Zonisamide Enhances Neurite Elongation of Primary Motor Neurons and Facilitates Peripheral Nerve Regeneration *In Vitro* and in a Mouse Model

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Abstract

No clinically applicable drug is currently available to enhance neurite elongation after nerve injury. To identify a clinically applicable drug, we screened pre-approved drugs for neurite elongation in the motor neuron-like NSC34 cells. We found that zonisamide, an anti-epileptic and anti-Parkinson's disease drug, promoted neurite elongation in cultured primary motor neurons and NSC34 cells in a concentration-dependent manner. The neurite-scratch assay revealed that zonisamide enhanced neurite regeneration. Zonisamide was also protective against oxidative stress-induced cell death of primary motor neurons. Zonisamide induced mRNA expression of nerve growth factors (BDNF, NGF, and neurotrophin-4/5), and their receptors (tropomyosin receptor kinase A and B). In a mouse model of sciatic nerve autograft, intragastric administration of zonisamide for 1 week increased the size of axons distal to the transected site 3.9-fold. Zonisamide also improved the sciatic function index, a marker for motor function of hindlimbs after sciatic nerve autograft, from 6 weeks after surgery. At 8 weeks after surgery, zonisamide was protective against denervation-induced muscle degeneration in tibialis anterior, and increased gene expression of *Chrne*, *Colq*, and *Rapsn*, which are specifically expressed at the neuromuscular junction. We propose that zonisamide is a potential therapeutic agent for peripheral nerve injuries as well as for neuropathies due to other etiologies.

Introduction

Motor paralyses due to damage to the peripheral and central nervous systems, especially those due to motor nerve damage, cause serious disabilities in ADL (activities of daily living), but no

effective treatment has been established for this condition. Local administration of neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), induces the axonal elongation of motor nerves and ameliorates motor deficits in model animals [1–4]. Similarly, laminins and cadherin-11 promote the axonal elongation of motor neurons [5–8]. These molecules, however, cannot be easily used in clinical settings, because local administration would be the only available option and their half-lives in our body are very short [9–11]. Recently, cell transplantation from different sources has been gaining attention as a therapy for nerve damage and therapeutic effects have been reported in model animals [12–18]. Cell transplantation is thus likely to be one of the promising modalities to treat motor nerve damage, if safety concerns are addressed [19]. Small molecule compounds that were reported to improve motor nerve damage include ibuprofen, a non-steroidal anti-inflammatory drug [20]; valproic acid, an anti-epileptic drug [21]; and Y27632, an inhibitor of Rho-associated kinase (ROK) [22]. Although these are promising compounds, none has yet been available in clinical settings.

Drug repositioning is a strategy for discovering new efficacies of existing drugs whose dosage, administration method, safety margin, adverse effects, etc., are already known, which enables fast application of the drug in clinical practice. Drug repositioning may drastically reduce the cost and time of drug development compared to conventional approaches [23, 24].

In this study, we screened 1,186 FDA-approved drugs to identify a clinically applicable compound that promotes neurite elongation of cultured motor neurons. Firstly, we performed extensive phenotypic screening with NSC34, a neuroblastoma and spinal motor neuron hybrid cell line. We then examined whether candidate compounds identified by the first screening promoted neurite elongation of mouse primary motor neurons. We finally identified that zonisamide, an anti-epileptic and anti-parkinsonian drug, enhanced neurite elongation and increased the number of branch points in primary motor neurons. Zonisamide also improved the viability of H_2O_2-treated primary motor neurons and induced mRNA expression of *Bdnf*, *Ngf*, *Ntf4*, *Ntrk1*, and *Ntrk2*. In addition, zonisamide enhanced nerve regeneration in a mouse model of sciatic nerve autograft. We propose that zonisamide is a promising compound for peripheral nerve injuries and neuropathies due to other etiologies.

Materials and Methods

Ethics statement

All animal experiments were approved by the Animal Care and Use Committee of Nagoya University. Mice were used to isolate primary spinal motor neurons at embryonic day 13.5 (E13.5) and to examine the effect of intragastric administration of zonisamide after sciatic nerve autograft.

Culture of NSC34 cells

NSC34 cells (mouse neuroblastoma-spinal cord hybrid cells displaying a multipolar motor neuron-like phenotype) that stably express the doxycycline-induced green fluorescent protein (GFP) construct (NSC34-pTetR12-TO/GFP) [25] were kindly provided by Dr. Shinsuke Ishigaki, Department of Neurology, Nagoya University Graduate School of Medicine. NSC34 cells were cultured in a humidified atmosphere of 95% air-5% CO_2 in a 37°C incubator in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific). Cells were seeded at 1.6×10^3 cells/well on a 96-well plate with the differentiation medium of DMEM/F12 (Invitrogen) containing 1% FBS and 2 mg/ml doxycycline to induce GFP expression. On day 1, the differentiation medium was changed to DMEM/F12 (Invitrogen) containing 1% NEAA (Non-Essential Amino Acid, MP Biomedicals)

and 2 mg/ml doxycycline, and the cells were incubated for 48 additional hours in the presence of 10 µM of 1,186 FDA-approved chemical compounds (Prestwick Chemical). Then, the nuclei of the cells were stained with the fluorescent dye Hoechst 33342 (1:100,000, Sigma Aldrich), and images of 16 fields per well were automatically taken with the ArrayScan VTI HCS Reader (Thermo Scientific Cellomics). The average neurite lengths in each well were automatically analyzed with the Neuronal Profiling v4.0 BioApplication (Thermo Scientific Cellomics).

Culture of primary spinal motor neurons

Primary cultures of mouse embryonic spinal motor neurons were dissociated from embryonic spinal cord at E13.5 of C57BL/6J mice. Zonisamide was first dissolved in 100% dimethyl sulfoxide (DMSO) to make 200 µM to 2 mM solutions, and then added to the culture medium at a final concentration of 1 to 20 µM. Culture media with or without zonisamide all contained 0.5% DMSO. Cells were seeded at 4.0×10^3 cells/well on a 96-well plate coated with poly-L-lysine and laminin (Asahi Techno Glass), and maintained in Sumilon neuron culture medium (Sumitomo Bakelite). Cells were incubated for 2 days and were fixed for 15 min with 4% para-formaldehyde in phosphate-buffered saline (PBS) at room temperature. Cells were washed in PBS once, blocked by 2% goat serum and 0.1% Triton-X in PBS, and then immunostained with mouse anti-tau-1 monoclonal antibody (1:500, MAB3420, Millipore) and the Alexa Fluor 555 goat anti-mouse secondary antibody (1:1000, Life Technologies). Neurites were automatically analyzed with the ArrayScan VTI HCS Reader, as stated above. The 200 longest neurites were considered to quantify neurite length and the number of neurite branch points. In addition, the number of cells with neurite length > 25 µm was counted in each well to calculate the ratio of neurite-bearing cells.

