR agonist, has been reported to exert hypotensive effects primarily under hypertensive conditions rather than normotensive states [14]. Administration of AVE 0991 at a dose of 10 mg/kg (i.p.) for 30 days did not affect blood pressure in normotensive mice [7]. Due to its inability to cross the intact blood–brain barrier, Ang-(1–7) has been administered via i.c.v. routes in studies investigating its central effects [13, 29, 32]. In contrast, AVE 0991 has been shown to easily cross the blood–brain barrier following i.p. administration [18, 33]. Furthermore, while Ang-(1–7) can bind to receptors other than MasR, such as AT2R [34] and MrgD [35], AVE 0991 binds selectively to MasR [36, 37]. Based on these findings in the present study, AVE 0991 was administered intraperitoneally at doses of 3 and 10 mg/kg.

Effects of AVE 0991 on Seizures

Beyond the classical RAS pathway, only a limited number of studies have examined the role of the ACE2/Ang-(1–7)/MasR axis in epilepsy. These have primarily focused on changes in RAS components in the brain following induction of different seizure models. In PTZ-induced single-dose seizures, transient increases in hippocampal Mas mRNA levels have been observed, which returned to baseline within 24 hours [12]. In the pilocarpine-induced epilepsy model, Ang-(1–7) levels increased during the acute and silent phases but decreased during the chronic phase [38]. In addition, regarding the therapeutic effect of the protective RAS pathway, a 28-day infusion of Ang-(1–7) reduced epileptic seizures in the pilocarpine-induced epileptic model [13].

In the present study, single-dose administration of AVE 0991 did not affect seizure parameters in PTZ-kindled mice. Despite the absence of acute studies examining Mas receptor agonism in epilepsy, its effects have been explored in different experimental models. When AVE 0991 is administered at the onset of heatstroke, molecular effects emerge within 4–6 hours, accompanied by marked hepatoprotection at biochemical, cellular, and histological levels by 9 hours [39]. In a study investigating how surgical trauma disrupts RAS balance in the brains of aged rats, particularly in the hippocampus, AVE 0991 administered immediately after surgery was found to exert molecular effects within the first 6 hours, while structural and functional effects became evident within 24 hours [40]. Similarly, when AVE 0991 was administered one hour after the induction of subarachnoid hemorrhage, its molecular effects began within 6 hours and resulted in significant improvements in oxidative stress, neuronal apoptosis, and neurological function at 24 hours [41]. In the current study, AVE 0991 was administered 30 minutes prior to seizure induction. Taken together, both previous reports and our findings suggest that the pharmacological effects of systemically administered AVE 0991 emerge in a time-dependent manner rather than occurring immediately. This delay is likely due to the kinetics of G protein-coupled receptor activation and subsequent intracellular signaling.

On the other hand, long-term administration of AVE 0991 mitigated seizure severity, delayed the onset of both the first myoclonic jerk and generalized seizures, and reduced the total duration of generalized seizures. To date, while the

impact of chronic AVE 0991 administration on epileptic seizures remains unexplored, only one study has examined the effects of Ang-(1–7) [13]. In the pilocarpin-induced epileptic model, 28-day i.c.v. infusion of Ang-(1–7) reduced the frequency of spontaneous motor seizures [13]. These findings suggest that MasR agonism plays a modulatory role in generalized epileptic seizures and may hold therapeutic potential. Nevertheless, the precise cellular mechanisms through which the protective axis of the RAS exerts its actions remain to be fully elucidated.

Effects of AVE 0991 on Anxiety-Like Behaviors and Cognitive Functions

Preclinical evidence from PTZ-induced kindling models demonstrates that neuropsychiatric comorbidities commonly observed in epilepsy patients are reliably replicated in rodents [24, 42]. However, behavioral outcomes vary by model; while a pilocarpine-induced model unexpectedly produced anxiolytic-like effects, evidenced by increased time spent in the open arms of the EPM [13], PTZ-induced kindling consistently yields anxiogenic phenotypes. Specifically, several studies have reported reduced time spent in the center of the OFT following PTZ administration [43, 44]. The discrepancy between pilocarpine and PTZ models is likely attributable to differences in the mechanisms of action of the epileptogenic agents employed. Consistent with the PTZ literature [24], mice in the present study exhibited increased anxiety-like behavior, demonstrated by reduced time spent in both the center of the OFT and the open arms of the EPM. Collectively, these data confirm that the PTZ-induced kindling model reliably mimics the anxiety-like comorbidities associated with epilepsy.

To date, only one study has explored the role of the ACE2/Ang-(1–7)/MasR axis in epilepsy-associated anxiety. In that study, Ang-(1–7) treatment reversed the pilocarpine-induced anxiolytic effect in rats, normalizing their behavior toward control levels [13]. Conversely, long-term administration of the MasR agonist AVE 0991 in our study increased both time spent in the open arms of the elevated plus maze and time spent in the center of the open field test, thereby significantly attenuating PTZ-induced anxiety-like behaviors. These seemingly opposing findings suggest that Mas receptor agonists may exert regulatory effects on anxiety-like behaviors associated with epilepsy. Nonetheless, further investigation utilizing varied doses, extended timelines, and mechanistic assessments is warranted to optimize its translational relevance.

