FK506 causes pain by upregulating $Na_V1.7$ channels in the spinal dorsal root ganglia of $Na_V1.7$–ChR2 mice

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Complete List of Authors:	Maruta, Toyoaki; Kumamoto University Faculty of Life Sciences School of Medicine, Anesthesiology; University of Miyazaki, Anesthesiology Shiraishi, Seiji; Hiroshima University, Community Medicine for Anesthesiology Kouroki, Satoshi; Miyazaki Daigaku, Anesthesiology Kurogi, Mio; Miyazaki Daigaku Hirata, Naoyuki; Kumamoto University Faculty of Life Sciences School of Medicine, Anesthesiology
Keywords:	FK506, tacrolimus, calcineurin inhibitor-induced pain syndrome, dorsal root ganglion, Na_V1.7, optogenetics
Abstract:	Calcineurin inhibitors, including tacrolimus (FK506), are used as immunosuppressive agents and can cause unexplained calcineurin inhibitor-induced pain syndrome (CIPS). We investigated how FK506 affects the expression of $Na_V1.7$, a voltage-gated Na^+ channel implicated in pain perception that is upregulated in dorsal root ganglion (DRG) neurons in several pain disorders. We generated a model of FK506-induced pain by administering FK506 to $Na_V1.7$–ChR2 mice, which exhibit light-responsive pain. To evaluate nociceptive responses, paw withdrawal threshold (PWT) was measured using the von Frey test. The optogenetic place aversion (OPA) and light irradiation paw withdrawal tests were also performed. On the 11th day of initial injection, DRGs were dissected from mice under anesthesia and analyzed for *$Na_V1.7$* expression using quantitative reverse transcription PCR (RT-qPCR). PWT was also measured for mice that received the selective $Na_V1.7$ inhibitor or vehicle. PWT was lower in FK506-treated mice than in those administered the vehicle on the 8th and 12th days after initial injection ($P < 0.05$). Mechanical hypersensitivity was reversible and peaked at around 10 days after FK506 administration. OPA and light irradiation paw withdrawal test results corroborated the hypersensitivity to light-responsivity. *$Na_V1.7$* mRNA levels in DRG were higher in FK506-treated mice than in those administered the vehicle on the 11th day ($P < 0.05$). A selective $Na_V1.7$ inhibitor reversed FK506-induced pain. Increased *$Na_V1.7$* expression in DRG neurons may be responsible for FK506-induced peripheral neuropathy. Our findings suggest that endogenous calcineurin regulates *$Na_V1.7$* expression. Thus, selective $Na_V1.7$ inhibition could be a potential therapeutic strategy for CIPS.

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Toyoaki Maruta[1,2*¶], Seiji Shiraishi[3¶], Satoshi Kouroki[2], Mio Kurogi[2], and Naoyuki Hirata[1]

[1] Department of Anesthesiology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan

[2] Department of Anesthesiology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

[3] Division of Community Medicine for Anesthesiology, Hiroshima University, Hiroshima, Japan

*Corresponding author

Toyoaki Maruta, Department of Anesthesiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Tyuoku, Kumamoto 860-8556, Japan

E-mail: mmctm2@yahoo.co.jp

¶ These authors contributed equally to this work

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Author Contributions

TM and SS designed the experiments. TM, SK, and MK performed the experiments and analyzed the data. TM and SS drafted the manuscript. NH and SS supervised the experimental approach and corrected the manuscript. All authors read and approved the final manuscript.

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Abstract

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Introduction

Calcineurin inhibitors, including tacrolimus (FK506) and cyclosporine, are commonly employed as immunosuppressive agents, particularly in transplantation medicine. Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase that regulates a multitude of physiological processes, including ion channel activity and immune function.[1,2] It is expressed at high levels in T cells and the nervous system, including the spinal dorsal horn and dorsal root ganglion (DRG).[1,2] Primary sensory neurons in the DRG receive signals produced by peripheral nerve endings that then incorporate and transmit them to the spinal cord.

