Title Page

Manuscript Category:

Original Research Article

Title:

Prevention and Alleviation of Penicillin-induced Epileptic Discharge by Transient Receptor Potential Vanilloid 4 (TRPV4) Antagonist in Mice: Effects Mediated by Extracellular Glutamate Control

Running Title:

TRPV4 Antagonist Suppresses Epileptic Discharges

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Abstract

Transient receptor potential vanilloid 4 (TRPV4) channels, expressed in presynaptic membranes and astrocytes, have attracted attention as factors involved in epilepsy. We investigated the role of TRPV4 channels as therapeutic targets for epilepsy. We injected Penicillin G (PG) into the cortex of wild-type (WT) mice or TRPV4 KO mice. We also injected RN 1734, a TRPV4 antagonist, before and after PG injection. We recorded epileptic discharges (EDs) and the concentration of extracellular glutamate in the mice. In WT mice, the glutamate concentration increased after PG injection and EDs occurred. In TRPV4 KO mice, the glutamate concentration and beta-band power of the EDs were lower than that in WT mice. When WT mice were administered RN 1734 prior to PG injection, both the glutamate concentration and spike amplitude at late phase following PG injection were comparable to those in TRPV4 KO mice. However, when RN 1734 was administered after the onset of EDs, the alleviation of EDs lasted for only 10 min, and the glutamate concentration did not decrease. RN 1734 modulated EDs in a timing-dependent manner, with significant effects during ongoing EDs and limited effects following pre-treatment. These results indicate that TRPV4 antagonists modulate glutamate dynamics in an activity-dependent manner.

(200 words)

19 Keywords: Transient receptor potential vanilloid 4, Penicillin G, extracellular glutamate
20 concentration, epileptic discharge, epilepsy
21

Introduction

The prevalence of epilepsy is relatively high (1/100), with 30% of the patients having refractory epilepsy.[1] Surgical treatment is selected for patients with refractory epilepsy; however, indications are limited depending on the epileptic focus. Therefore, it is necessary to develop novel therapies for refractory epilepsy.

The transient receptor potential vanilloid 4 (TRPV4) channel is expressed in different cells, such as those in the brain, epithelia of the trachea, and urothelial cells in the bladder.[2] The TRPV4 channel is a polymodal protein activated by temperatures of 25 °C - 37 °C, hypoosmotic conditions, or mechanical stress. Inactivation of the TRPV4 channel suppresses abnormal epileptiform electroencephalograms in a generalized seizure model.[3–5] Therefore, the TRPV4 channel has received considerable attention as a potential target for novel anti-epileptic drugs. The mechanism underlying the anti-epileptic effects of TRPV4 channel inactivation is currently under investigation.

Many studies have revealed a relationship between extracellular glutamate levels and epileptic seizures.[6] Extracellular glutamate levels are elevated in patients with medial temporal lobe epilepsy,[7] and glutamate transporter-deficient mice (GLT1) exhibit fatal

epileptic seizures.[8] Increased local concentrations of glutamate contribute to the onset and persistence of seizures and can be caused by a seizure-induced release from the presynaptic membrane, inadequate reuptake of astrocytic glutamate transporters, or production by activated astrocytes.[2,9] TRPV4 channels are involved either in the production of glutamate from activated astrocytes or in its release from presynaptic membranes; however, which of the two processes is more involved in seizure generation remains unknown.

The TRPV4 channel in astrocytes has recently become the focus of attention as a factor involved in epilepsy and brain edema. TRPV4 channel activation causes astrocytes to release ATP, which excites the surrounding astrocytes and triggers neuronal activity in the synapses.[10] For example, the loop diuretic bumetanide, a promising new antiseizure drug, acts on astrocytes.[6]

This study aimed to elucidate the mechanisms underlying the antiepileptic effects of TRPV4 channel inactivation. We obtained electrocorticograms (ECoGs), measured extracellular glutamate concentrations in the same lesion and examined their correlation using a TRPV4 channel antagonist and TRPV4 channel KO mice.

Materials & Methods

Animal model

We used wild-type (WT) or TRPV4KO C57BL/6 mice aged 8–12 weeks and weighing 28–32 g (Japan SLC Inc., Fukuoka, Japan). To minimize variability due to estrous cycle–related hormonal fluctuations, which can significantly affect neuronal excitability and seizure thresholds, all animals included were male. All animals were kept under standard laboratory conditions in a temperature- and humidity-controlled room (23 ± 2°C, 55 ± 5%, respectively) on a 12-h light/dark cycle (lights on at 8:00 a.m.). Animals had ad libitum access to food and water.

Animal care and experimental procedures were approved by the Experimental Animal Care and Use Committee of the Yamaguchi University School of Medicine, Japan (24-053). All experiments were performed according to the guidelines of the Japan Association for Laboratory Animal Facilities of the National University Corporation.

Placement of electrodes, catheters, and probes

The animals were anesthetized with urethane (1.75 mg/10 ml/kg, administered intraperitoneally), and their heads were fixed using a stereotaxic instrument. Rectal temperature was adjusted to 37 ± 2 °C with a heating pad, and cortical temperature was maintained at 30 °C ± 2 °C. We made two burr holes (1.0 mm diameter each) at 1.0 mm posterior and 2.5 mm bilateral from the bregma. The left burr hole was used to place the ECoG electrodes and the injection cannula (0.4 mm diameter × 40 mm length and 3 μl volume, EIM-40, Eicom, Japan). A right burr hole was used to place the ECoG electrodes to measure the ECoG data without drug injection. We placed two ECoG reference electrodes into the epidural space on the bilateral somatosensory cortices and cerebellar area. A thin thermocouple (IT-24; Physitemp, Tokyo, Japan) was placed. A ground electrode was placed on the tail.

Drugs

Drugs were injected through the left cannula without mixing. The control mice received PG (penicillin G, which induces epileptic seizures by inhibiting GABAA receptors and lowering seizure thresholds; Meiji, Japan) and 10% DMSO (dimethyl sulfoxide, solvents; Merck KGaA, Darmstadt, Germany), whereas the treatment group of mice received PG and RN 1734 (selective antagonist of the TRPV4 channel, Merck KGaA,

94 Darmstadt, Germany). We used a 10 µl Hamilton syringe with a 26-gauge removable
95 needle (1701RN-7758-02; Hamilton, Reno, NV). The 1 µl internal cavity of the
96 removable needle was filled with 1 µl PG (dissolved in 0.9% saline at 200 IU/µl), and
97 the 10 µl syringe body was filled with 1 µl PG and 8.6 µl RN 1734, separated with 0.4
98 µl air.[11]
99 Next, the injection cannula and Hamilton syringe were connected through a Teflon tube
100 (JT-10; 50 cm long, 4 µl volume, Eicom, Japan). The injection cannula was inserted to a
101 depth of 0.75 mm from the brain surface. PG was administered intracortically for 10
102 min at a rate of 0.1 µl/min using a microinjection pump (ESP-64, Eicom, Japan),
103 beginning 30 min after the start of the ECoGs recording. DMSO (10 %) or RN 1734
104 (dissolved in 10 % DMSO, DMSO: Merck KGaA in saline) was administered
105 intracortically for 10 min, with RN 1734 administration starting 90 min after the PG
106 injection, at a rate of 0.1 µl/min using a microinjection pump (Fig. 1A).
107
108 The spike amplitudes of PG-induced epileptic discharges (EDs) were averaged every 5
109 min after the start of ECoG. In preliminary experiments, PG-induced EDs began to
110 spike at 50 min, peaked at 90 min, and lasted for over 120 min. Therefore, we decided
111 to evaluate the EDs for 5 min, 85-90 min after PG injection. In addition, because we

confirmed that the spike amplitude of EDs decreased immediately after RN 1734 injection, we set the period for evaluating the effect of RN 1734 to 5 min immediately after RN 1734 injection.