Cognitive impairments in epilepsy are associated with disrupted neuronal network integrity, hippocampal inflammation, and impaired synaptic plasticity [45]. PTZ-induced seizures impair learning and memory, with reduced spontaneous alternation in the Y-maze serving as a marker of spatial memory deficits [44]. The Ang-(1–7)/Mas axis has been shown to enhance cognition by promoting synaptic plasticity and long-term potentiation, with clinical evidence supporting the cognitive benefits of RAS modulation [46–48]. Consistent with these findings, AVE 0991 restored spontaneous alternation performance in PTZ-kindled mice, suggesting that activation of the protective RAS axis may alleviate epilepsy-related cognitive impairment.

Effect of AVE 0991 on MasR, NR2B, and BDNF mRNA Expressions

In models of neurodegenerative and metabolic diseases, reductions in MasR levels have been associated with cognitive impairment, and MasR agonists such as AVE 0991 have been shown to reverse these decreases [16, 49, 50]. In a high-fat diet-induced diabetic cognitive impairment model, reductions in hippocampal Ang-(1–7) and MasR expression were significantly ameliorated by AVE 0991 treatment [49]. Similarly, in an Alzheimer's disease model, AVE 0991 administration dose-dependently increased Mas1 mRNA and protein levels and improved cognitive performance [16]. These studies indicate that MasR activation can promote structural and functional recovery, particularly within cognitive circuits. Indeed, in other neurodegenerative conditions, such as Huntington's disease models, RAS modulators have been reported to increase MasR or AT2R expression [51]. Collectively, these findings suggest that the

Mas receptor constitutes a key component of the protective adaptive response in both seizure-related and neurodegenerative processes.

On the other hand, in PTZ-induced acute seizures, Mas mRNA expression transiently increases in the hippocampus, particularly in the dentate gyrus, within 2–6 hours after seizure onset and returns to basal levels by 24 hours, indicating a seizure-specific response that is absent when seizures are pharmacologically prevented despite PTZ administration [12]. Consistent with the dynamic and phase-dependent regulation of the RAS, MasR expression has been reported to increase during the early (silent) phase of epilepsy but remain unchanged during the chronic phase, while long-term Ang-(1–7) administration reduces MasR levels in pilocarpine-induced temporal lobe epilepsy [13, 38]. In the present study, tissues were harvested 24 hours after the final seizure challenge, a time point at which transient seizure-induced MasR expression typically returns to baseline. Accordingly, the lack of differences in MasR levels between the PTZ control and healthy control groups is consistent with previous reports and supports the transient nature of seizure-induced MasR upregulation. In contrast, chronic AVE 0991 administration significantly increased MasR mRNA expression in both the cortex and the hippocampus, suggesting that prolonged MasR activation not only stimulates receptor activity but also enhances its transcription. These findings indicate that chronic MasR agonism may reinforce the protective RAS axis by sustaining MasR expression, thereby promoting neuroprotective adaptations against epileptogenesis.

NMDA receptors are key mediators of excitatory neurotransmission and play a central role in epileptogenesis [52]. PTZ-induced seizures increase extracellular glutamate levels, leading to excessive NMDA receptor activation, particularly of the NR2B subunit, and calcium-dependent excitotoxicity [53, 54]. While some studies report decreased NR2B expression in PTZ models [55], most demonstrate increased cortical and hippocampal NR2B levels that persist for days after seizures in both rats and mice [53, 56, 57]. Consistently, the present study observed elevated NR2B in PTZ-induced mice, likely reflecting enhanced excitatory drive and excitotoxic burden. In addition, evidence suggests that the protective arm of the RAS, particularly the Ang-(1–7)/Mas axis, can modulate NMDA receptor function [46, 58, 59]. Ang-(1–7) has been shown to normalize stress-induced changes in phosphorylated NR2B via MasR-dependent mechanisms, and functional interactions between MasR and NMDA receptors have been demonstrated, including reduced NMDA receptor activity following Ang-(1–7)/Mas activation [58]. In line with these findings, long-term AVE 0991 administration reduced NR2B levels in the PTZ group, suggesting that MasR activation may attenuate seizure-related excitatory imbalance and restore synaptic homeostasis, potentially through both direct modulation of NMDA signaling and indirect reduction of seizures.

Brain-derived neurotrophic factor (BDNF) is a CREB-regulated neurotrophin activated downstream of calcium influx via NMDA receptors and voltage-gated calcium channels, linking synaptic activity to neurotrophic signaling [53, 60–62]. Although PTZ-induced seizure studies report variable results, most kindling models show increased BDNF expression across brain regions and species [56, 63]. Consistently, the present study observed elevated BDNF mRNA in the cortex and hippocampus following PTZ kindling. Additionally, the RAS, particularly the Ang-(1–7)/MasR axis, modulates BDNF signaling and exerts neuroprotective effects [31, 51, 64]. In the present study, AVE 0991 attenuated PTZ-induced BDNF upregulation, suggesting that MasR activation helps normalize seizure-related neurotrophin dysregulation, potentially by stabilizing upstream excitatory pathways involving NMDA/NR2B-mediated calcium influx and downstream CREB-BDNF signaling.