The use of calcineurin inhibitors is associated with unexplained severe pain, often referred to as calcineurin inhibitor-induced pain syndrome (CIPS), which is characterized by burning and episodic severe pain sensitivity in the lower extremities of patients.[2–8] Although rare, CIPS is increasingly being recognized as a serious complication caused by calcineurin inhibitors. In animal CIPS models, calcineurin inhibitors have been reported to induce pain hypersensitivity via activation of synaptic *N*-methyl-D-aspartate (NMDA) receptors.[1,9] Despite the use of Ca^{2+} channel blockers and gabapentinoids as analgesics,[10] the molecular mechanism underlying CIPS remains unclear and its treatment is challenging.

Voltage-gated sodium channels (VGSCs) are crucial for electrogenesis in excitable cells. $Na_V1.7$, a VGSC subtype encoded by *SCN9A*, plays a critical role in pain signal transduction in humans.[11–17] Genetic studies have recognized $Na_V1.7$ dysfunction in human pain disorders. Inherited gain-of-function missense mutations in $Na_V1.7$ occur in primary erythromelalgia,[13,17–19] and recessively inherited loss-of-function mutations in *SCN9A* result in channelopathy-associated insensitivity to pain.[13–15,20–22] $Na_V1.7$ is selectively expressed in

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DRG neurons and sympathetic ganglia, particularly being abundantly expressed in small-diameter DRG neurons and preferentially expressed in nociceptors and during evoked action potential firing in Aβ- and C-fibers.[23–28] $Na_V1.7$ is also implicated in pain perception in small animal models of pain. $Na_V1.7$ expression is elevated in the DRG neurons of diabetic neuropathy,[29] chronic constrictive injury (CCI),[27] and paclitaxel-induced peripheral neuropathy rat models.[30,31]

In a previous study, we demonstrated that treatment of cultured bovine adrenal chromaffin cells with FK506 or cyclosporine increased $Na_V1.7$ expression.[32,33] Furthermore, erythromelalgia has been reported in patients receiving cyclosporine.[34,35,36] Based on these findings, we aimed to investigate whether CIPS is involved in the upregulation of $Na_V1.7$ in DRG neurons in a FK506-induced pain model, which was generated in light-responsive pain ($Na_V1.7$–ChR2) mice previously developed by us.[37–39] This study provides novel information about the contribution of $Na_V1.7$ to CIPS.

Materials and Methods

Animal characteristics and pharmacological treatments

$Na_V1.7$–ChR2 mice, weighing approximately 25–30 g, were used in this study. These mice were generated as previously described.[37–39] All the mice were individually housed in a temperature- and humidity-controlled environment with a 12-h light–dark cycle and permitted free access to food and water. This study was conducted in strict accordance with the guidelines for the Proper Conduct of Animal Experiments (Science Council of Japan) and approved by the Experimental Animal Care and Use Committee (2024-511). Male mice, aged 2–6 months, were used. All efforts were made to minimize the number of animals used and

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their suffering. Mice in each group were randomly selected, and the experimenter blinded to the mouse group.

The experimental protocol is illustrated in Figure 1. We used a FK506-induced neuropathic pain model reported by Huang et al.[40] FK506 (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in dimethyl sulfoxide (DMSO) and phosphate-buffered saline at 0.3 mg/mL. FK506 (3 mg/kg) was intraperitoneally (i.p.) administered to mice daily for one week under 2–3% sevoflurane anesthesia. Mice in the vehicle group were i.p. injected with the solvent vehicle (30% DMSO) daily for one week. The von Frey test was performed before and after (1, 4, 8, 12, 16, 20, and 24 days) FK506 or vehicle injection. On the 11th day after initial injection, the mice were decapitated after inhalational sevoflurane-induced anesthesia, and their DRGs then dissected. *$Na_V1.7$* expression was measured using reverse transcription-PCR (RT-PCR). The optogenetic place aversion (OPA) test was simultaneously performed with the von Frey test. The light irradiation test was performed before FK506 injection and on the 11th day after initial FK506 injection. To determine the analgesic effects of DS-1971a, a selective $Na_V1.7$ inhibitor, the von Frey test was performed before FK506 injection, as well as before and 2 h after DS-1971a or vehicle (0.5% methylcellulose) administration on the 11th day after initial FK506 injection.