Power analysis of the ECoGs

The ECoGs were amplified using a bioamplifier (EX-1; Dagan Corporation, Minneapolis, MN, USA) and analyzed using an A/D converter at a sampling rate of 2 kHz (PowerLab 8/30; AD Instruments, Castle Hill, Australia) for 120 min of continuous recording (30 min for the stabilization of ECoG basic rhythms and 90 min for ECoG measurements after PG injection). The ECoG recordings used a low-frequency filter (0.1 Hz), a high-frequency filter (10 kHz), and a notch filter (off). The beta-band wave (14-24 Hz) is a useful indicator of epileptic seizures in the cortex, showing significant differences before and after the antiepileptic effect of focal brain cooling. Therefore, we calculated the frequency band intensity of the beta band after fast Fourier transform of the ECoGs. Spike waves in abnormal epileptiform ECoGs were defined as those with spike durations of less than 100 ms. To assess TRPV4 inhibition by RN 1734, we evaluated ECoG recordings using three different doses of RN 1734 (1 mM, 3 mM, and 10 mM) along with PG. The control group received PG or 10% DMSO. All results are

expressed as mean ± standard error of the mean (SEM). Differences between baseline and pre-injection, and pre- and post-injection in the same groups for the power intensity of the beta-band wave were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test or Wilcoxon test, with $p < 0.05$ indicating significance. Differences between WT and TRPV4KO mice for the power intensity of the beta-band wave at baseline were assessed using paired t-tests, with $p < 0.05$ considered significant.

Measurement of glutamate concentration

Extracellular glutamate levels were measured using glutamate biosensors (Pinnacle Technology, Inc., Part #7004-Glutamate, Lawrence, KS, USA) according to previously published methods. The WT and TRPV4 mice were anesthetized and fixed as in the first examination, and the glutamate biosensor was inserted into the primary somatosensory cortex (S1) through the left bur hole, which corresponded to the cortical region receiving PG injection and consistently exhibiting epileptiform discharges on EcoG recordings. This region was selected to enable direct spatial correlation between electrophysiological activity and extracellular glutamate concentration.

The stereotaxic coordinates of the biosensor were determined according to the Paxinos and Franklin mouse brain atlas and were as follows: anteroposterior (AP), −1.0 mm

148 from the bregma; mediolateral (ML), ±2.5 mm from the midline; and dorsoventral
149 (DV), −2.0 mm from the cortical surface. The biosensor was carefully advanced to the
150 target depth to minimize tissue damage. We began recording the EcoG immediately
151 after insertion of the biosensor. Ten min later, PG was injected for 10 min, and RN 1734
152 was administered 60 min after PG injection (Fig. 2A). We also measured the
153 concentration of extracellular glutamate in parallel with ECoG recordings using the
154 biosensor. We evaluated the concentration of extracellular glutamate and intensity of the
155 beta-band wave for 10 min during the same two periods: 10 min during PG injection
156 and 50 min after PG injection, when the spike amplitude of the EDs is observed.
157
158 Differences in the concentration of extracellular glutamate or the intensity of beta-band
159 waves between groups were assessed using Student's T test or Mann-Whitney U test,
160 with $p < 0.05$ considered significant.
161
162 We analyzed the relationship between extracellular glutamate and the intensity of the
163 beta-band waves using the t-test and the Pearson product-moment correlation
164 coefficient.
165

166 Prophylactic administration of RN 1734 and seizure scoring

167 Finally, to evaluate the effect of TRPV4 inactivation on not only EDs but also epileptic
168 seizures, we performed an awake examination using the following procedure. WT or
169 TRPV4 mice were anesthetized with sevoflurane (3% for induction, 1.5% for
170 maintenance), and ECoGs were recorded. We measured extracellular glutamate levels
171 using a biosensor in parallel with the ECoG recordings, as described earlier. We
172 administered 10% DMSO or RN 1734 (dissolved in 10% dimethyl sulfoxide: DMSO:
173 Merck KGaA in saline) intracortically for 10 min at a rate of 0.1 µl/min using a
174 microinjection pump, 30 min after the stabilization with sevoflurane. PG was injected
175 30 min later. We recorded ECoGs 150 min after stabilization with sevoflurane,
176 interrupted the sedation to awaken the mice, and continued video monitoring (Fig. 3A).
177 Based on previous studies, [3,4] we analyzed the relationship between extracellular
178 glutamate levels and seizure scores (stage 1: ear and facial twitching; stage 2:
179 convulsive twitching axially through the body; stage 3: myoclonic jerks and rearing;
180 stage 4: turning over onto the side, wild running, and wild jumping; stage 5: generalized
181 tonic-clonic seizures; and stage 6: death) using the t-test and Pearson product-moment
182 correlation coefficient. The severity of the epileptic seizures was evaluated using seizure

183 scores. Differences in seizure scores between groups were assessed using Student's t-
184 test or Mann-Whitney U test, with $p < 0.05$ considered significant.
185
186 Statistical Analysis
187 All data are presented as mean ± standard error of the mean (SEM).
188 Normality of data distribution was assessed using the Shapiro–Wilk test, and
189 homogeneity of variance was evaluated using Levene's test.
190
191 For comparisons between two independent groups, Student's t-test was applied when
192 data were normally distributed with equal variances; otherwise, the Mann–Whitney U
193 test was used. For paired comparisons within the same group, paired t-tests or Wilcoxon
194 signed-rank tests were performed, as appropriate.
195
196 For comparisons among more than two groups at a single time point, one-way analysis
197 of variance (ANOVA) followed by Dunnett's post hoc test was conducted. When
198 heterogeneity of variance was detected, Welch's ANOVA was applied. When the
199 assumption of normality was not satisfied, the Kruskal–Wallis test was used as a non-
200 parametric alternative.

Time-course data for beta-band power and extracellular glutamate concentrations were analyzed using two-way repeated-measures ANOVA, with group as the between-subject factor and time as the within-subject factor. When significant main effects or group - time interactions were detected, post hoc multiple comparisons with appropriate correction were performed.