This study has several limitations. First, only the effects of the MasR agonist AVE 0991 were investigated, while the impact of MasR antagonism on seizure parameters, behavior, and molecular outcomes was not assessed; such experiments would provide a more comprehensive understanding of the role of MasR signaling in epilepsy pathophysiology. Second, AVE 0991 was evaluated exclusively in a kindled model, and its potential effects on epileptogenesis were not explored, limiting conclusions regarding disease-modifying or preventive properties. Third,

behavioral assessments of learning and memory were limited, and more comprehensive testing using paradigms such as passive avoidance or the Morris water maze could better clarify its cognitive effects. Fourth, only mRNA expression levels of MasR, NR2B, and BDNF were analyzed, without corresponding protein-level validation. However, mRNA changes often correlate with protein expression; this is not always the case, preventing definitive conclusions at the protein level. Finally, interactions between the RAS and GABAergic signaling were not examined, and including GABA receptor analyses in future studies may help elucidate potential cross-talk between these pathways in epilepsy.

In conclusion, this study provides substantial evidence for the role of the ACE2/Ang-(1–7)/MasR axis in the pathophysiology of epilepsy. It demonstrates that the Mas receptor agonist AVE 0991 exerts multifaceted protective effects against seizure activity and seizure-associated behavioral and molecular alterations. Our findings indicate that MasR activation not only reduces seizure severity and alleviates anxiety-like behaviors and cognitive deficits but also contributes to synaptic homeostasis by regulating epileptogenesis-related molecular markers, including NR2B and BDNF. Furthermore, the upregulation of MasR expression following long-term AVE 0991 treatment suggests that the protective RAS arm may support adaptive and neuroprotective remodeling processes in epileptic tissue. Taken together, these results highlight MasR-centered pharmacological approaches as promising therapeutic targets for epilepsy and its associated comorbidities, and emphasize the importance of future studies that employ diverse epilepsy models, dosing regimens, and timing protocols to more clearly define the therapeutic potential of this axis.

Declarations

Author Contributions

Author Contributions Conceptualization: GA and BO; Methodology: GA, BO and ETA; Formal analysis and investigation: GA, BO, ETA, EST, MA, and EA; Writing-original draft preparation: GA, BO and ETA; Supervision: GA; Writing-review and editing: GA, BO, ETA, EST, MA, and EA.

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Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval

All experimental procedures were approved by the Ondokuz Mayıs University Local Ethics Committee for Animal Experiments with decision number 2023/16.

Conflict of interest

The authors declare no conflicts of interest.