Scratch assay

Primary motor neurons were seeded at 8.0×10^4 cells/cm^2 $\times$ 0.7 cm^2/well in the Nun Lab-Tek Chamber Slide (Thermo Scientific) coated with poly-L-lysine, and were cultured in Sumilon neuron culture medium for 48 h. Neurites of primary motor neurons were wounded with a linear scratch by a 200-µl sterile pipette tip. The cells were immediately rinsed with PBS and cultured for 48 additional hours in Sumilon neuron culture medium with 0, 1, 10, or 20 µM zonisamide. The cells were then fixed in 4% paraformaldehyde and immunostained with neuron-specific ß-III tubulin monoclonal antibody (1:1000, MAB1195, R&D systems) and with the Alexa Fluor 488 goat anti-mouse secondary antibody (Life Technologies). Images of the scratched areas were taken with a microscope (Olympus LX71), and the length of regenerated neurites of the primary motor neurons at the scratched area was automatically measured by the MetaMorph software (Universal Imaging).

Time-lapse imaging of axonal elongation of primary motor neurons

Primary motor neurons were seeded at 6.0×10^3 cells in each well of a 96-well plate and cultured in Sumilon neuron culture medium to induce neurite elongation. Phase contrast microscopic images were automatically taken using the IncuCyte ZOOM Live Cell Imaging System (Essen Bioscience) every 8 h for 3 days. Neurite length was automatically analyzed with the IncuCyte's NeuroTrack software.

MTS assay

Cell viability was estimated with the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega), which represents mitochondrial reductase activity. Primary motor

neurons were initially cultured in Sumilon neuron culture medium for 24 h, and then switched to DMEM/F12 with 0.5% of FBS for 24 h. Variable concentrations (2 to 20 μM) of zonisamide were added to the medium. After 1 h, cells were exposed to 100 μM H_2O_2 for 24 h. The cells were incubated with MTS reagent for 2 h and MTS signals were quantified with a microplate reader (PowerScan HT, DS Pharma Biomaterial). MTS signals were normalized to those without H_2O_2 treatment.

Total RNA extraction and real-time RT-PCR analysis

Total RNA was isolated using Trizol (Thermo Fisher Scientific) from primary motor neurons cultured with 0, 1, or 10 μM zonisamide. Total RNA was similarly isolated from a 3-mm segment of the sciatic nerve, and from the tibialis anterior muscle. First strand cDNA was synthesized with ReverTra Ace (Toyobo). We quantified mRNA expression of *Bdnf*, *Ngf*, *Ntf4*, *Ntrk1*, *Ntrk2*, *Map2*, *Mapt*, *Gap43*, *Chrne*, *Colq*, and *Rapsn* using LightCycler 480 Real-Time PCR (Roche) and SYBR Green (Takara). mRNA levels were normalized to *Gapdh*. The primer sequences are shown in S1 Table.

Western blotting

Zonisamide (0, 10, 20 μM) was added to primary motor neurons 4 h after starting the cultures. The cells were lysed on day 2 or 3 with a buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0, 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 100 mM NaF, 10 mM sodium pyrophosphate, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate. Total proteins were dissolved in 1x Laemmli buffer, separated on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel, and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membrane was washed in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and blocked for 1 h at room temperature in TBS-T with 3% skim milk. The membrane was incubated overnight at 4°C either with an antibody (dilution 1:1000) for anti-Erk1/2 (#4696, Cell Signaling), anti-phosphorylated Erk1/2 (#4370, Cell Signaling), anti-JNK1/2/3 (#9926, Cell Signaling), or anti-phosphorylated JNK1/2/3 (#4668, Cell Signaling). The membrane was washed three times for 10 min with TBS-T and incubated with a secondary mouse anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP, GE Healthcare, 1:6000) for 1 h at room temperature. The blots were detected with the Amersham ECL Western blotting detection reagent (GE Healthcare) and quantified with the Image J program (http://imagej.nih.gov/ij/index.html).

Mouse model of sciatic nerve autograft

Adult male C57BL/6J mice (8 weeks old; 19.5 to 22.0 g) were purchased from Charles River. Mice were anesthetized with isoflurane and the left sciatic nerve was exposed through gluteal muscle incision under sterile conditions. The sciatic nerve was transected at two sites: (i) 3 mm distal to the first branch entering into biceps femoris (the proximal transected site) and (ii) 3 mm further distal to the proximal site (the distal transected site). The excised 3-mm nerve segment was moved aside to confirm complete dissection at two sites, then placed back to the original position. Perineuriums were sutured with 10–0 black nylon thread at the proximal and distal sites under microscopy. The remaining thread was later used as a marker to locate the transected sites when the mice were sacrificed. To reduce the toxicity of DMSO, we first made 60 mg/ml zonisamide in 100% DMSO, and then diluted it in olive oil to make 3 mg/ml zonisamide in 5% DMSO. Control solution (5% DMSO) or 30 mg/kg/day zonisamide (3 mg/ml x

~0.2 ml) was intragastrically administered using a disposable sonde needle (flexible type, Fuchigami) once a day starting from a day after surgery up to 1 or 8 weeks later when the mice were sacrificed. We analyzed myelination of sciatic nerves in six mice in each group. A 6-mm nerve segment was excised by cutting at the proximal transected site and at 3 mm distal to the distal transected site 1 week after the sciatic nerve autograft. Then the fibers were fixed in 2% paraformaldehyde/2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.3 overnight at 4°C, postfixed in 2% osmium tetroxide for 3 h, and dehydrated in ascending concentrations of ethanol. After Epon embedding, semithin cross sections (1 μm) were obtained 0.7 mm distal to the distal transected site, stained with alkaline Toluidine blue (Sigma), and examined under a microscope (FSX100, Olympus). The number and area of myelinated axons in the sciatic nerve were manually counted using Image J software in a blinded manner.

Motor functions were assessed every week in three mice in each group by the walking track analysis, in which footprints of both hindlimbs were recorded when mice were allowed to walk freely on a runway [26]. Sciatic function index (SFI) was calculated by measuring the width and length of footprints of both legs in a blinded manner. If there is no difference between the cut and uncut hindlimbs, the SFI is 0, whereas the SFI is -100 when the cut hindlimb is completely paralyzed [27]. Before taking footprint records, the mice were trained to walk through the run-way several times. Footprints were recorded on six to eight sheets each time. Eight weeks after the sciatic nerve autograft, the whole gastrocnemius muscle and a 3 mm sciatic nerve segment from the distal transected site to 3 mm further distal to it were isolated for extraction of total RNA. Similar specimens were isolated from the uncut hindlimb. Tibialis anterior muscles were also isolated and fixed with 100% cooled methanol for hematoxylin and eosin staining.

Statistical analysis

Unpaired Student's t-test, one-way or two-way ANOVA, and post-hoc Tukey honestly significant difference (HSD) test were performed by SPSS ver. 21 (IBM). P values of 0.05 or less were considered statistically significant.