Correlations between electrophysiological parameters (beta-band power or spike amplitude) and extracellular glutamate concentrations were evaluated using the Pearson product–moment correlation coefficient for normally distributed data, and corresponding p-values are reported.

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University). A two-tailed p value < 0.05 was considered statistically significant.

Results

218 TRPV4 antagonist decreases PG-induced ECoGs amplitudes and beta-band power in a

219 dose-dependent manner.

220

221 Fig. 1A shows the protocol used to study the suppressive effects of TRPV4 deficiency

222 on PG-induced EDs. RN 1734, a TRPV4 antagonist, decreased the left-side ECoG

223 amplitudes and beta-band power in a concentration-dependent manner (Fig. 1B-E). In

224 WT mice, the suppressive effect of RN 1734 on EDs lasted for approximately 10 min,

225 after which the spikes resumed. In contrast, the EDs of the TRPV4 KO mice were low

226 from the start of the experiment (Fig. 1E, F). Heart rate data indicated no significant

227 differences in the depth of anesthesia between WT (n = 24; 563.2 ± 6.37 bpm) and

228 TRPV4 KO mice (n =6; 523.7 ± 13.2 bpm) (Mann-Whitney U test, p = 0.26, Fig.1H).

229 No significant differences in brain temperature were seen between WT and TRPV4 KO

230 mice (30.2 ± 0.09 °C vs. 30.3 ± 0.13 °C, respectively, p = 0.55, Student's t-test; Fig. 1I).

231

232 The transitions in the beta-band power every 5 min differed between the PG + DMSO

233 and PG + RN 1734 groups (Fig. 1J). The beta-band power in the PG + DMSO group

234 increased 60 min after PG injection. (Basal: 0.06 ± 0.02 μV^2, pre-injection: 3.4 ± 0.3

235 μV^2, p = 0.0313, Wilcoxon test; Fig. 1J). DMSO was injected into the epileptiform

236 focus 60 min after the PG injections, and no differences were seen in the beta-band
237 power before and after the injections (pre-injection: 3.4 ± 0.3 μV^2, post-injection: 2.9 ±
238 0.3 μV^2, p = 0.26, Dunnett test; Fig. 1J). In contrast, RN 1734 (1 or 3 or 10 mM)
239 reduced the beta-band power (pre-injection: 3.4 ± 0.8 μV^2, post-injection: 2.7 ± 0.8
240 μV^2, p = 0.0313, Wilcoxon test; pre-injection: 4.3 ± 0.6 μV^2, post-injection: 1.9 ± 1.0
241 μV^2, p = 0.046, Dunnett test; and pre-injection: 4.7 ± 1.3 μV^2, post-injection: 0.8 ± 0.4
242 $\mu V^{2,}$ p = 0.0313, Wilcoxon test; Fig. 1J).
243 Normality was confirmed in each treatment group (1 mM, 3 mM, 10 mM RN 1734)
244 using the Shapiro–Wilk test (all $p > 0.05$), whereas Levene's test indicated
245 heterogeneity of variance among groups ($p = 0.018$). Therefore, Welch ANOVA was
246 applied, revealing a significant group effect on the pre–post change (Δ) ($F = 7.37$, $p =$
247 0.0016). Consistently, the non-parametric Kruskal–Wallis test also demonstrated a
248 significant difference between the groups ($H = 17.52$, $p = 0.00055$). Furthermore, trend
249 analysis showed a strong positive correlation between dose and Δ (Spearman's $\rho = 0.84$,
250 $p < 0.001$), confirming a clear dose-dependent effect.
251
252 TRPV4 antagonist does not affect PG-induced beta-band power in TRPV4 KO mice
253

254 RN 1734 injection reduced PG-induced ECoG amplitudes and beta-band power in WT
255 mice, but not in TRPV4 KO mice (Fig. 1F, G). The PG-induced beta-band was
256 significantly lower in the TRPV4 KO mice than in the WT mice, as well as mice treated
257 with PG + 10% DMSO or 10 mM RN 1734 ($3.4 \pm 0.3\ \mu V^2$ vs. $1.7 \pm 0.6\ \mu V^2$,
258 respectively, $p=0.02$, and $4.7 \pm 1.3\ \mu V^2$ vs. TRPV4 KO: $1.7 \pm 0.4\ \mu V^2$, respectively,
259 $p=0.03$, Student's t-test; Fig. 1B, F, J).
260
261 Both WT and TRPV4 KO mice received DMSO and 10 mM RN 1734 injections into
262 the epileptiform focus 60 min after PG injections. No differences were seen in the beta-
263 band power before and after injection (pre-injection: $1.7 \pm 0.6\ \mu V^2$, post-injection: $1.6 \pm$
264 $0.5\ \mu V^2$, $p = 0.95$, and pre-injection: $1.7 \pm 0.4\ \mu V^2$, post-injection: $2.7 \pm 0.3\ \mu V^2$, $p =$
265 1.00, Dunnett test; Fig. 1F, G, J). Moreover, RN 1734 did not suppress the beta-band
266 power in TRPV4 KO mice. The TRPV4 antagonist dose-dependently decreased the PG-
267 induced ECoG amplitudes and beta-band power.
268
269 TRPV4 antagonist does not affect PG-induced extracellular glutamate concentrations in
270 TRPV4 KO mice
271

Fig. 2A shows the protocol used to study the correlation between the power of the beta-bands and extracellular glutamate concentration. RN 1734 decreased the ECoG amplitude and beta-band power in WT mice, but not in TRPV4KO mice (Fig. 2B, C). Changes in beta-band power and extracellular glutamate concentrations every 5 min in WT and TRPV4 KO mice are shown in Figures 2D and 2E, respectively. In WT mice, the extracellular glutamate concentration first increased after PG injection, followed by an increase in beta-band power (Fig. 2D, E). In contrast, compared to the WT mice (n = 4), the TRPV4 KO mice (n = 4) had significantly lower baseline extracellular glutamate concentrations (287.2 ± 44.5 mV vs. 119.2 ± 26.6 mV; p = 0.029, Mann-Whitney U test; Fig. 2F). RN 1734 (10 mM) injection into the EDs focus 60 min after PG administration significantly reduced beta-band power in WT mice (pre-injection: 2.4 ± 0.7 μV^2, post-injection: 0.8 ± 0.3 μV^2, p = 0.0063), whereas no significant change was observed in TRPV4 KO mice (pre-injection: 0.5 ± 0.3 μV^2, post-injection: 0.4 ± 0.2 μV^2, p = 0.97, Dunnett test; Fig. 2G). This lack of effect in TRPV4 KO mice was likely attributable to the already low baseline beta-band power in these animals. In contrast, RN 1734 administration did not significantly alter extracellular glutamate concentrations in either group when administered after the onset of epileptic discharges (WT mice, pre-injection: 55.9 ± 14.5 mV, post-injection: 62.3 ± 8.9 mV, p = 0.98;

TRPV4 KO mice, pre-injection: 8.7 ± 2.9 mV, post-injection: 4.1 ± 2.5 mV, $p = 0.88$, Dunnet test; Fig. 2H).