Disclosures

None

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Figures

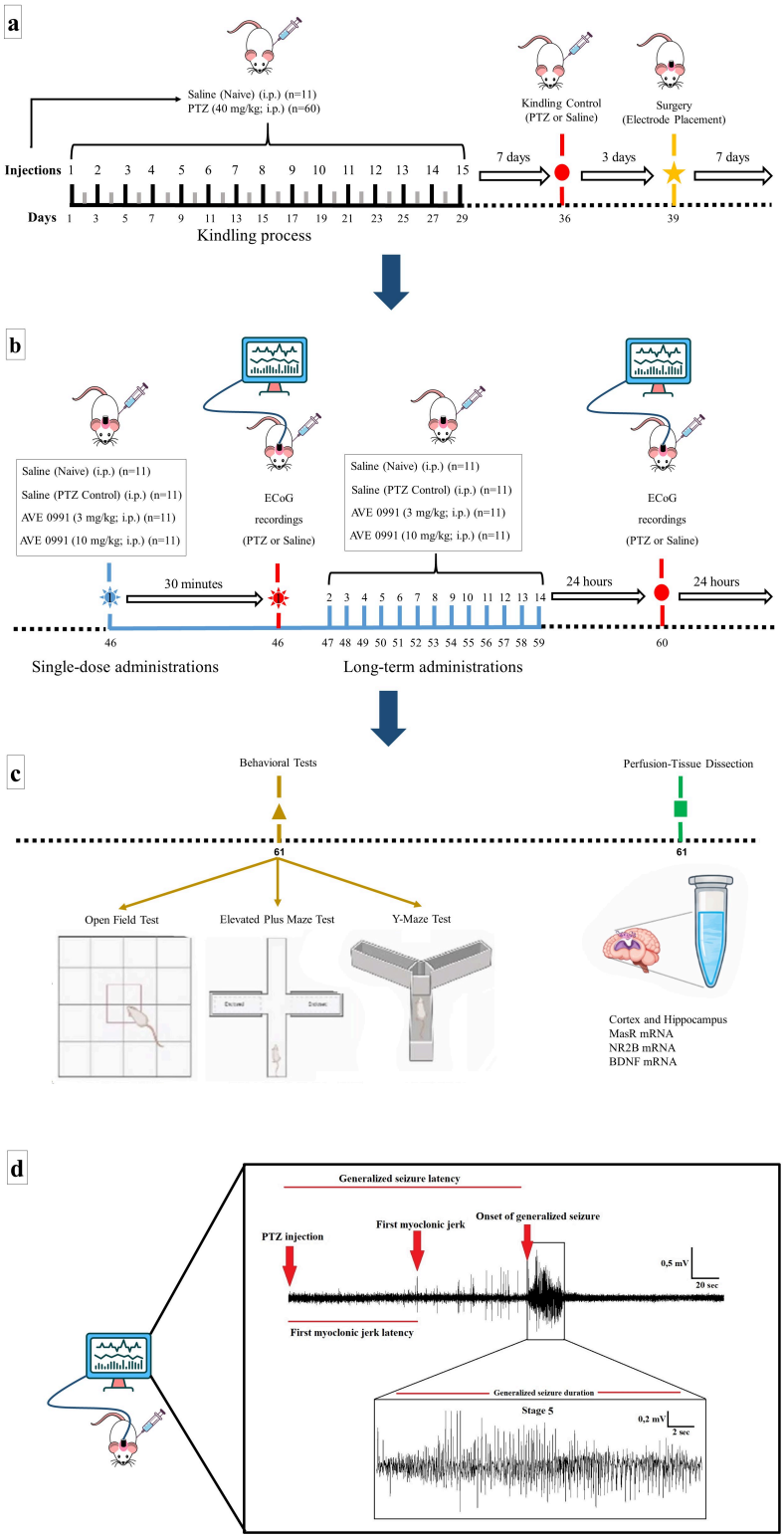


Figure 1

a-c) Timeline of experimental design. d) Representative seizure trace demonstrating the analyzed ECoG parameters.

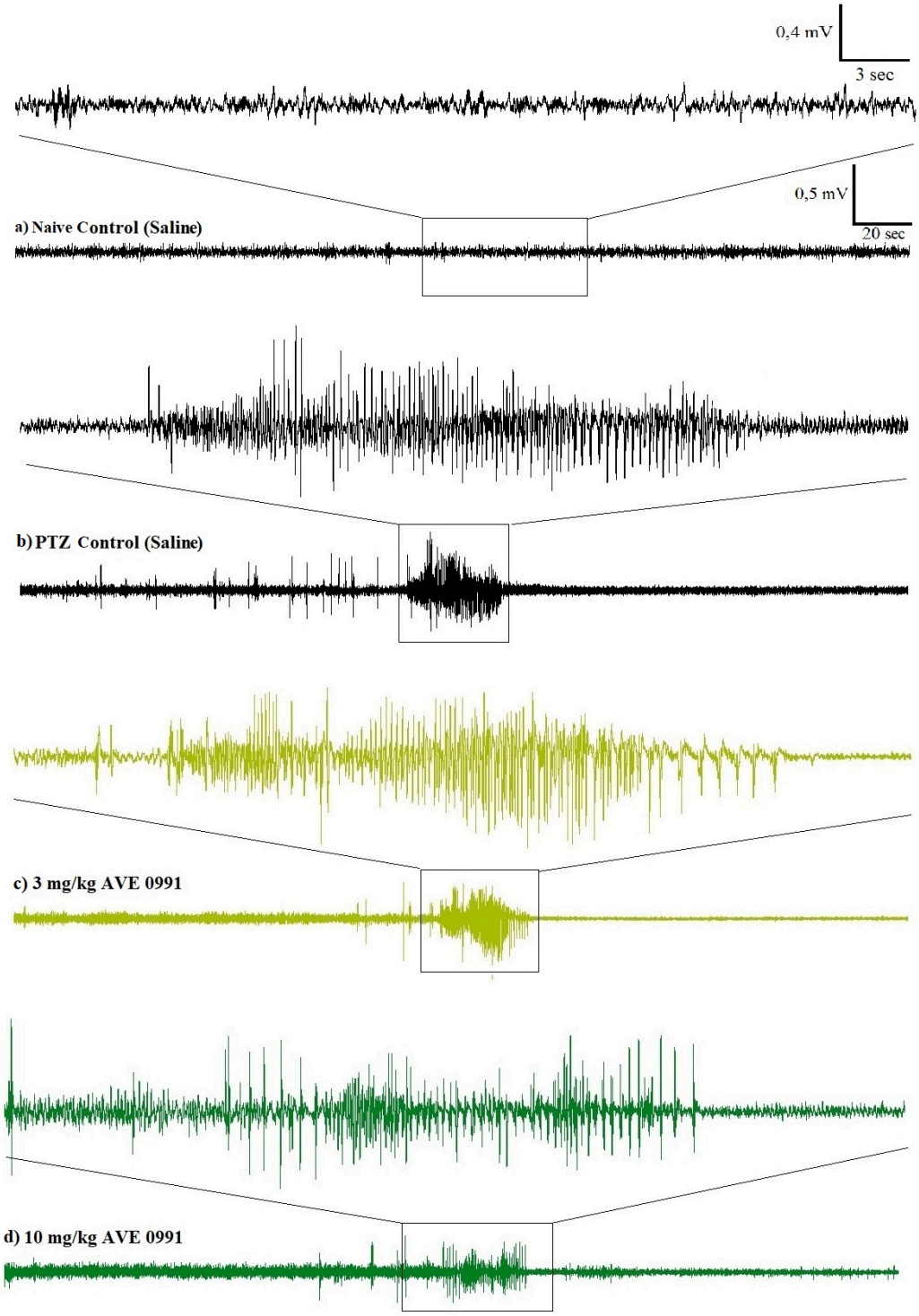


Figure 2

Representative ECoG recordings obtained after PTZ or saline injection administered 24 hours following the 14th treatment with saline or AVE 0991 (3 or 10 mg/kg).