Results

Zonisamide enhances neurite elongation of NSC34 motor neuron-like cells

To search for off-label effects of pre-approved drugs on neurite elongation of GFP-expressing NSC34 cells, we added 10 μM of 1,186 pre-approved drugs to NSC34 cells that were induced to differentiate into neurons. After 48 h, the average neurite lengths were automatically measured using the Cellomics Array Scan System. Each drug was tested four to sixteen times. A limited number of drugs after the first round of screening were added to primary motor neurons isolated from the spinal cords of C57/BL6 mice at E13.5. The cells were fixed after 48 h of culture, and the axons were fluorescently immunostained for Tau, followed by automatic measurement of the neurite lengths using the ArrayScan VTI HCS Reader. We found that zonisamide consistently elongated neurites of primary motor neurons (S1 Fig).

We next looked into the dose response effects of zonisamide on neurite elongation of spinal motor neurons. We found that zonisamide enhanced axonal elongation in a concentration dependent manner in NSC34 cells (Fig 1A) and primary motor neurons (Fig 1B). Additionally, the number of branch points in the axons of primary motor neurons was also increased in a dose-dependent manner (Fig 1C). However, the outgrowth ratios of primary motor neurons, which were the ratios of neurite-bearing neurons, were not enhanced by zonisamide treatment (Fig 1D).
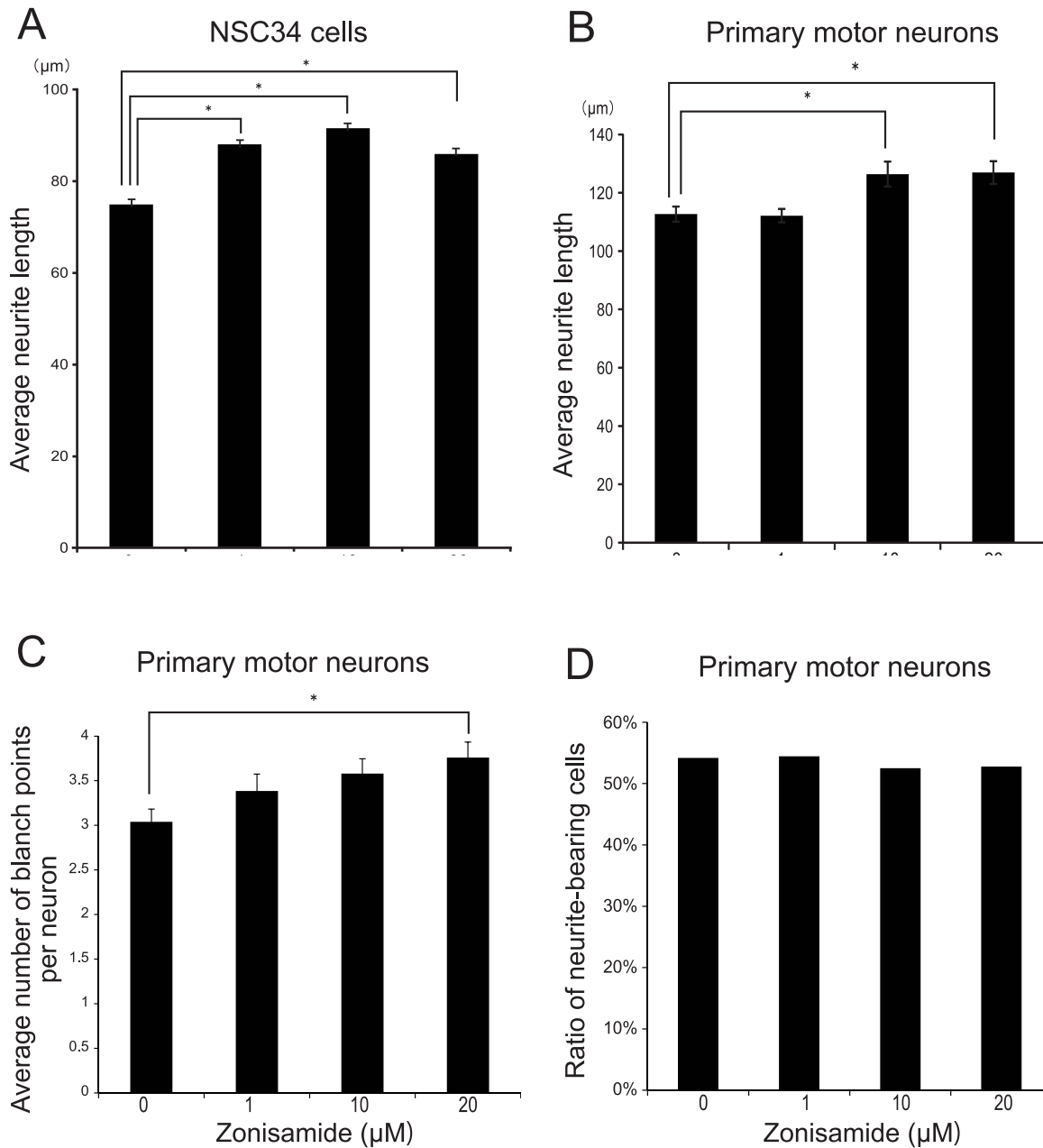
Fig 1. Zonisamide increases neurite length and the number of branch points in NSC34 cells and mouse primary spinal motor neurons. (A, B)
Mouse NSC34 motor neuron-like cells **(A)** and primary motor neurons isolated from the spinal cord of mouse embryos at E13.5 **(B)** were differentiated for 48 h in the presence of the indicated concentrations of zonisamide. The neurite lengths of more than 200 cells were automatically measured with the ArrayScan. *$p < 0.01$ by one-way ANOVA followed by Tukey HSD. **(C)** The number of branch points of more than 200 differentiation-induced primary motor neurons was automatically measured with the ArrayScan. *$p < 0.01$ by one-way ANOVA followed by Tukey HSD. **(D)** The number of differentiation-induced primary motor neurons with neurite lengths of more than 20 µm was divided by the total number of cells under the microscope to calculate the ratio of neurite-bearing cells. No statistical significance was observed by one-way ANOVA. Mean and SE are indicated for all the panels.

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Zonisamide enhances neurite regeneration of primary motor neurons

To determine whether zonisamide enhances regeneration of axons, a scratch assay was conducted using mouse primary motor neurons. Primary motor neurons were differentiated for 48 h to extend neurites. A network of neurites on the culture plate was linearly scratched off

with a 200-µl sterile pipette tip. Variable concentrations of zonisamide (0, 1, 10, or 20 µM) were added to the medium and the lengths of the neurites that elongated into the cutouts were automatically quantified with MetaMorph image analysis software. The lengths of the regenerated neurites elongated into the cutouts were increased in a dose-dependent manner (Fig 2). Zonisamide was thus able to enhance neurite regeneration in primary motor neurons.