Estimation of mechanical sensitivity using the von Frey test

Mechanical sensitivity was examined by determining the paw withdrawal threshold (PWT) using an electronic von Frey esthesiometer (IITC Life Science Inc., Woodland Hills, CA, USA) fitted with a polypropylene tip. Each adult mouse was placed in a 10 cm × 10 cm suspended chamber with a metallic mesh floor. After acclimating the mice for 30 min, the

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polypropylene tip was perpendicularly applied to the plantar surface of the right and left hind paws with sufficient force for 3–4 s. Brisk withdrawal or paw flinching was considered a positive response. The pain threshold was calculated as the mean of three measurements.

The analgesic effect of DS-1971a on FK506-induced neuropathic pain was determined using the von Frey test. One side of the hind paws of mice was tested for sensitivity to mechanical stimulus before FK506 injection, as well as before and 2 h after DS-1971a or vehicle administration on the 11th day after initial FK506 injection. DS-1971a (10 and 100 mg/kg) in 0.5% methylcellulose or a vehicle (0.5% methylcellulose) was orally administered. The settings for DS-1971a administration were previously determined in a preliminary study.[41]

RT-PCR of DRG samples

Following euthanasia with sevoflurane, DRG samples from each mouse were obtained and dissected. Total cellular RNA was isolated from homogenized DRG samples via acid guanidinium thiocyanate-phenol-chloroform extraction using TRIzol reagent (Total RNA Isolation Reagent; Invitrogen, Carlsbad, CA, USA). The quality and quantity of the extracted RNA were assessed based on the optical density ratio at 260 and 280 nm measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). We obtained 500–1000 ng/μL RNA from DRG samples and used 2 μg total RNA to synthesize the cDNA template. RT-PCR was performed in a 20-μL reaction mixture using a first-strand cDNA synthesis kit (SuperScript II Reverse Transcriptase; Invitrogen), following the manufacturer's instructions. PCR amplification was then performed on a thermal cycler (Veriti Thermal Cycler; Thermo Fisher Scientific) in a 20-μL reaction mixture containing

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EmeraldAmp MAX PCR Master Mix (TAKARA Bio Inc., Shiga, Japan), 1 μL (estimated 100 ng) cDNA template, and 0.4 μM forward and reverse primers. The following primers synthesized by Macrogen Global Headquarters (Seoul, Korea) were used for the PCR assays: $Na_V1.7$-forward (5′-agatgcaacagcctctacca-3′), $Na_V1.7$-reverse (5′-gagtttggcatagacctccgt-3′), β-actin-forward (5′-cgtaaagacctctatgccaaca-3′), and β-actin-reverse (5′-cggactcatcgtactcctgct-3′). The PCR protocol comprised an initial denaturation step (10 min at 95°C), followed by 35 cycles (10 s at 98°C, 30 s at 60°C, and 60 s at 72°C) for $Na_V1.7$ and 27 cycles (10 s at 98°C, 30 s at 55°C, and 60 s at 72°C) for β-actin, and a final extension step (90 s at 72°C). The PCR products were separated via electrophoresis on a 2% agarose gel, and the bands visualized using a LAS-4000 lumino image analyzer (Fujifilm, Tokyo, Japan).

Assessment of aversive behavior

Aversive behavior upon optogenetic stimulation was assessed using an OPA system (Bioresearch Center, Nagoya, Japan),[37–39] which consisted of two chambers (20 cm × 24 cm) connected through an entrance. Each chamber floor was lit by a 20 × 24 array of LEDs of two different colors—green (530 nm) and blue (470 nm). To eliminate bias due to the natural preference for dark environments, both chambers were uniformly illuminated at a power of 7 mW during the test. After habituating the mice to the chambers for 10 min with the LEDs switched off, each mouse was allowed to move freely for a further 10 min in the chambers with the LED switched on. The position of each mouse while the LEDs were turned on was recorded using a video camera and analyzed with BIOBSERVE Viewer 2 software. The percentage of time spent in each chamber during the 10-min observation period was determined.