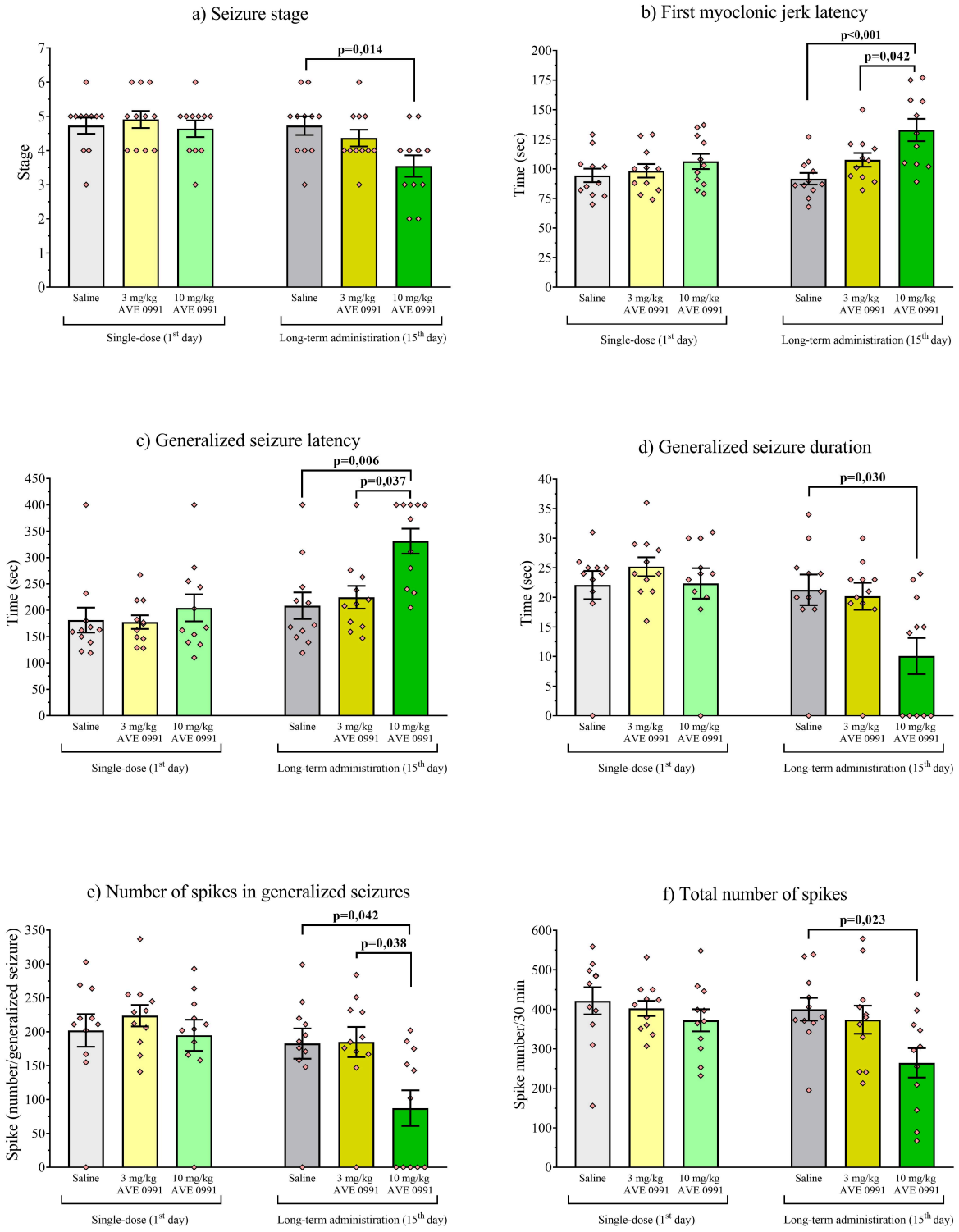


Figure 3

Seizure parameters of the experimental groups. a) Seizure stage, b) First myoclonic jerk latency, c) Generalized seizure latency, d) Generalized seizure duration, e) Number of spikes in generalized seizures, f) Total number of spikes.