Zonisamide is unlikely to initiate neurite outgrowth, but is able to enhance neurite elongation in primary motor neurons

After zonisamide was administered to mouse primary motor neurons, the temporal profile of the axonal elongation was automatically traced with the IncuCyte ZOOM Live Cell Imaging System (Fig 3). Zonisamide had no overt effect up to 40 h after its administration. However, zonisamide-treated cells exhibited the neurite elongation effect after 40 h. We observed that 10 µM zonisamide had a greater effect than 1 µM throughout the observation period. These results suggest that zonisamide has an enhancing effect on neurite elongation rather than on the initiation of neurite outgrowth.

Zonisamide is protective against oxidative stress in primary motor neurons

Primary motor neurons were treated with zonisamide for 1 h and then exposed to 100 µM H_2O_2 for 24 h to induce oxidative stress-mediated cell death. Subsequently, the number of viable primary motor neurons was quantified with an MTS assay. Zonisamide increased the number of viable cells in a dose-dependent manner (Fig 4). Zonisamide thus provided a neuroprotective effect against oxidative stress in primary motor neurons.

Zonisamide upregulates expression of neurite elongation-related genes

We added 0, 1, 10, or 20 µM zonisamide to primary motor neurons and evaluated mRNA expression levels by quantitative RT-PCR. Zonisamide increased the mRNA levels of the neurotrophins *Bdnf*, *Ngf*, and *Ntf4* on days 2 and 3, although dose-response effects were not always observed (Fig 5). The mRNA levels of the receptors *Ntrk1* (a receptor for NGF) and *Ntrk2* (a receptor for BDNF and NT4-4/5) were also upregulated by zonisamide, again with equivocal dose-response effects. mRNA levels of the structural proteins *Map2* (enriched in dendrites), *Mapt* (enriched in axons), and *Gap43* (enriched in the growth cone), however, were not upregulated by zonisamide up to day 3. These results suggest that zonisamide induces expression of neurotrophins and their receptors.

We also looked into phosphorylation of Erk1/2 and JNK1/2/3, and found that zonisamide suppressed phosphorylation of Erk1/2 in NSC34 cells and primary motor neurons (S2 Fig). In contrast, zonisamide minimally enhanced phosphorylation of JNK1/2/3 in NSC34 cells, but had no effect in primary motor neurons (S2 Fig).

## Zonisamide facilitates nerve regeneration in a mouse model of sciatic nerve autograft

To examine the effects of zonisamide on nerve regeneration *in vivo*, we made a mouse model of sciatic nerve autograft by transecting and suturing the left sciatic nerve at two sites that were 3 mm apart. Zonisamide (30 mg/kg/day) was intragastrically administered to the model mice every day from a day after surgery. One week after surgery, analysis of a cross section of the sciatic nerve 0.7 mm distal to the distal transected site demonstrated that the number of axons remained unchanged but the area of axons was increased 3.9-fold (Fig 6A–6D). We also looked
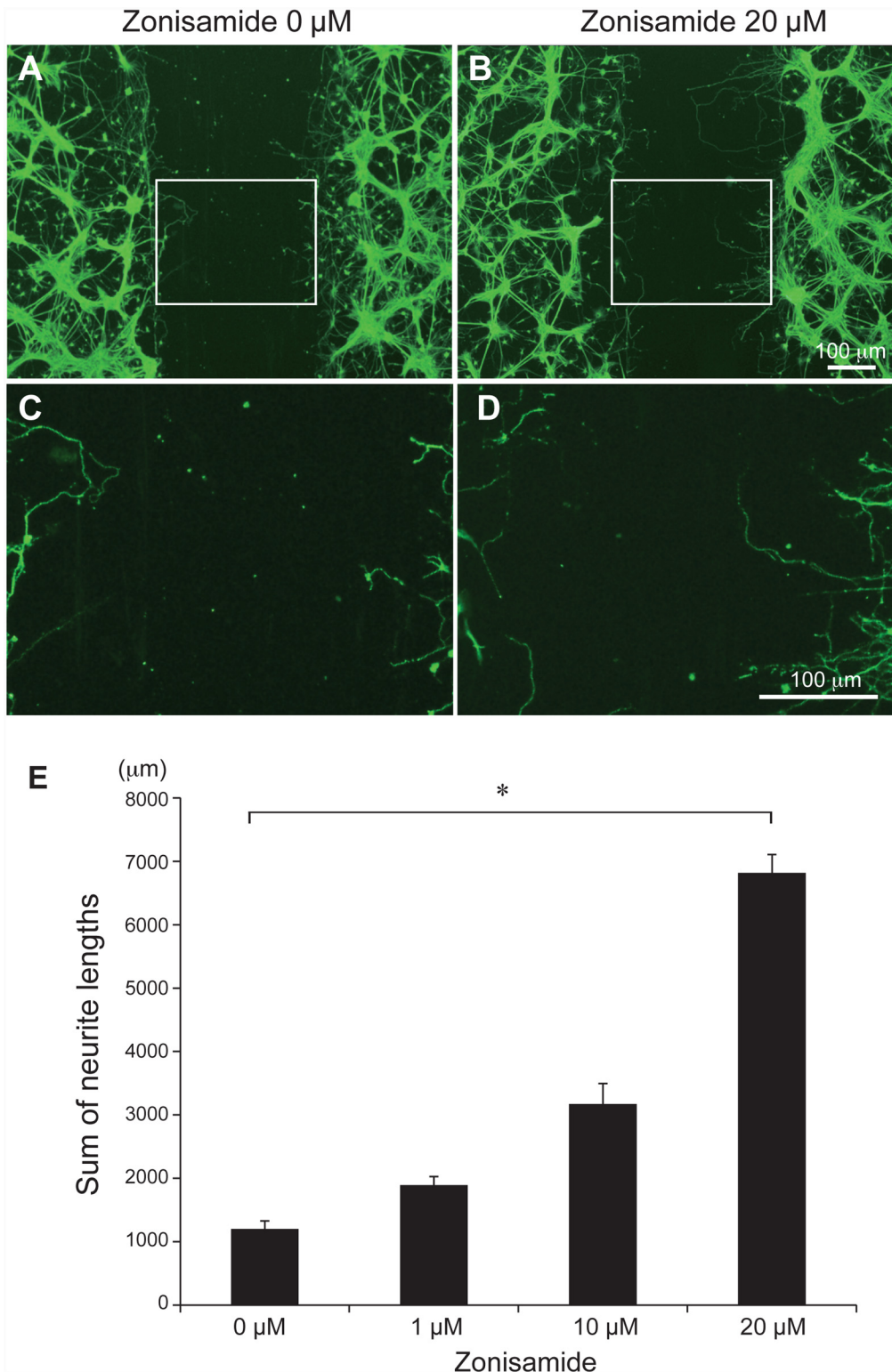
Fig 2. Zonisamide induces regeneration of neurites in primary motor neurons. (A-D) Differentiated primary motor neurons were scratched to evaluate their ability to regenerate neurites. Newly elongated neurite lengths into the scratched area after 48 h were automatically measured by the MetaMorph. Boxed areas in **(A)** and **(B)** are magnified in **(C)** and **(D)**. **(E)** The sum of regenerated neurite lengths in the square area (250 µm × 1000 µm) within the scratched area are calculated in four visual fields and the mean and SE are indicated. *p < 0.05 by one-way ANOVA followed by Tukey HSD.