A two-way repeated-measures ANOVA revealed a significant main effect of group ($F(1,6) = 10.45$, $p = 0.0179$) and time ($F(19,114) = 19.10$, $p < 0.001$), as well as a significant group - time interaction ($F(19,114) = 6.01$, $p < 0.001$), indicating that the temporal changes in extracellular glutamate concentration differed significantly between groups. For beta-band power, a two-way repeated-measures ANOVA demonstrated a significant main effect of time ($F(19,114) = 6.93$, $p < 0.001$) and a significant group - time interaction ($F(19,114) = 2.26$, $p = 0.0044$), whereas the main effect of group did not reach statistical significance ($F(1,6) = 4.43$, $p = 0.0799$). These results indicate that the temporal profile of beta-band activity differed significantly between groups.

From the time of PG injection to the appearance of EDs, the maximum concentration of extracellular glutamate significantly correlated with the beta-band power of the abnormal epileptiform ECoGs immediately before RN 1734 injection ($r^2 = 0.67$, $p = 0.014$, Pearson product-moment correlation coefficient: Fig. 2I), which tended to increase as the concentration of glutamate increased with PG infusion.

TRPV4 antagonist pre-injection and TRPV4 KO decrease EDs and extracellular glutamate.

Fig. 3A shows the protocol used to study the correlation between extracellular glutamate concentration and seizures. Representative changes in ECoG amplitudes and beta-band power showed that RN 1734 injection decreased PG-induced ECoG amplitudes and beta-band power in both WT and TRPV4 KO mice (Fig. 3B-D). Changes in spike amplitude and extracellular glutamate concentration every 5 min in the two groups of mice are shown in Figures 3E and 3F, respectively. In the WT mice (n = 6), the extracellular glutamate concentrations increased significantly in response to PG injection in the DMSO group (95.9 ± 15.3 mV vs. 193.0 ± 29.3 mV, respectively, p = 0.029, Paired t-test; Fig. 3G), but not in the 10 mM RN 1734 group (95.9 ± 15.3 mV vs. 111.9 ± 12.1 mV, respectively, p = 0.80, Student's t-test; Fig. 3G). In contrast, in the TRPV4 KO group (n = 4), the extracellular glutamate concentrations did not increase significantly in response to PG injection both in the DMSO group (95.9 ± 15.3 mV vs. 88.8 ± 10.4 mV, respectively, p = 0.51, Student's t-test; Fig. 3G) and in the 10 mM RN 1734 group (111.9 ± 12.1 mV vs. 88.8 ± 10.4 mV, respectively, p = 0.20, Student's t-test; Fig. 3G). In the WT mice, PG-induced spike amplitudes were higher in the DMSO

325 group than in the 10 mM RN 1734 group as well as the DMSO group in TRPV4 KO
326 mice (-2.8 ± 0.2 mV vs. -0.9 ± 0.3 mV, respectively, p = 0.0025, Student's t-test, and -
327 2.8 ± 0.2 mV vs. -0.9 ± 0.2 mV, respectively, p = 0.0018, Student's t-test; Fig.3H). PG-
328 induced spike amplitudes in the 10 mM RN 1734 group were comparable in the WT and
329 TRPV4 KO mice (-0.9 ± 0.3 mV vs. -0.9 ± 0.2 mV, respectively, p = 0.97, Student's t-
330 test; Fig. 3G).
331 For extracellular glutamate in Fig. 3F, a two-way repeated-measures ANOVA revealed
332 a significant main effect of time ($F(19,114) = 5.55$, $p < 0.001$), whereas neither the main
333 effect of group ($F(2,6) = 1.60$, $p = 0.2775$) nor the group - time interaction ($F(38,114) =$
334 1.03, $p = 0.445$) reached statistical significance. These results indicate that extracellular
335 glutamate concentration changed over time following PG administration, but its
336 temporal profile did not differ significantly among groups under this experimental
337 condition. In contrast, for spike amplitude in Fig. 3E, no significant main effects of
338 group ($F(2,6) = 0.41$, $p = 0.681$) or time ($F(19,114) = 0.95$, $p = 0.523$), and no
339 significant group - time interaction ($F(38,114) = 1.02$, $p = 0.446$) were observed. These
340 findings suggest that the spike amplitude did not exhibit significant group-dependent or
341 time-dependent changes in this experimental setting.

342 The maximum concentration of glutamate from the PG injection to the appearance of
343 EDs was significantly correlated with the spike amplitude intensity of the abnormal
344 epileptiform ECoGs (WT: $r^2 = 0.94$, $p = 0.0066$, TRPV4 KO mice: $r^2 = 0.87$, $p = 0.032$,
345 Pearson product-moment correlation coefficient: Fig. 3J), which tended to decrease as
346 the concentration of glutamate increased with PG injection.
347
348 TRPV4 antagonist and TRPV4 KO both suppress PG-induced seizure.
349
350 The WT mice treated with DMSO had higher seizure scores compared to (a) WT mice
351 treated with 10 mM RN 1734 group (4.3 ± 0.3 vs. 2.3 ± 0.5, respectively, $p = 0.026$,
352 Mann-Whitney U test, Fig. 3I) as well as (b) DMSO-treated TRPV4 KO mice (4.3 ± 0.3
353 vs. 2.0 ± 0.4, $p = 0.017$, Mann-Whitney U test; Fig.3I). Hence, both TRPV4 inactivation
354 and knockout reduced the seizure scores.
355
356 **Discussion**
357 In this study, we demonstrated the suppressive effects of TRPV4 inactivation on EDs,
358 which led to the suppression of epileptic seizures. We also examined the correlation
359 between the ECoGs and extracellular glutamate concentrations to elucidate the

360 mechanisms underlying the anti-epileptic effects of TRPV4 inactivation. The key
361 findings of our study were as follows: (1) RN 1734, a TRPV4 antagonist, suppressed
362 EDs for approximately 10 min and epileptic seizures in a dose-dependent manner (Figs
363 1 and 3). (2) ED intensity and seizure scores were significantly lower in TRPV4 KO
364 mice than in WT mice, and RN 1734 was naturally ineffective in TRPV4 KO mice. (3)
365 The PG-induced increase in extracellular glutamate levels preceded an increase in beta-
366 band power. The effect of the TRPV4 antagonist on elevated beta-band power was
367 temporary, lasting only for approximately 10 min, whereas its effect on elevated
368 extracellular glutamate concentration was weak (Fig. 2). (4) Administration of a TRPV4
369 antagonist before PG injection was associated with lower beta-band power and
370 extracellular glutamate concentration at late phase following PG administration.
371 To determine the optimal RN 1734 concentrations that would have an effect equivalent
372 to TRPV4 deficiency (as in TRPV4 KO mice), we first examined the dose-dependent
373 inhibition of drug-induced EDs by the TRPV4 antagonist RN 1734 at 10 mM, which
374 significantly suppressed drug-induced EDs. Consistent with previous in vitro studies
375 demonstrating the inhibitory effects of TRPV4 antagonists on neuroexcitation, we found
376 that RN 1734 suppressed the power of EDs in vivo.
377

Our data also show that the reduction in the power of drug induced EDs was comparable between TRPV4 KO mice and mice treated with TRPV4 antagonists. Although previous studies have shown that TRPV4 deficiency suppresses the power of EDs, its effect on epileptic seizures has not yet been determined. [12,13] However, our results demonstrated that the inactivation or deficiency of TRPV4 suppressed EDs and lowered seizure scores.