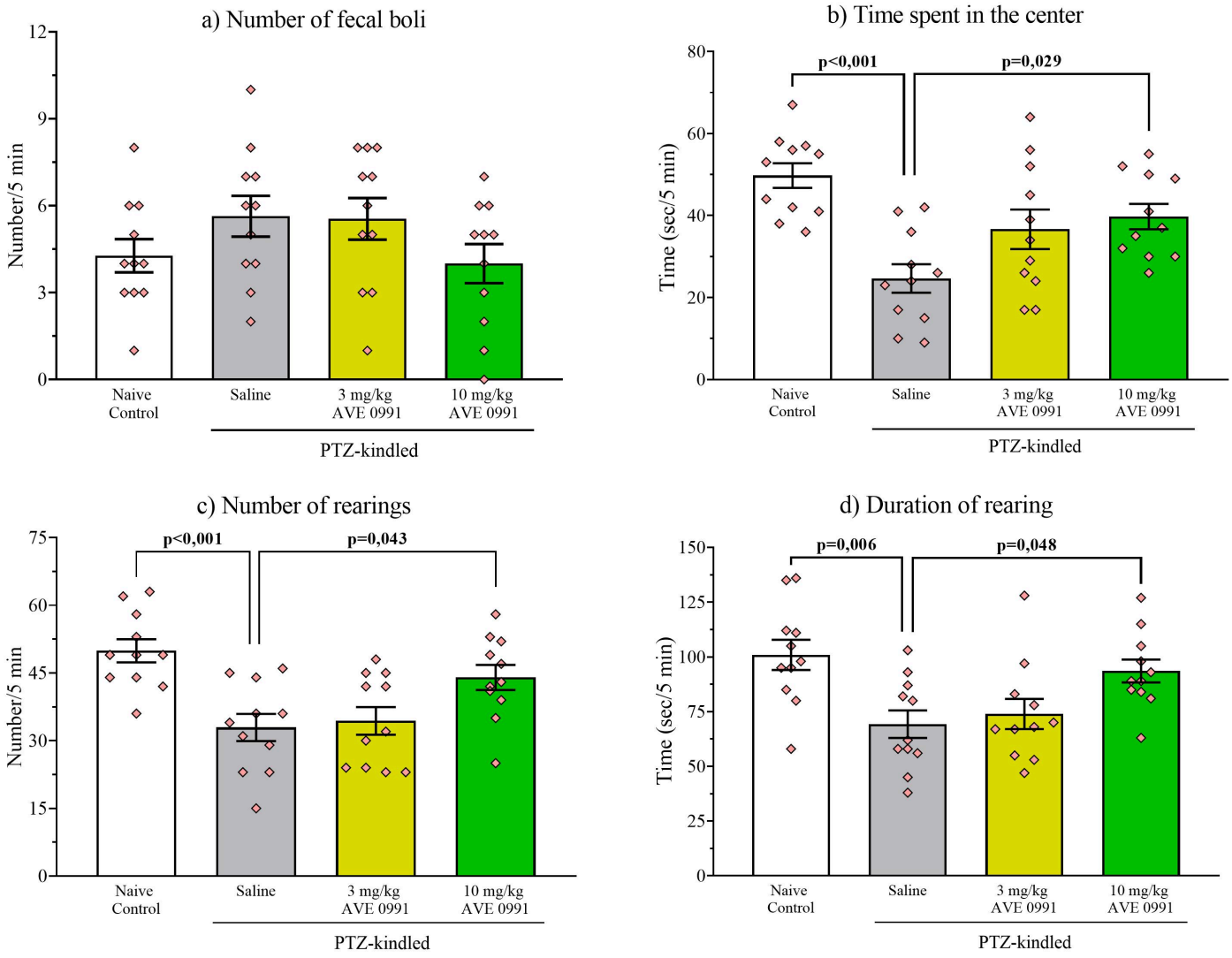


Figure 4

Open field test parameters of experimental groups. a) Number of fecal boli, b) Time spent in the center, c) Number of rearings, d) Duration of rearing.