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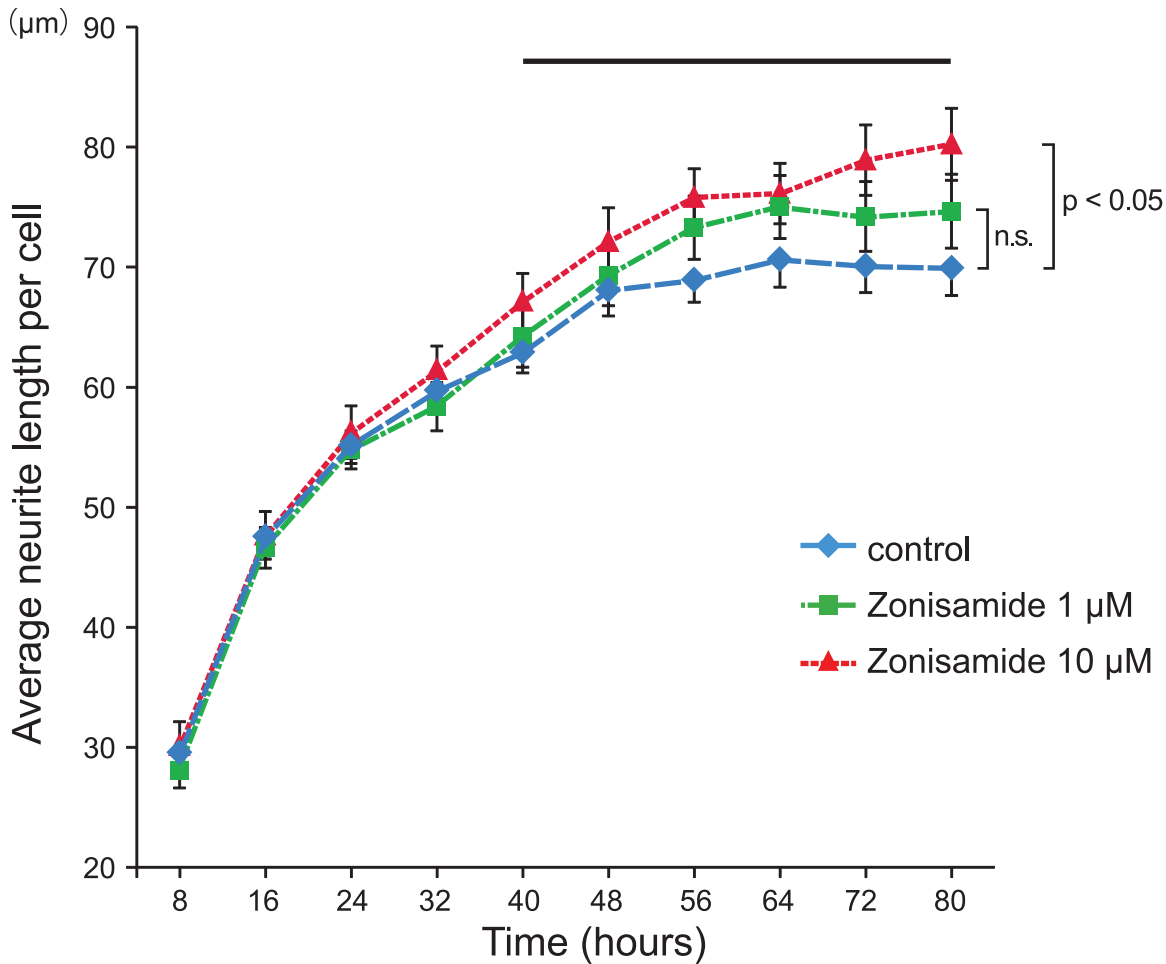
Fig 3. Temporal profile of zonisamide-induced neurite elongation of primary motor neurons. Average neurite lengths of differentiation-induced primary motor neurons were automatically measured every 8 h by the IncuCyte. Mean and SE of the average neurite length per cell in three culture dishes are indicated. The two-way ANOVA analysis was applied from 40 to 80 h (the bar on the top) after adding the differentiation medium. The statistical difference is indicated on the right side of the lines. n.s., not significant.

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into the long-term effects of zonisamide on motor functions and the targeted muscles (tibialis anterior and gastrocnemius). The sciatic function index (SFI), a marker for motor function of hindlimbs after sciatic nerve autograft [27], was significantly improved from 6 to 8 weeks after surgery in zonisamide-treated mice (Fig 6E). Eight weeks after surgery, histological and morphometric analyses of cross sections of tibialis anterior muscle showed that zonisamide accelerated recovery from denervation atrophy (Fig 6F and 6G). In addition, gene expression of *Chrne* [28], *Colq* [29], and *Rapsn* [30], which are specifically expressed at the neuromuscular junction, were increased in zonisamide-treated mice (Fig 6H). We also examined gene expression in a sciatic nerve segment distal to the distal transected site (S3 Fig). Eight weeks after surgery, mRNA levels of the neurotrophins *Ngf* and *Ntf4*, but not *Bdnf*, were increased in zonisamide-treated mice (S3 Fig). mRNA levels of their receptors *Ntrk1* and *Ntrk2* were also increased by zonisamide, although gene expression of *Ntrk1* was low and variable in both uncut and cut hindlimbs (S3 Fig). We analyzed the same genes in primary motor neurons, and observed similar upregulation of neurotrophins and their receptors by zonisamide (Fig 5). In contrast to primary motor neurons, mRNA levels of the structural proteins *Map2*, *Mapt*, and *Gap43* were
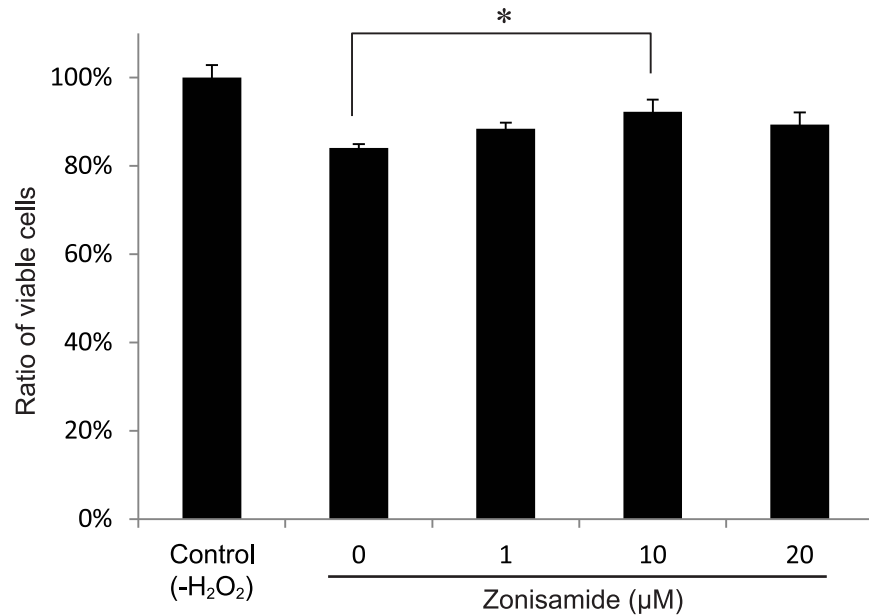
Fig 4. Zonisamide rescues cell death due to oxidative stress. Primary motor neurons in DMEM/F12 with 0.5% FBS were treated with variable concentrations of zonisamide. After 1 h, cells were exposed to 100 μM hydrogen peroxide (H_2O_2) for 24 h. The number of viable cells was estimated by the MTS assay and was normalized to that without H_2O_2 (control). Mean and SE are indicated (n = 6). *p < 0.05 by one-way ANOVA followed by Tukey HSD.

