Supplementary Information for

Vesicular nucleotide transporter is a molecular target of eicosapentaenoic acid for neuropathic and inflammatory pain treatment

Yuri Kato, Kengo Ohsugi, Yuto Fukuno, Ken Iwatsuki, Yuika Harada, and Takaaki Miyaji

*Takaaki Miyaji
Email: miyaji-t@okayama-u.ac.jp

This PDF file includes:

- Materials and Methods
- Figures S1 to S5
- Legend for Movie S1
- SI References

Other supplementary materials for this manuscript include the following:

- Movie S1
Supplementary Information

Materials and Methods

Chemicals

Lipid metabolites were purchased from Cayman Chemical (Ann Arbor, MI, USA). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were dissolved in ethanol to a concentration of 250 mg/mL and diluted with saline to a concentration of 0.1 mg/mL for in vivo experiments. The EPA metabolites were provided by Prof. Hirotaka Imai and Dr. Taro Sakamoto at Kitasato University. ATP disodium salt and ADP disodium salt were purchased from Sigma Aldrich (St. Louis, MO, USA), dissolved to 200 mM in 40 mM MOPS-Tris (pH 7.0) buffer, and diluted with saline for in vivo experiments. Pregabalin and duloxetine were purchased from Sigma Aldrich and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively, and dissolved and diluted with saline for in vivo experiments. DAMGO and CTOP were purchased from Sigma Aldrich and Abcam (Cambridge, UK) respectively, dissolved in ethanol (stock concentrations: 5 mg/mL and 2 mg/mL, respectively), and diluted with saline for in vivo experiments. The control experiments were conducted by adding the same concentration of ethanol corresponding to the lipid metabolites in the reaction mixture, Krebs-Ringer bicarbonate buffer, and saline.

Complementary DNA (cDNA)

Human vesicular nucleotide transporter (VNUT) (Accession No. NM001302643.1) cDNAs were cloned using polymerase chain reaction and confirmed using DNA sequencing.

Δψ Measurement by fluorescence quenching

We assayed Δψ (inside-positive) by measuring the fluorescence quenching of oxonol V (Sigma Aldrich), as previously described (1). The reaction mixture (450 µL) consisting of 1 µg of protein incorporated into proteoliposomes, 20 mM MOPS-Tris (pH 7.0), 140 mM potassium acetate, 5 mM magnesium acetate, 10 mM KCl, and 1 µM oxonol V was incubated for 50 s at 27°C. The reaction was initiated by adding 2 µM valinomycin in the absence or presence of EPA and was terminated by adding 2 µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Control activity was then subtracted from the quench in the presence of CCCP.

Neurotransmitter release from neurons

Rat fetal hippocampal neurons were isolated and cultured at 2.0 × 10^5 cells per 3.5-cm dish in Neurobasal medium (GIBCO, Waltham, MA, USA) supplemented with 0.5 mM glutamine, 100 units/mL of penicillin, 100 µg/mL of streptomycin, 0.25 µg/mL of fungizone, and B27 supplement (GIBCO) in 5% CO2/95% air at 37°C, as previously described (1, 2). The cultured neurons were washed three times with Krebs-Ringer bicarbonate buffer composed of 128 mM NaCl, 1.9 mM KCl, 1.2 mM KH2PO4, 1.3 mM MgSO4, 26 mM NaHCO3, 10 mM D-glucose, 10 mM HEPES-NaOH (pH 7.4), 2.4 mM CaCl2, and 0.2% (w/v) bovine serum albumin. After the incubation of cells in Krebs-Ringer bicarbonate buffer at 37°C for 3 h, 55 mM KCl was added to stimulate neurotransmitter release. After incubation at 37°C for 20 min, Krebs-Ringer bicarbonate buffer was collected and centrifuged. The supernatant content of ATP was measured using an ATP bioluminescence assay kit (Sigma Aldrich), and those of glutamate, aspartate, GABA, and glycine were measured by ultra-high-performance liquid chromatography using Accucore™-150-C18 (2.1 mm × 150 mm, 2.6 µm; Thermo Fisher Scientific, Waltham, MA, USA) and fluorescence detection.

Surgery for partial sciatic nerve ligation

C57BL/6 mice (male, 7–9 weeks old, 22–30 g at the time of the test) underwent unilateral ligation of approximately half of the sciatic nerve high in the thigh, as previously described (3).
The von Frey test
C57BL/6 mice were acclimatized to an elevated metal mesh floor chamber (10.0 × 16.0 × 9.0 cm) for 60 min before the von Frey test. Mechanical hyperalgesia was assessed by measuring the left hind-paw withdrawal response to stimulation with a series of von Frey filaments (0.04–2.0 g; Aesthesio) presented perpendicular to the plantar surface (1). We determined the 50% paw withdrawal threshold using Dixon’s up-down method (4).

Plantar test
C57BL/6 mice were acclimatized to an elevated acrylic observation chamber (14.0 × 17.0 × 11.0 cm) for 60 min before the plantar test. Heat hyperalgesia was assessed using a Hargreaves radiant heat apparatus (Ugo Basile, Lombardy, Italy) (1). The heat source, a mobile infrared photobeam, was positioned under the plantar surface of the left hind paw. The cutoff was set to 20 s to prevent tissue damage in untreated control mice.