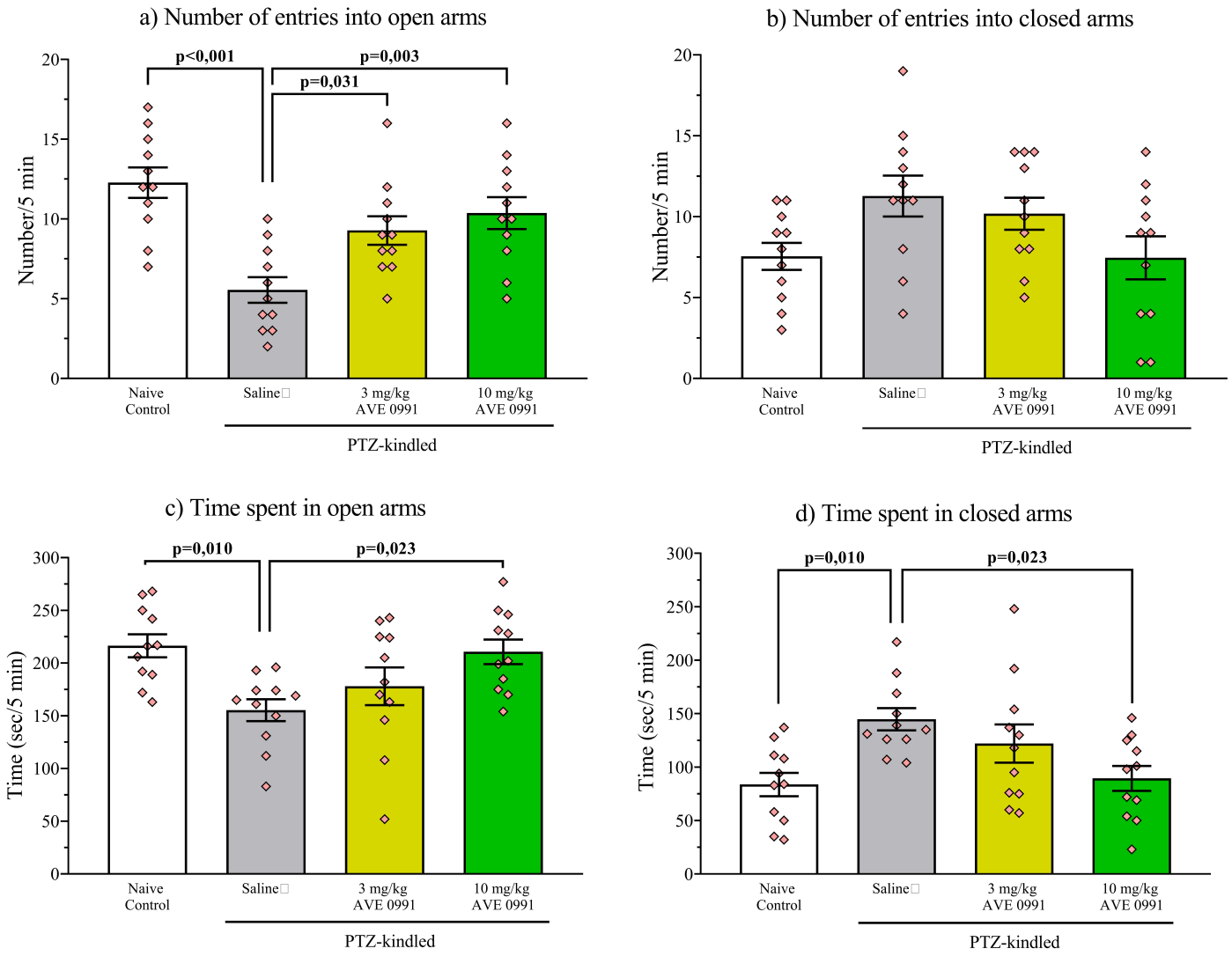


Figure 5

Elevated plus maze parameters of experimental groups. a) Number of entries into open arms, b) Number of entries into closed arms, c) Time spent in open arms, d) Time spent in closed arms.