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increased in model mice (S3 Fig). Taken together, our mouse model showed that zonisamide enhanced axonal regeneration and functional recovery of the sciatic nerve.

Discussion

Here, we identified that the widely prescribed anti-epileptic and anti-Parkinsonian drug, zonisamide, enhanced neurite elongation in NSC34 cells and primary motor neurons. In addition, zonisamide increased the number of axonal branch points, enhanced neurite regeneration, and rescued H_2O_2-induced cell death of primary motor neurons. We also confirmed that zonisamide improved nerve regeneration and functional recovery in a mouse model of sciatic nerve autograft. Zonisamide is an anti-epileptic agent widely used for adjunctive treatment of partial seizures in adults and is an effective anti-parkinsonian agent especially for resting tremor [31]. The antiepileptic effects of zonisamide are accounted for by inhibition of sodium channels [32], inhibition of T-type calcium channels [33, 34], indirect inhibition of glutamate receptors [35], suppression of GABA/benzodiazepine receptors [36, 37], and enhancement of release of the inhibitory neurotransmitter GABA [36, 37]. In addition, zonisamide has other pharmacological effects, such as protective effects for dopaminergic neurons [38], neuroprotection against cerebral ischemia [39], an anti-oxidant effect in epilepsy [40], and a radical scavenging action [41, 42]. Furthermore, zonisamide attenuated degeneration of motor neurons and loss of astrocytes in a model mouse of amyotrophic lateral sclerosis [43], although the underlying mechanisms remain unsolved. The effect of zonisamide on sparing motor axons, however, has not been examined to date.

We found that mRNA levels of neurotrophins (*Bdnf*, *Ngf*, and *Ntf4*) and their receptors (*Ntrk1* and *Ntrk2*) were upregulated by zonisamide in primary motor neurons and in model mice. A recent report showed that TrkB agonists promoted axonal regeneration in a mouse
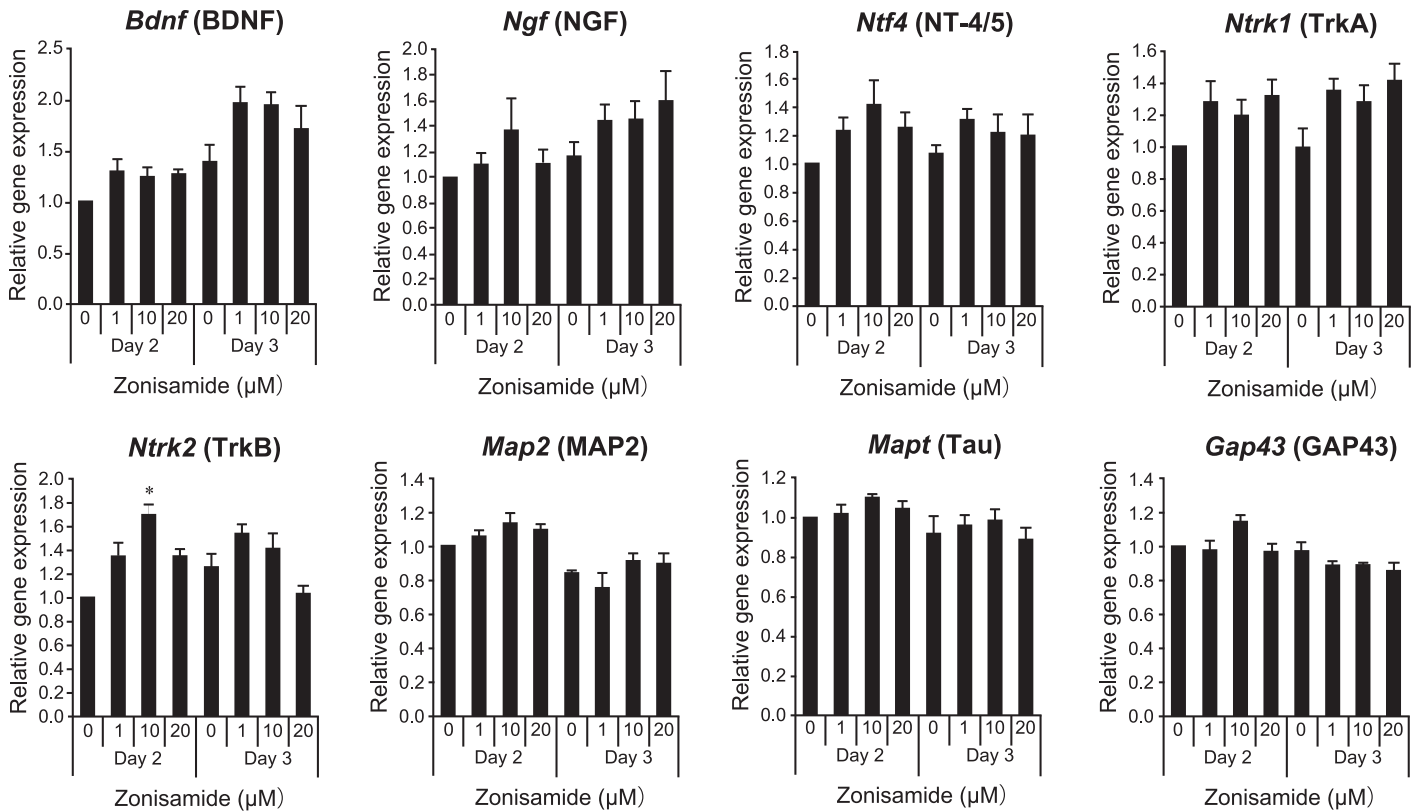
Fig 5. Effects of zonisamide on mRNA levels of nerve growth factors and their receptors, as well as neurite markers in primary motor neurons.
Gene expression in primary motor neurons was quantified by quantitative RT-PCR on days 2 and 3 after adding the differentiation medium. The indicated concentration of zonisamide was added 8 h after adding the differentiation medium. Gene expression was normalized to *Gapdh* and to cells without zonisamide. Mean and SE are indicated (*n* = 4). *p < 0.05 by one-way ANOVA followed by Tukey HSD.