The suppressive effect of TRPV4 deficiency on EDs is consistent with previous reports showing that TRPV4 deficiency contributes to the lower power of EDs induced by kindling or hyperthermia. [12,13] To the best of our knowledge, this is the first study to report the suppressive effects of TRPV4 antagonists on EDs in a cortical focal epilepsy model. Excitability was significantly reduced in the neurons of TRPV4 KO mice, with a -5 mV decrease in the resting membrane potential, [8] compared to that in the neurons of WT mice. These results indicate that TRPV4 KO neurons require larger depolarization to evoke firing than WT neurons. Consistent with these studies, we demonstrated the effect of TRPV4 suppression on EDs in vivo using an antagonist. However, if the only effect of TRPV4 suppression on EDs was to lower the resting membrane potential and increase the threshold, the same effect should be expected if the antagonist was

396 administered before or after PG administration. In contrast, in our experiments, RN
397 1734-mediated ED suppression lasted for only approximately 10 min when
398 administered after PG, suggesting the involvement of other mechanisms that sustain
399 seizures.
400 In addition, when the entire time course was evaluated using a two-way repeated-
401 measures ANOVA, a significant group - time interaction was observed only when the
402 TRPV4 antagonist was administered after the onset of epileptiform discharges, whereas
403 no significant group-dependent effects were detected when the antagonist was
404 administered prior to PG injection. These findings indicate that the effects of TRPV4
405 inhibition are not uniform across time, and suggest that TRPV4 is preferentially
406 involved in the temporal progression and maintenance of ongoing epileptiform activity
407 rather than in seizure initiation. This phase-dependent effect of TRPV4 inhibition
408 suggests that the underlying mechanisms are not limited to neuronal excitability alone,
409 but may involve activity-dependent regulation of extracellular glutamate.
410 We cannot conclude the origin of glutamate, which increased with PG stimulation and
411 was modulated with the TRPV4 antagonist, because we did not use astrocyte-specific
412 knockout mice or antagonists. We suggest the following mechanisms based on our
413 limited results: glutamate released from neurons works as a neurotransmitter; thus, an

414 overdose should immediately cause ED. Conversely, astrocytes regulate extrasynaptic
415 glutamate, and persistent accumulation of glutamate lowers the ED threshold. In the
416 present study, the peak increase in extracellular glutamate levels preceded the peak
417 increase in ED (Fig. 2B, 3 B). This suggests that PG may disturb astrocytic regulation
418 of glutamate rather than enhance neuronal release of glutamate. The deficiency of
419 TRPV4 and the administration of the TRPV4 antagonist prior to PG injection (Fig. 3C-
420 D, 3F-G) may have inhibited both neuronal and astrocytic glutamate increases. Of these,
421 the effect on astrocytic TRPV4 channels may be significant, since the suppression of
422 neurons was not necessary in the low-glutamate condition. In contrast, the antagonist
423 administered during ED was limited to the ED (Fig. 1C-E, 2 B). The TRPV4 antagonist
424 suppressed new glutamate production but did not decrease glutamate levels (Fig. 2B,
425 2E, 2H). Thus, although a slight decrease in neurotransmitters in glutamatergic neurons
426 would temporarily decrease ED, the threshold for ED would remain low. Other
427 plausible explanations include rapid drug metabolism or diffusion out of the target
428 region, short receptor-binding kinetics, fast local clearance due to the injection method,
429 and the intrinsic self-sustaining mechanisms of ED. Our speculation regarding these
430 mechanisms is partly supported by previous reports that TRPV4-negative hippocampal
431 neurons or astrocytes have lower excitability of postsynaptic cells in the glutamatergic

nervous system and lower extracellular glutamate concentrations in astrocytes. [8,10,12] In addition to exploring the differential roles of TRPV4 in neurons and glial cells and its downstream circuit mechanisms, research on the pharmacokinetics of RN 1734 in the brain is required.

When these ANOVA results are interpreted in the context of glutamate regulation, they support the hypothesis that astrocytic, rather than purely neuronal, mechanisms contribute to the phase-specific role of TRPV4 during seizures. Astrocytic TRPV4 channels are known to be activated by activity-dependent changes such as ionic imbalance, cell swelling, and mechanical stress, which progressively intensify during ongoing epileptiform activity. The presence of a significant group - time interaction only after seizure onset is therefore consistent with the notion that astrocytic TRPV4-mediated glutamate release becomes functionally relevant during the propagation and maintenance phases of seizures, promoting pathological extracellular glutamate accumulation rather than triggering seizure initiation.

The limitations of this study include the fact that the group sizes of 4–6 mice per experiment were underpowered to account for the biological variability observed in the electrophysiological recordings and glutamate assays. Second, a vehicle group using 10% DMSO was used as the solvent for the TRPV4 antagonist; however, a sham group

using physiological saline was not established. Therefore, the potential influence of DMSO cannot be completely excluded, and the results should be interpreted with caution.

Third, a large volume of DMSO was required because of the poor solubility of RN 1734. Although we kept the infusion rate very low to minimize pressure-related injury, a total intracranial injection volume of 2 μl in the mouse brain was large and may induce local edema, cellular damage, or nonspecific drug diffusion, potentially confounding the electrophysiological results. To develop TRPV4 antagonists for clinical use, we must present histological evidence demonstrating that the injection does not cause significant tissue damage, undertake tracer experiments to delineate the actual spread of the injected solution, and produce data on drug solubility.

Fourth, owing to differences in solubility, distribution, and duration, other selective TRPV4 antagonists (citral, RN-9893, and GSK2193874) may yield more sustained suppression after seizure onset. Future studies should directly compare these compounds across dose–response and timing paradigms.

Conclusion

TRPV4 inactivation suppressed EDs and reduced seizure severity in a cortical focal epilepsy model. The effects of TRPV4 inhibition were dependent on the timing of intervention, with significant modulation of glutamate dynamics and beta-band activity during ongoing PG-induced seizures, and limited but significant differences observed at the peak phase following pre-treatment.