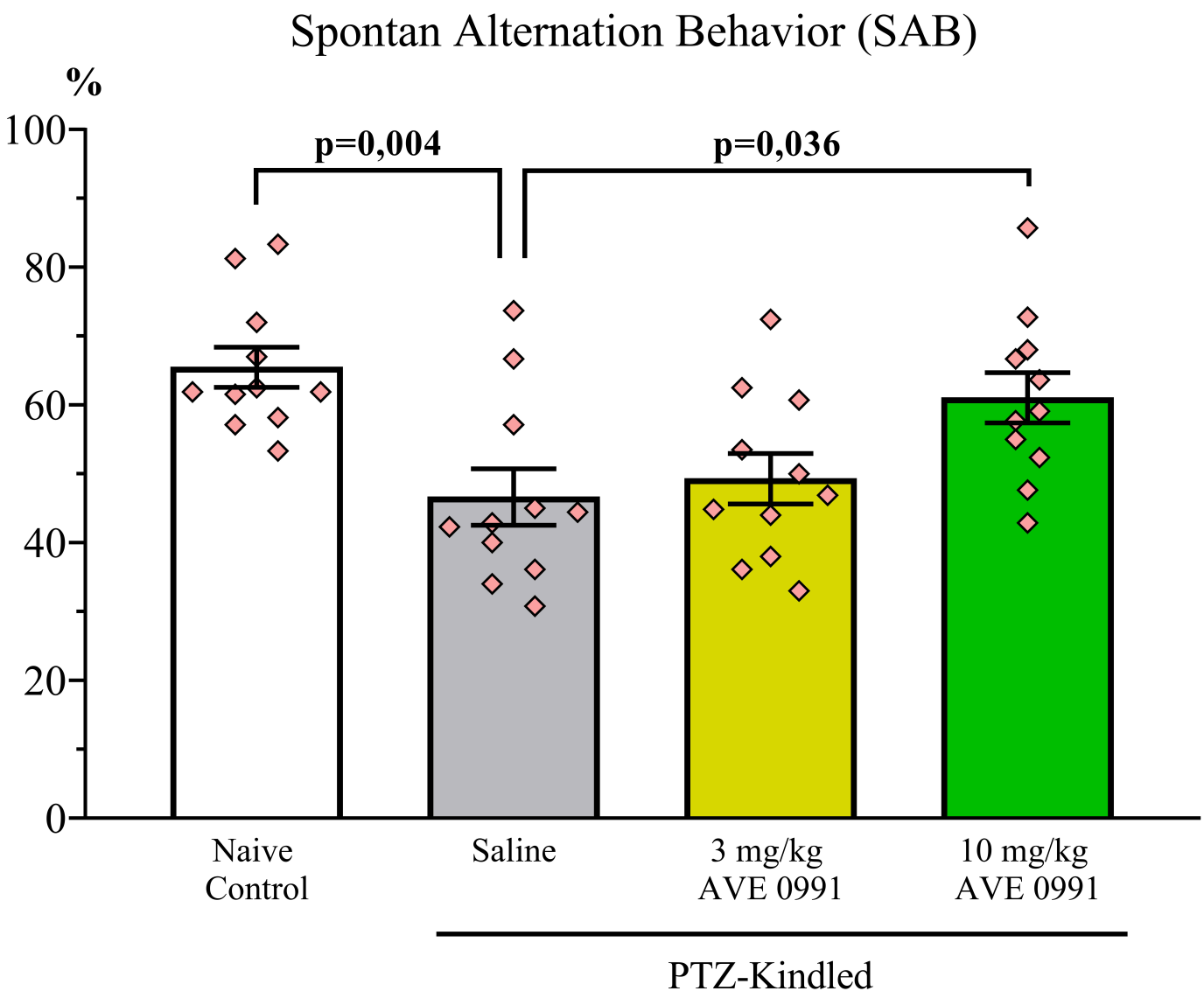


Figure 6

Spontaneous Alternation Behavior (SAB) of the Y-maze test.

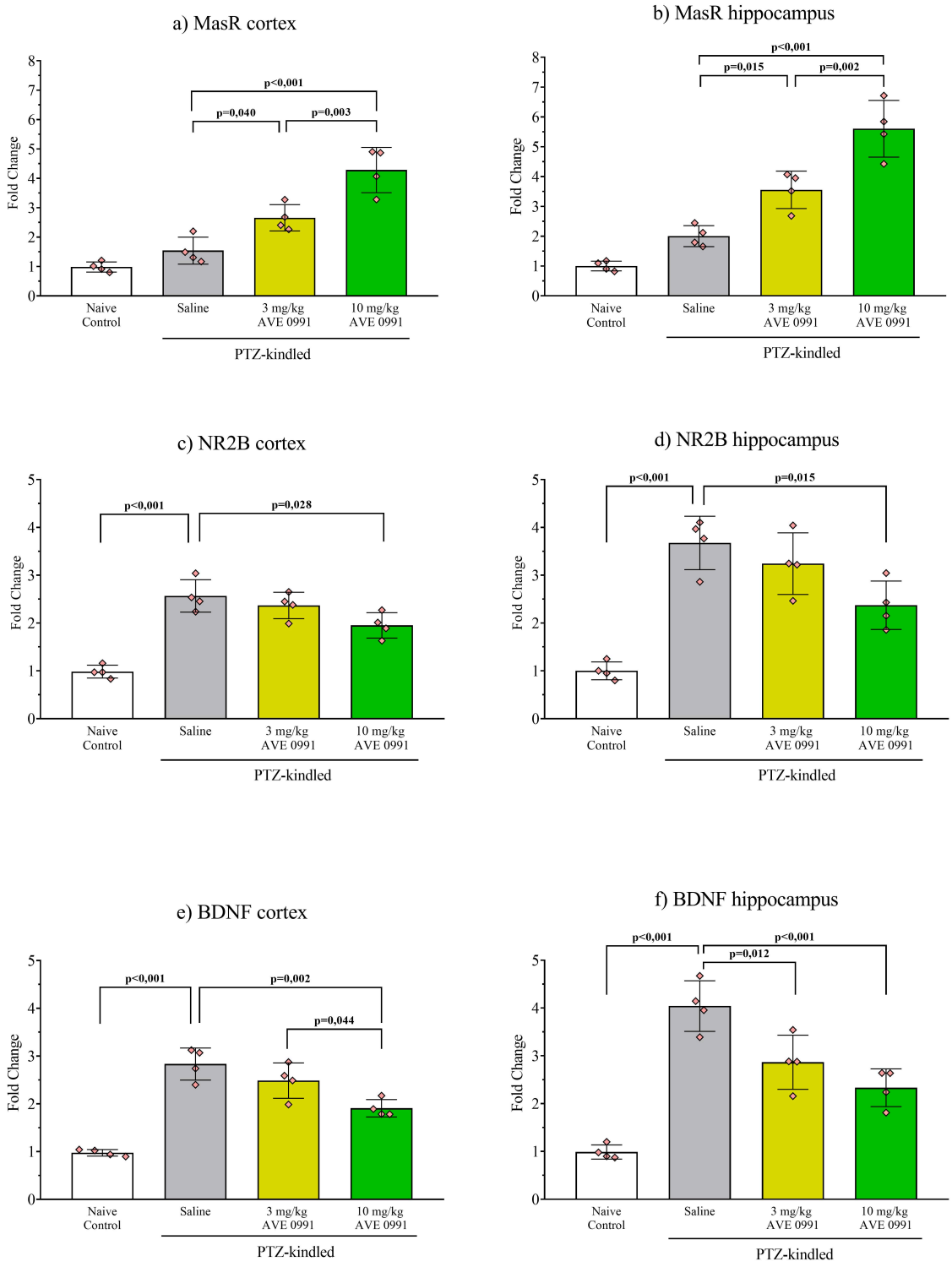


Figure 7

qPCR parameters of the study groups. a-b) MasR mRNA levels in cortex and hippocampus, c-d) NR2B mRNA levels in cortex and hippocampus, e-f) BDNF mRNA levels in cortex and hippocampus.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- SupplementalFigures.docx