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model of peripheral nerve injury [44]. Enhanced expression of *Bdnf* and *Ntrk2* by zonisamide may partly account for the enhanced neurite elongation in primary motor neurons and enhanced axonal regeneration in model mice. Activation of Erk1/2 [45, 46] and JNK1/2/3 [47, 48] are observed in neurite outgrowth. The attenuation of Erk1/2 phosphorylation by zonisamide and lack of effect on JNK1/2/3 phosphorylation by zonisamide in primary motor neurons were thus unexpected. In addition to neurite outgrowth, activation of Erk is also observed in apoptosis of neuroblastoma cells [49]. Consistent with the role of Erk in apoptosis, inhibition of Erk activation by a MEK inhibitor protected against focal cerebral ischemia [50, 51] as well as against oxidative stress in a mouse neuronal cell line and rat primary cortical neurons [52]. Accordingly, the attenuated phosphorylation of Erk1/2 by zonisamide that we observed might have conferred the neuroprotective effect in H_2O_2-treated primary motor neurons.

Gene expression of structural proteins (*Map2*, *Mapt*, and *Gap43*) were not upregulated by zonisamide in primary motor neurons, but were increased in model mice. Similarly, zonisamide upregulated genes specifically expressed at the neuromuscular junction (*Chrne*, *Colq*, and *Rapsn*). We observed the effects of zonisamide for up to 3 days in primary motor neurons. In contrast, we observed the effects of zonisamide for 8 weeks in model mice. Three days were likely to be sufficient to observe the effects of zonisamide on neurotrophins and their receptors, but this period was too short to observe the effects on structural proteins.

The maximum clinically used dose of zonisamide for patients with epilepsy is 600 mg/day. Assuming that the human body weight is 60 kg, 600 mg/day is equivalent to 10 mg/kg/day. We
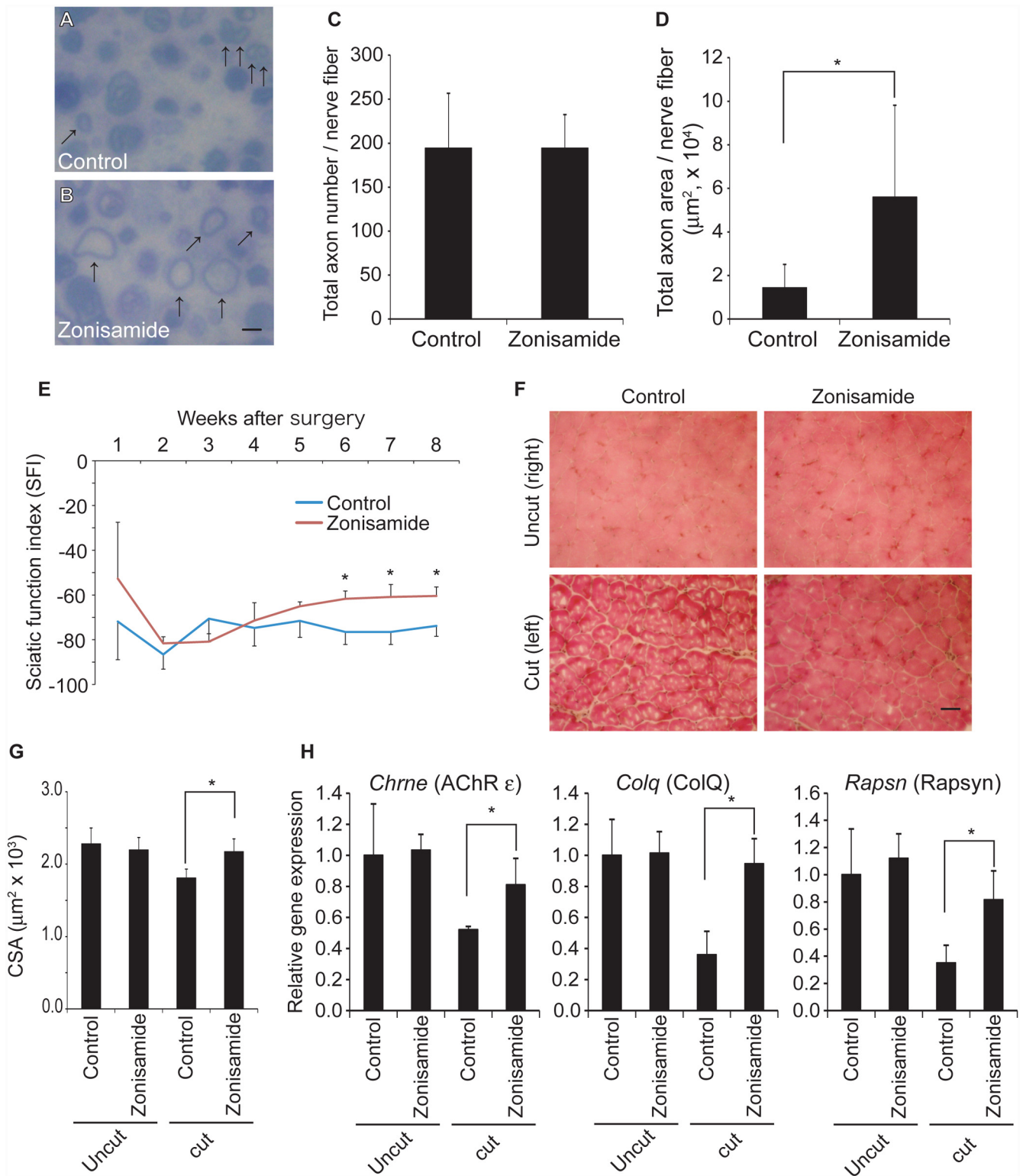
Fig 6. Effects of zonisamide on a mouse model of sciatic nerve autograft. Model mice were made by cutting the left sciatic nerve at two sites 3 mm apart. Mice (*n* = 9 in each group) were intragastrically given either control solution or 30 mg/kg/day zonisamide every day from 1 day after surgery up to 1 week **(A-D)** or 8 weeks **(E-H)** when mice were sacrificed. **(A, B)** Six mice in each group were scarified 1 week after surgery. Representative images of Toluidine blue-stained cross sections of the sciatic nerve 0.7 mm distal to the distal transected site are shown. Myelinated axons are indicated by small arrows. Bar = 20 μm. **(C, D)** Blinded morphometric analysis of the number **(C)** and area **(D)** of myelinated axons in a single sciatic nerve preparation 0.7 mm distal to

the distal transected site. Mean and SD are indicated ($n = 6$ mice in each group). $*p < 0.05$ by Student's t-test. **(E)** Temporal profile of sciatic function indices (SFIs) of footprints of mice treated with control solution or 30 mg/kg/day zonisamide every day from 1 day after surgery. Mean and SD of three mice in each group are indicated. Note that the SFIs are improved in zonisamide-treated mice from 6 weeks after sciatic nerve autograft. $*p < 0.05$ by Student's t-test. **(F)** Representative images of hematoxylin and eosin staining of cross sections of tibialis anterior 8 weeks after surgery. Note fewer target fibers and larger fiber sizes in zonisamide-treated tibialis anterior. Bar = 30 μm. **(G)** Blinded morphometric analysis of cross-sectional areas (CSA) of muscle fibers of tibialis anterior 8 weeks after surgery. Mean and SD are indicated ($n > 98$ in each group). $*p < 0.05$ by Student's t-test. **(H)** Quantitative RT-PCR of *Chrne*, *Colq*, and *Rapsn* expression in gastrocnemius muscles with uncut and cut sciatic nerves 8 weeks after surgery. Gene expression normalized to *Gapdh* on the uncut right side of mice taking control solution was set at 1.0, and relative gene expression is indicated by mean and SD ($n = 3$ in each group). $*p < 0.05$ by Student's t-test.