Acknowledgments

We would llike to thank Prof. Makoto Tominaga, who supplied TRPV4KO mice from the Thermal Biology Group, Exploratory Research Center on Life and Living Systems (ExCELLS), Okazaki, Japan. We would also like to thank Editage (www.editage.jp) for providing excellent assistance with the English language editing.

Funding Statement:

This work was supported by Grants-in-Aid for Scientific Research C (grant no. 22K09235 to S.N.) and Grants-in-Aid for Young Scientists (21K15269 and 23K14356 to H.M.) from the Japan Society for the Promotion of Science (JSPS).

Ethics Statement:

All animal experiments were approved by the Experimental Animal Care and Use Committee of Yamaguchi University School of Medicine, Japan (not applicable) and conducted in accordance with the Declaration of Helsinki (as revised in Brazil 2013) and institutional guidelines.

Conflict of Interest;

The authors declare no conflict of interest.

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585

Figure Legends

Fig. 1 Suppressive effect of TRPV4 deficiency on PG-induced EDs.

(A) Experimental protocol. Mice were anesthetized with urethane. The power of the beta-band in ECoGs was investigated for 5 min during the basal activity (25-30 min) and the pre-(85-90 min) and post-(90-95 min) PG injection periods. Changes in ED in WT mice injected with (B) PG + 10% DMSO (n = 6), (C) PG + 1 mM RN 1734 (n = 6), (D) PG + 3 mM RN 1734, and (E) PG + 10 mM RN 1734. Changes in ED in TRPV4 KO mice injected with (F) PG + 10% DMSO (n = 6) and (G) PG + 10 mM RN 1734. The WT and KO mice were compared for (H) heart rate and (I) brain temperature. (J) Summary of the investigation of beta-band bands at baseline (white) and with pre-injection (orange) and post-injection (green). The results are shown as means ± standard errors of the mean.

*p<0.05, **p<0.01, Student's t-test, †p<0.05, ††p<0.01, †††p<0.001, Dunnet test, §p<0.05, Wilcoxon test, x symbols: individual data points.

NS, not significant; TRPV4, transient receptor potential vanilloid 4; PG, penicillin G; EDs, epileptic discharge; ECoG, electrocorticogram; RN 1734, TRPV4 antagonist; WT, wild-type mice; KO, knockout.

Fig. 2 Correlation between the power of beta-band bands and extracellular glutamate concentration.

(A) Experimental protocol. Mice were anesthetized with urethane. The power of beta-band in ECoGs and the extracellular glutamate concentration were investigated every 5 min (0-100 min); during the basal activity (5-10 min) and the pre-(75-80 min) and post-(80-85 min) PG injection periods. Shown are the changes in EDs, a spectrum view of beta-band power, and the extracellular glutamate concentration in WT mice (n = 4) treated with (B) PG + 10 mM RN 1734 (n = 4), and in TRPV4 KO mice (n = 5) treated with (C) PG + 10 mM RN 1734. The WT and KO mice were compared for (D) beta-band power, (E) extracellular glutamate concentration, and (F) maximum glutamate concentration every 5 min. (G) Beta-band power and (H) extracellular glutamate concentrations were measured before (orange) and after (green) RN 1734 injection in WT and KO mice. (I) Correlation between the power of beta-band bands and extracellular glutamate concentration in WT (white round) and KO (blue triangle) mice. The results are shown as mean± standard error of the mean; *$p<0.05$, **$p<0.01$, Student's t-test, #$p<0.05$, Mann Whitney U test, NS, no significant; r^2, the square of the Pearson product-moment correlation coefficient, x symbols: individual data points.

622 Fig. 3 Correlation between extracellular glutamate concentration and seizures.

623 (A) Experimental protocol. Mice were anesthetized with sevoflurane. The beta-band

624 power in ECoGs and the extracellular glutamate concentration were investigated every

625 5 min (0-100 min) during the basal activity (5-10 min) and the pre-(75-80 min) and

626 post-(80-85 min) PG injection periods. Shown are the changes in EDs and a spectrum

627 view of beta-band power and the extracellular glutamate concentration in WT mice

628 treated with (B) 10% DMSO + PG (n = 3), (C) 10 mM RN 1734 + PG (n = 3), and

629 TRPV4 KO mice treated with (D) 10% DMSO + PG (n = 4). (E) Beta-band power (F)

630 and extracellular glutamate concentrations were measured every 5 min in WT mice

631 treated with 10% DMSO (black) and 10 mM RN 1734 (grey), as well as in TKPV4 KO

632 mice treated with 10 % DMSO (blue). (G) The ratio of pre-PG injection to post-PG

633 injection extracellular glutamate concentration, (H) spike amplitude, and (I) seizure

634 scores were measured in WT mice treated with 10% DMSO (white) and 10 mM RN

635 1734 group (grey), as well as in TKPV4 KO mice treated with 10 % DMSO (blue). (J)

636 The correlation between spike amplitude and the ratio of pre-PG injection to post-PG

637 injection in extracellular glutamate concentration was measured in WT (white round)

638 and KO (blue triangle) mice.

639 The results are shown as mean ± standard error of the mean; *p<0.05, **p<0.01,

640 ***p<0.001, Student's t-test, #p<0.05, Mann Whitney U test, ††p<0.01, †††p<0.001,

641 Dunnett-t test, NS, not significant; r^2, the square of the Pearson product-moment

642 correlation coefficient, x symbols: individual data points.