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Light irradiation test

To determine light-responsive hypersensitivity due to FK506-induced hyperalgesia, a light irradiation paw withdrawal test[37–39] was performed before FK506 injection and on the 11th day after initial FK506 injection. Mice were habituated for 1 h in transparent cubicles (10 cm × 6.5 cm × 6.5 cm) set atop a 5 mm-thick glass floor and separated from each other with opaque dividers. Acute nocifensive behaviors were elicited using a pulsing LED light (465 nm blue light at 10 Hz; Doric Lenses Inc., Quebec, Canada) set at different intensities and aimed at the plantar surface of the hind paw. Light intensity was determined using a light power meter (LPM-100). As the power meter measures light intensity in mW, the light density in mW/mm^2 was calculated by dividing the light intensity by the illuminated area in square millimeters (48 mm^2). The mice underwent a total of five trials of 1 s each, with 5-s intervals between trials. The percentage of trials during which hind paw withdrawal or paw licking occurred was recorded.

Experimental design and statistical analysis

Each behavioral experiment was performed for $n \geq 10$ animals, and RT-PCR performed for $n = 5$ animals. Data were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's HSD test. The results are presented as mean ± standard deviation (SD). Statistical significance was set at $P < 0.05$. The statistical software, JMP Pro 17 (SAS Institute, Inc., Cary, NC, USA) for Macintosh, was used for the analyses.

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Results

Mechanical hyperalgesia induced by FK506 treatment

To examine whether FK506 treatment induces mechanical hyperalgesia, we performed the von Frey test. As shown in Figure 2, compared with the vehicle group, significant mechanical hypersensitivity was observed in FK506-treated mice on the 8th and 12th days after initial FK506 injection ($P < 0.05$). This hypersensitivity was reversible and peaked between the 8th and 12th days after initial FK506 injection.

Upregulation of $Na_V1.7$ *expression by FK506 treatment*

To confirm the upregulation of *$Na_V1.7$* expression upon FK506 treatment, we examined *$Na_V1.7$* mRNA levels in the DRGs of FK506- or vehicle-treated mice (Figure 3). *$Na_V1.7$* mRNA levels in the FK506-treated group were significantly upregulated on the 11th day after initial FK506 injection compared with those in the vehicle-treated group ($P = 0.007$). On the 24th day, the levels were significantly reduced compared with those measured on the 11th day ($P = 0.01$).

Optogenetic behavior test

As increased *$Na_V1.7$* expression is expected to be accompanied by upregulated expression of the light-responsive channel, channelrhodopsin 2 (ChR2), in $Na_V1.7$−ChR2 mice (which are light-responsive pain mice), we verified the hypothesis that enhanced light-responsivity leads to stronger nociceptive pain upon light exposure. To investigate the change in light-responsivity due to FK506 treatment, we performed OPA and light irradiation hind paw withdrawal tests. As shown in the OPA test (Figure 4a), the time spent by FK506-treated

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mice in the blue floor room was significantly shorter than that spent by the vehicle group mice on the 8th and 12th days after initial FK506 injection, which was the same time when peak mechanical hypersensitivity was observed in the von Frey test ($P < 0.05$).

The FK506-treated mice were subjected to a light irradiation hind paw withdrawal test before and after (on the 11th day after initial FK506 injection) FK506 treatment. Figure 4b shows the leftward shift of the light intensity-withdrawal response curve due to FK506 treatment, indicating that the FK506 treatment made the mice hypersensitive to light.

Analgesic effect of DS-1971a on FK506-induced neuropathic pain

To investigate the analgesic effect of DS-1971a on FK506-induced neuropathic pain, we performed the von Frey test before FK506 injection (Pre), as well as before and 2 h after DS-1971a or vehicle administration on the 11th day after initial FK506 injection. At 10 and 100 mg/kg, DS-1971a completely relieved FK506-induced mechanical hypersensitivity (Figure 5).

Discussion

As previously reported,[40] FK506 treatment resulted in the induction of reversible neuropathic pain (Figure 2). Mechanical hypersensitivity peaked at around the 10th day after initial FK506 administration. Furthermore, as observed in previous in vitro studies,[32,33] *$Na_V1.7$* expression was elevated in DRGs during the onset of neuropathic pain (Figure 3). FK506-induced pain could be effectively treated with a selective $Na_V1.7$ inhibitor (Figure 5). These findings suggest that increased $Na_V1.7$ expression plays a pivotal role in the pathogenesis of FK506-induced pain.