Insulin tolerance test
C57BL/6 mice (male, 7–17 weeks old, 20–32 g at the time of the test) were fasted for 4 h and were injected intravenously with saline or 1 mg/kg EPA. The mice were immediately injected intraperitoneally with 0.75 units of human insulin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)/kg body weight. The blood glucose level was measured using a Glucose Pilot System (Iwai Chemicals Company Ltd., Tokyo, Japan). Insulin sensitivity (% of control) was estimated by the ratio of blood glucose before and after insulin injection.
Fig. S1. Structure of polyunsaturated fatty acids (PUFAs) used in the inhibitor screening
Fig. S2. Time course of the effects of eicosapentaenoic acid (EPA) and EPA ethyl ester on neuropathic pain

(A-B) Von Frey test performed in a time-dependent manner after intravenous injection of saline (yellow circles), EPA (A) (red circles), or EPA ethyl ester (B) (red circles) in wild-type mice at 3 d after the paclitaxel injection (n = 5–6). Control experiments used saline + 0.04% and 0.1% ethanol corresponding to the solvent comprising 1 mg/kg EPA and EPA ethyl ester, respectively. Data represent mean ± standard error of the mean. *P < 0.05, **P < 0.01 (one-way analysis of variance followed by Dunnett’s test).
**Fig S3. Eicosapentaenoic acid (EPA) prodrug also attenuates neuropathic pain**

EPA ethyl ester is a prodrug, which is metabolized to EPA and ethanol by carboxylesterase in most cells, to facilitate absorption and distribution of EPA. Von Frey test was performed 1 h after the intravenous injection of saline or EPA ethyl ester at the indicated concentrations in wild-type mice at 3 d after paclitaxel injection, n = 5 mice. The control experiment used saline + 0.1% ethanol corresponding to the solvent comprising 1 mg/kg EPA ethyl ester. Data represent mean ± standard error of the mean. **P < 0.01 (one-way analysis of variance followed by Dunnett's test).
Fig S4. Mu opioid receptor antagonist does not inhibit eicosapentaenoic acid (EPA)-evoked analgesic effect

(A) Von Frey test was performed 15 min (time of maximal effect) after the intrathecal injection of saline or 5 ng DAMGO at 3 d after intraperitoneal injection of paclitaxel in wild-type mice, n = 6–7. (B) The von Frey test was performed 45 min (time of the maximal effect) after the intravenous injection of saline or 1 mg/kg EPA at 3 d after intraperitoneal injection of paclitaxel in wild-type mice, n = 6–10. The 1 ng CTOP was intrathecally injected 10 min before intrathecal injection of DAMGO or intravenous injection of EPA in wild-type mice. The control experiment used saline + 0.04% ethanol corresponding to the solvent of 1 mg/kg EPA. Data represent mean ± standard error of the mean. *P < 0.05, **P < 0.01; NS, not significant (one-way ANOVA followed by Dunnett’s test or two-tailed paired Student’s t-test).
Fig S5. Eicosapentaenoic acid (EPA) attenuates nerve injury-induced neuropathic pain via VNUT inhibition

(A) Von Frey test performed at the indicated time after intravenous injection of saline or EPA at the indicated concentrations in wild-type mice and vesicular nucleotide transporter (VNUT)$^{-/-}$ mice at 10 d after nerve injury, $n = 7–8$ mice. (B) Von Frey test was performed 45 min after the intravenous injection of saline or EPA at the indicated concentrations in wild-type mice and VNUT$^{-/-}$ mice at 10 d after nerve injury, $n = 7–8$ mice. The control experiment used saline + 0.04% ethanol corresponding to the solvent of 1 mg/kg EPA. Data represent mean ± standard error of the mean. **$P <0.01$; NS, not significant (one-way ANOVA followed by Dunnett's test or two-tailed paired Student's $t$-test).
Legend for Movie S1

Movie S1. Eicosapentaenoic acid (EPA) causes no change in locomotor activity

The movie was recorded 45 min (saline or 1 mg/kg EPA; the first half), 1 h (1 mg/kg EPA ethyl ester; the second half), 3 h (1 mg/kg pregabalin), or 1 h (30 mg/kg duloxetine) after intravenous injection of the indicated compounds in wild-type mice, as the times of maximal effect.

SI References

1. Y. Kato et al., Identification of a vesicular ATP release inhibitor for the treatment of neuropathic and inflammatory pain. Proc. Natl Acad. Sci. U. S. A. 114, E6297–E6305 (2017).
2. G. A. Banker, W. M. Cowan, Rat hippocampal neurons in dispersed cell culture. Brain Res. 126, 397–442 (1977).
3. Z. Seltzer, R Dubuner, Y. Shir, A novel behavioral model of neuropathic pain disorder produced in rats by partial sciatic nerve injury. Pain 43, 450–456 (1990).
4. W. J. Dixon, Efficient analysis of experimental observations. Annu. Rev. Pharmacol. Toxicol. 20, 441–462 (1980).