Fig. 1

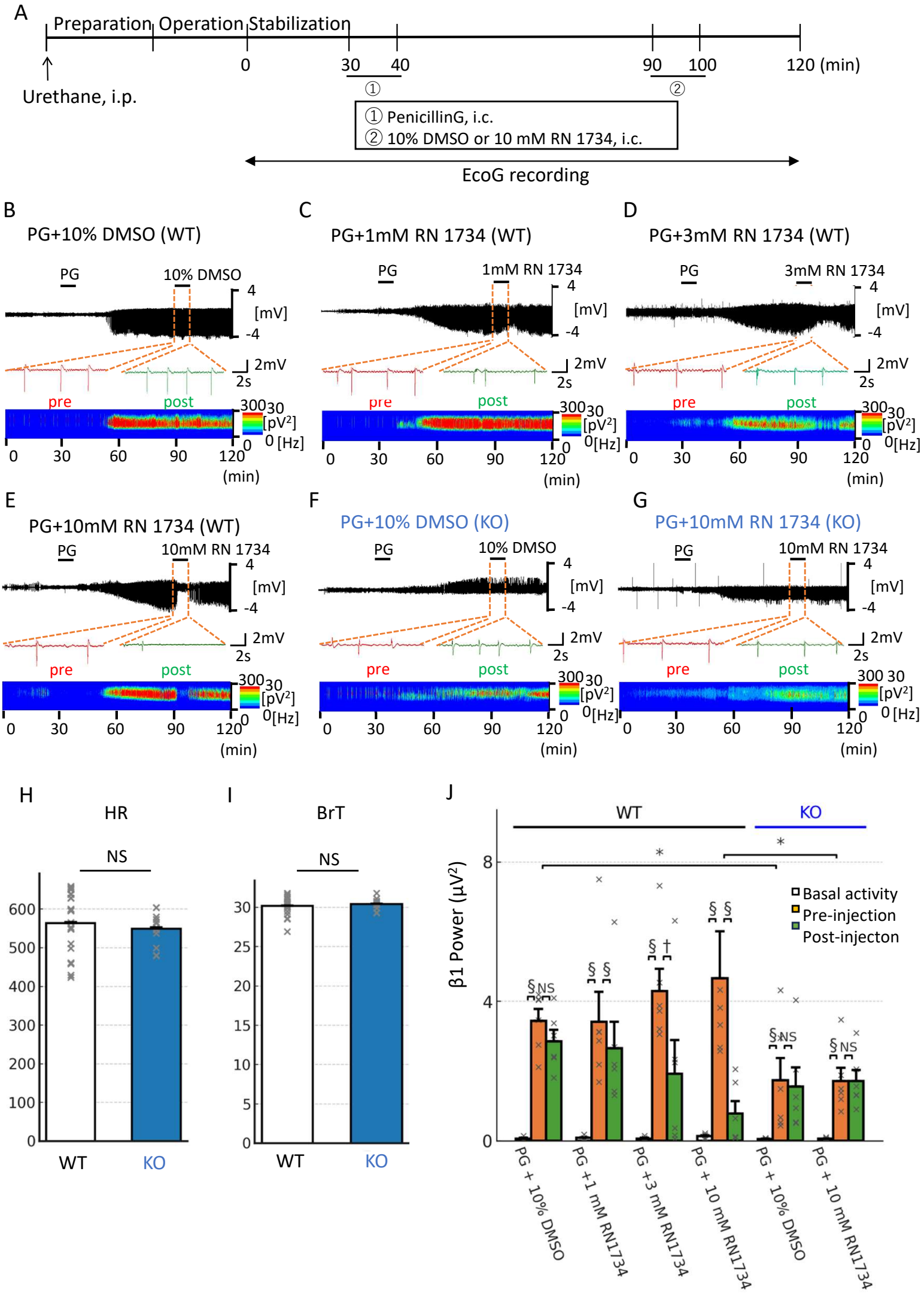

A
Preparation
Operation
Stabilization
0
30
40
90
100
120 (min)
Urethane, i.p.
① PenicillinG, i.c.
② 10% DMSO or 10 mM RN 1734, i.c.
EcoG recording
B
PG+10% DMSO (WT)
C
PG+1mM RN 1734 (WT)
D
PG+3mM RN 1734 (WT)
E
PG+10mM RN 1734 (WT)
F
PG+10% DMSO (KO)
G
PG+10mM RN 1734 (KO)
PG
10% DMSO
1mM RN 1734
3mM RN 1734
10mM RN 1734
pre
post
[mV]
2mV
2s
[pV²]
[Hz]
(min)
H
HR
I
BrT
NS
WT
KO
J
WT
KO
β1 Power (μV²)
Basal activity
Pre-injection
Post-injecton
PG + 10% DMSO
PG +1 mM RN1734
PG +3 mM RN1734
PG + 10 mM RN1734
PG + 10% DMSO
PG + 10 mM RN1734