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Cyclosporine and FK506 form a complex with the immunophilins, cyclophilin A and FK506-binding protein 12 kDa (FKBP12), thereby inhibiting the phosphatase activity of calcineurin[42] and consequently preventing the dephosphorylation of transcription factors belonging to the nuclear factor of activated T cells (NFAT) family in T cells. Dephosphorylation is essential for the nuclear translocation of NFAT, which in turn activates genes encoding various cytokines, including interleukin-2.

Although uncommon, severe pain symptoms induced by calcineurin inhibitors, termed CIPS, are characterized by burning and episodic severe pain sensitivity in the lower extremities, frequently accompanied by distress during standing and walking.[7,8] In studies on CIPS model animals, gabapentinoids (α2δ-1 inhibitors),[40] glutamate NMDA receptor (NMDAR) antagonists,[9] and casein kinase-2 (CK2) inhibitors[1] have been demonstrated to restore pain sensitivity. This may be due to calcineurin inhibition enhancing the activity of presynaptic and postsynaptic NMDARs in the spinal dorsal horn.[9,40,43] The α2δ-1 subunit forms a complex with phosphorylated NMDARs and enhances their activity.[40,43] CK2, a serine/threonine protein kinase, enhances NMDAR activity similar to effect of calcineurin.[1,43] Gabapentinoids, including pregabalin and gabapentin, are clinically employed for CIPS treatment.[2,4–8] Despite evidence suggesting that calcineurin also regulates voltage-gated Ca^{2+} and TRPV1 channels, their association with CIPS remains unproven.[43]

Our findings indicate that FK506 induces *$Na_V1.7$* expression in the DRG. This is the first study to demonstrate the involvement of VGSC in an FK506-induced pain model. In clinical practice, selective $Na_V1.7$ inhibitors may prove effective for CIPS treatment. Previous genetic studies have indicated that $Na_V1.7$ is a key player in the processing of human pain, and it has thus become a focus in research as a therapeutic target for pain

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treatment.[13,15,16,44] $Na_V1.7$ expression was reported to increase in animal models of inflammation, diabetes, and CCI,[27,29,45] and a selective $Na_V1.7$ inhibitor could reduce inflammatory and neuropathic pain in mice.[16,41,46,47] Our results provide the first direct evidence that FK506 induces a significant increase in *$Na_V1.7$* expression in DRGs. Furthermore, we examined nociceptive behavior after administering DS-1971a, a selective $Na_V1.7$ inhibitor.[41] PWT was significantly increased after FK506 administration, highlighting the potential of $Na_V1.7$ inhibitors as new targets for CIPS treatment.

The expression of VGSCs is regulated by a variety of mediators. $Na_V1.7$ expression is reportedly affected by TNF-α levels and extracellular signal-regulated kinase phosphorylation in the DRGs.[29,48,49] In addition, nerve growth factor and glial cell-derived neurotrophic factor can upregulate the expression of Na^+ channels in the DRG.[50] These findings suggest that $Na_V1.7$ is involved in the FK506-mediated induction of neuropathic pain. Further studies are required to characterize the mechanisms underlying the upregulation of dorsal ganglionic $Na_V1.7$ after FK506 administration.

In the present study, we demonstrated that light-responsive hypersensitivity occurs at the onset of neuropathic pain using a light-responsive pain mouse model (Figure 4). This is likely not solely attributable to the increased *$Na_V1.7$* expression observed; the design of genetic modification in $Na_V1.7$–ChR2 mice may likely result in an increase in ChR2 expression occurring concurrently with the increase in $Na_V1.7$ levels.[36–38] This finding indicates that $Na_V1.7$–ChR2 mice can be used to screen for changes in the expression of *$Na_V1.7$*.

This study had several limitations. First, although sex-related differences in pain threshold may exist, we did not focus on these differences in the current study; we

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customarily used male mice, as was done in previous reports.[9,38,41] Second, we concluded that FK506-induced $Na_V1.7$ upregulation contributes to pain induction based on the increased $Na_V1.7$ mRNA levels detected via RT-PCR, enhanced light-responsive pain expected from $Na_V1.7$ upregulation, and attenuation of FK506-induced pain by a $Na_V1.7$ inhibitor. Although additional data obtained from western blotting analysis or voltage-clamp recordings would provide multifaceted confirmation of $Na_V1.7$ upregulation, these were not performed in the present study. Third, we demonstrated $Na_V1.7$ upregulation using a mouse model that induces pain with FK506 administration; however, we considered that this cannot be directly applied to the pathogenesis of CIPS in humans. Further research, including clinical studies, is necessary to elucidate the pathogenesis of CIPS in humans. Fourth, calcineurin is a dephosphorylating enzyme; therefore, its inhibition maintains protein phosphorylation. Phosphorylation of $Na_V1.7$ or other molecules is likely involved in CIPS. However, the present study did not investigate these possibilities.

Conclusion

We found that $Na_V1.7$ was upregulated in the DRG of FK506-induced pain mice, and that its inhibition attenuated FK506-induced hyperalgesia. These findings provide new insights into the physiological function of calcineurin in pain transmission via the regulation of $Na_V1.7$ at the DRG level. This information advances our understanding of the molecular mechanisms underlying CIPS and may help in developing a new strategy to deal with CIPS.

Statements and Declarations

Ethical considerations

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This study was conducted in strict accordance with the guidelines for the Proper Conduct of Animal Experiments (Science Council of Japan) and approved by the Experimental Animal Care and Use Committee.

Consent to participate

Not applicable

Consent for publication

Not applicable

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1. *In vivo* experimental design. (a) FK506 or a vehicle (30% dimethyl sulfoxide [DMSO]) was intraperitoneally (i.p.) injected into mice daily for one week. The von Frey test was performed before and after (1, 4, 8, 12, 16, 20, and 24 days) injecting FK506 or the vehicle. On the 11th day after initial FK506 or vehicle injection, dorsal root ganglia (DRGs) were dissected from mice in each group, and $Na_V1.7$ expression measured using reverse transcription PCR (RT-PCR). The optogenetic place aversion (OPA) test was simultaneously performed with the von Frey test. A light irradiation test was performed before FK506 injection and on the 11th day after initial FK506 injection. (b) To determine the analgesic effects of DS-1971a, the von Frey test was performed before FK506 injection, as well as before and 2 h after administering DS-1971a or a vehicle (0.5% methylcellulose) on the 11th day after initial FK506 injection.

Figure 2. Paw withdrawal test (von Frey test). The von Frey test was performed before (Pre) and after (1, 4, 8, 12, 16, 20, and 24 days) injecting FK506 or a vehicle (30% dimethyl sulfoxide [DMSO]). The hind paw withdrawal data were analyzed using two-way analysis of variance, (ANOVA) followed by Tukey's HSD test. All results are presented as mean ± standard deviation (SD) for 10 or more animals. *$P < 0.05$, compared with the vehicle group.

Figure 3. Reverse transcription PCR (RT-PCR) for $Na_V1.7$ mRNA expression in dorsal root ganglion (DRG). $Na_V1.7$ mRNA expression in DRG neurons measured using RT-PCR. β-actin was used as a positive control to confirm successful mRNA extraction and equal loading of samples. Relative levels of $Na_V1.7$ mRNA/β-actin mRNA are shown. Data were analyzed

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using two-way analysis of variance (ANOVA), followed by Tukey's HSD test, and presented as mean ± standard deviation (SD) for five animals.

Figure 4. Optogenetic place aversion (OPA) and light irradiation hind paw withdrawal tests. (a) The OPA test was performed before (Pre) and after (1, 4, 8, 12, 16, 20, and 24 days) injecting FK506 or a vehicle. Length of stay in the blue light floor room (%) was analyzed using an unpaired *t*-test. All results are presented as mean ± standard deviation (SD) for 10 or more animals. **P* < 0.05, compared with the vehicle group. (b) The blue light irradiation hind paw withdrawal test was performed before (Pre-FK506) and after (Post-FK506; on the 11th day after FK506 initial injection) FK506 treatment. Red arrows indicate a leftward shift of the curve due to FK506 treatment. Data were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's HSD test. All results are presented as mean ± standard deviation (SD) for 10 or more animals. **P* < 0.05, compared with Pre-FK506.