Fig. 2

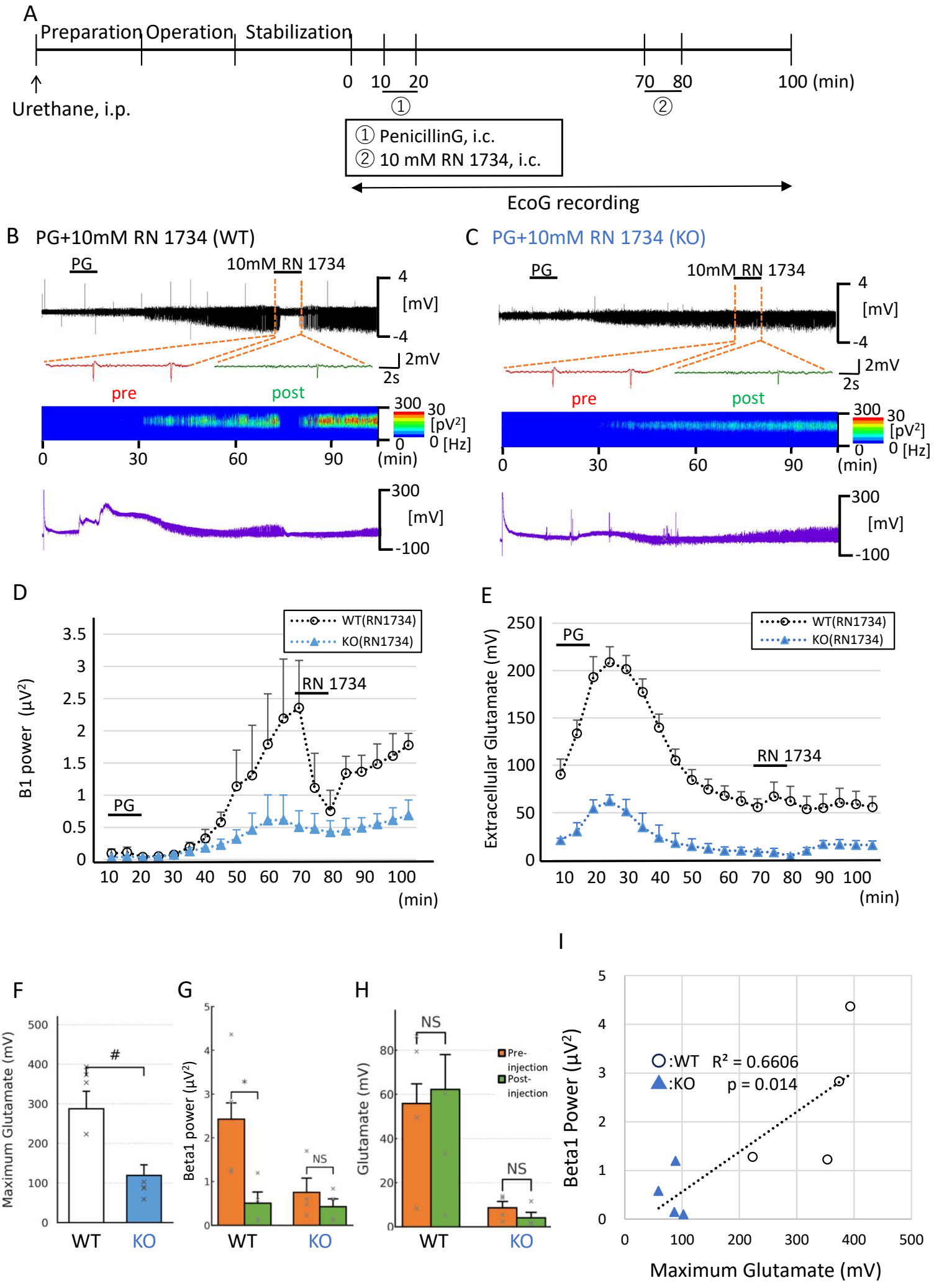
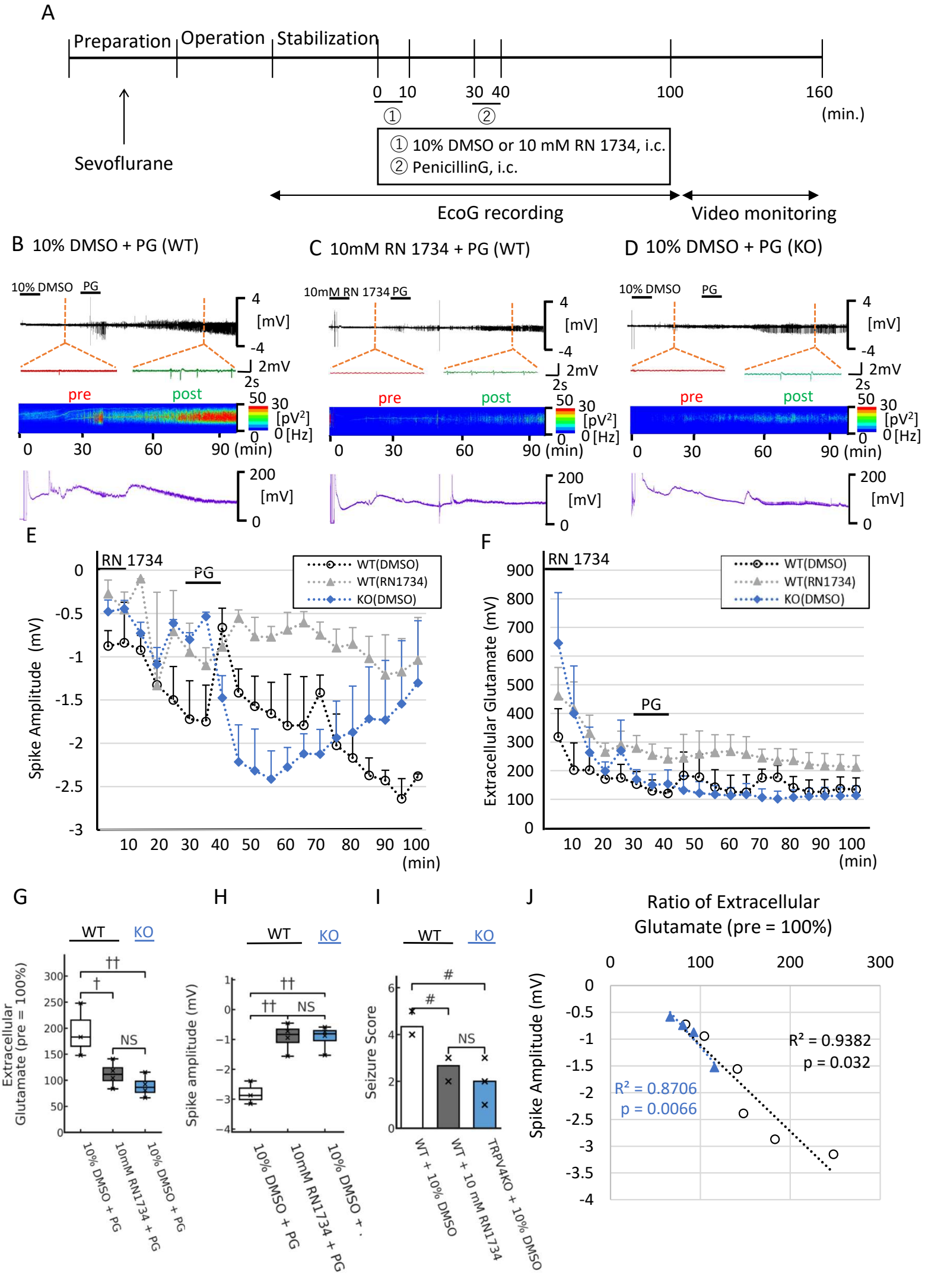

A
Preparation
Operation
Stabilization
Urethane, i.p.
0
10
20
70
80
100 (min)
① PenicillinG, i.c.
② 10 mM RN 1734, i.c.
EcoG recording
B PG+10mM RN 1734 (WT)
C PG+10mM RN 1734 (KO)
PG
10mM RN 1734
4
[mV]
-4
2mV
2s
pre
post
300
30
[pV²]
0 [Hz]
0
30
60
90
(min)
300
[mV]
-100
D
E
WT(RN1734)
KO(RN1734)
RN 1734
B1 power (μV²)
Extracellular Glutamate (mV)
(min)
F
G
H
I
Maximum Glutamate (mV)
Beta1 power (μV²)
Glutamate (mV)
Pre-injection
Post-injection
NS
#
*
WT
KO
:WT R² = 0.6606
:KO p = 0.014
Beta1 Power (μV²)
Maximum Glutamate (mV)

Fig. 3


A
Preparation
Operation
Stabilization
0
10
30
40
100
160
(min.)
①
②
Sevoflurane
① 10% DMSO or 10 mM RN 1734, i.c.
② PenicillinG, i.c.
EcoG recording
Video monitoring
B 10% DMSO + PG (WT)
C 10mM RN 1734 + PG (WT)
D 10% DMSO + PG (KO)
10% DMSO
PG
10mM RN 1734
4
[mV]
-4
2mV
2s
pre
post
50
30
[pV²]
0 [Hz]
0
30
60
90 (min)
200
[mV]
0
E
RN 1734
PG
WT(DMSO)
WT(RN1734)
KO(DMSO)
Spike Amplitude (mV)
0
-0.5
-1
-1.5
-2
-2.5
-3
10
20
30
40
50
60
70
80
90
100
(min)
F
RN 1734
PG
WT(DMSO)
WT(RN1734)
KO(DMSO)
Extracellular Glutamate (mV)
900
800
700
600
500
400
300
200
100
0
10
20
30
40
50
60
70
80
90
100
(min)
G
WT
KO
Extracellular Glutamate (pre = 100%)
300
250
200
150
100
50
0
††
†
NS
10% DMSO + PG
10mM RN1734 + PG
10% DMSO + PG
H
WT
KO
Spike amplitude (mV)
1
0
-1
-2
-3
-4
††
††
NS
10% DMSO + PG
10mM RN1734 + PG
10% DMSO + .
I
WT
KO
Seizure Score
6
4
2
0
#
#
NS
WT + 10% DMSO
WT + 10 mM RN1734
TRPV4KO + 10% DMSO
J
Ratio of Extracellular Glutamate (pre = 100%)
Spike Amplitude (mV)
0
100
200
300
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
R² = 0.9382
p = 0.032
R² = 0.8706
p = 0.0066