Figure 5. Analgesic effect of DS-1971a on FK506-induced neuropathic pain. The von Frey test was performed before FK506 injection (Pre), as well as before and 2 h after administering DS-1971a or a vehicle (0.5% methylcellulose) on the 11th day after initial FK506 injection. Data were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's HSD test. All data are presented as mean ± standard deviation (SD) for 10 animals. $^{*}P < 0.05$, compared with Pre; $^{\dagger}P < 0.05$, compared with the vehicle group.

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Fig 1

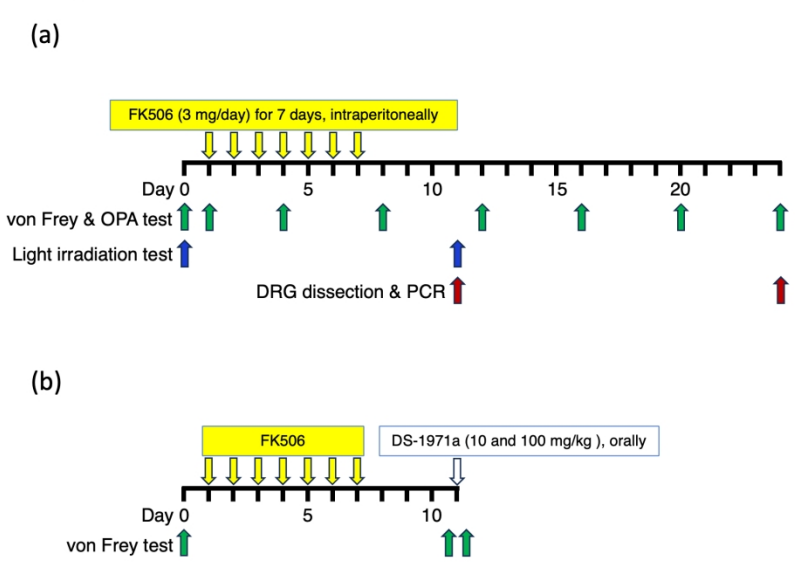

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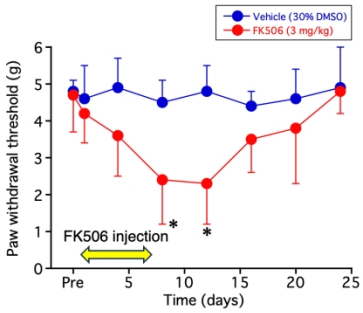
Fig 2



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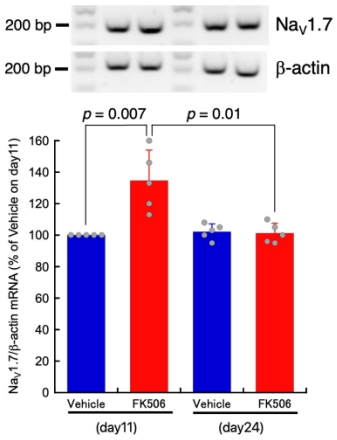

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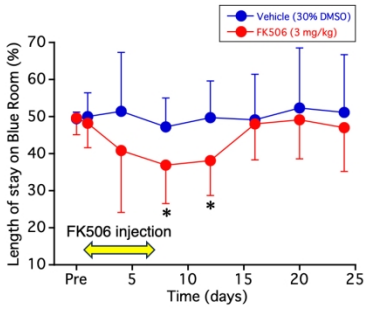
Fig 4

(a) OPA test



(b) Light irradiation hind paw withdrawal test

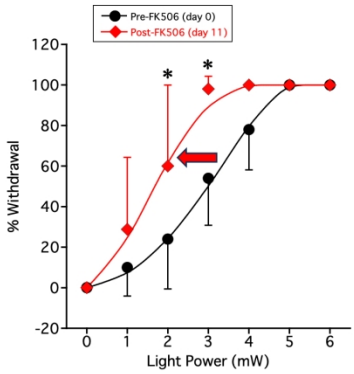


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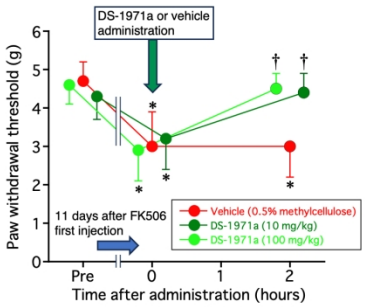
Fig 5



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