used three times more zonisamide (30 mg/kg/day) for the model mice. The human threshold of maximum dosage, the TDI (tolerable daily intake), is extrapolated from the rodent threshold, the NOAEL (no observed adverse effect level), by dividing the NOAEL by the UF (uncertainty factor) [53]. As the default UF value is 10 [54, 55], the three times greater dosage of zonisamide that we used in the mouse model will be readily extrapolated to human studies. As zonisamide has long been used without major adverse effects, we hope that zonisamide is a clinically applicable therapeutic drug for peripheral nerve injuries and neuropathies due to other etiologies, although additional animal and human studies are required.

Supporting Information

S1 Fig. Effects of zonisamide on neurite elongation of NSC34 cells and primary motor neurons. Average neurite lengths in 16 wells are normalized to those of controls and are individually plotted. Mean and SD are indicated. Neurite lengths were automatically estimated by the ArrayScan VTI HCS Reader.
(EPS)

S2 Fig. Western blotting of phosphorylated and total Erk1/2 in NSC34 cells (A) and primary motor neurons (B), as well as phosphorylated and total JNK1/2/3 in NSC34 cells (C) and primary motor neurons (D). Representative blots of three or more experiments are shown. Ratios of phosphorylated to total Erk1/2 and JNK1/2/3 are normalized to those of the controls and are indicated below the blots.
(EPS)

S3 Fig. Effects of zonisamide on mRNA levels of nerve growth factors and their receptors, as well as neurite markers in sciatic nerve in a mouse model of sciatic nerve autograft. Quantitative RT-PCR of the indicated genes in a sciatic nerve segment from the distal transected site to 3 mm further distal to it 8 weeks after sciatic nerve autograft. Gene expression normalized to *Gapdh* on the uncut right side of mice taking control solution was set at 1.0, and relative gene expression is indicated by mean and SD ($n = 3$ in each group). $*p < 0.05$ by Student's t-test. Note that all gene expression tended to be increased with zonisamide treatment on the autograft side, except for *Bdnf* and *Ntrk1*, for which gene expression was low and variable.
(EPS)

S1 Table. Primer sequences for quantitative RT-PCR.
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: HN NI K. Ohno. Performed the experiments: HY BO HN KI MT HI KN K. Ohta. Analyzed the data: HY BO HN. Contributed reagents/materials/analysis tools: HY BO HN KI MT K. Ohta. Wrote the paper: HY BO K. Ohno. Supervision of the research project: AM SI NI K. Ohno.

References

1. Terenghi G. Peripheral nerve regeneration and neurotrophic factors. J Anat. 1999; 194 (Pt 1):1–14. Epub 1999/05/05. PMID: 10227662; PubMed Central PMCID: PMC1467889.

2. Namiki J, Kojima A, Tator CH. Effect of brain-derived neurotrophic factor, nerve growth factor, and neurotrophin-3 on functional recovery and regeneration after spinal cord injury in adult rats. J Neurotrauma. 2000; 17(12):1219–31. Epub 2001/02/24. PMID: 11186234.

3. Coumans JV, Lin TT, Dai HN, MacArthur L, McAtee M, Nash C, et al. Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins. J Neurosci. 2001; 21(23):9334–44. Epub 2001/11/22. PMID: 11717367.

4. Boyd JG, Gordon T. A dose-dependent facilitation and inhibition of peripheral nerve regeneration by brain-derived neurotrophic factor. Eur J Neurosci. 2002; 15(4):613–26. Epub 2002/03/12. PMID: 11886442.

5. Tomaselli KJ, Reichardt LF. Peripheral motoneuron interactions with laminin and Schwann cell-derived neurite-promoting molecules: developmental regulation of laminin receptor function. J Neurosci Res. 1988; 21(2–4):275–85. Epub 1988/10/01. doi: 10.1002/jnr.490210220 PMID: 2975342.

6. Powell SK, Kleinman HK. Neuronal laminins and their cellular receptors. Int J Biochem Cell Biol. 1997; 29(3):401–14. Epub 1997/03/01. PMID: 9202420.

7. Brandenberger R, Kammerer RA, Engel J, Chiquet M. Native chick laminin-4 containing the beta 2 chain (s-laminin) promotes motor axon growth. J Cell Biol. 1996; 135(6 Pt 1):1583–92. doi: 10.1083/jcb. 135.6.1583 PMID: 8978824; PubMed Central PMCID: PMC2133966.

8. Marthiens V, Gavard J, Padilla F, Monnet C, Castellani V, Lambert M, et al. A novel function for cadherin-11 in the regulation of motor axon elongation and fasciculation. Mol Cell Neurosci. 2005; 28 (4):715–26. doi: 10.1016/j.mcn.2004.12.001 PMID: 15797718.

9. Pardridge WM, Kang YS, Buciak JL. Transport of human recombinant brain-derived neurotrophic factor (BDNF) through the rat blood-brain barrier in vivo using vector-mediated peptide drug delivery. Pharm Res. 1994; 11(5):738–46. Epub 1994/05/01. PMID: 8058646.

10. Poduslo JF, Curran GL. Glycation increases the permeability of proteins across the blood-nerve and blood-brain barriers. Brain Res Mol Brain Res. 1994; 23(1–2):157–62. Epub 1994/04/01. PMID: 8028478.

11. Poduslo JF, Curran GL. Permeability at the blood-brain and blood-nerve barriers of the neurotrophic factors: NGF, CNTF, NT-3, BDNF. Brain Res Mol Brain Res. 1996; 36(2):280–6. Epub 1996/03/01. PMID: 8965648.

12. Uemura T, Takamatsu K, Ikeda M, Okada M, Kazuki K, Ikada Y, et al. Transplantation of induced pluripotent stem cell-derived neurospheres for peripheral nerve repair. Biochem Biophys Res Commun. 2012; 419(1):130–5. doi: 10.1016/j.bbrc.2012.01.154 PMID: 22333572.

13. Nakamura M, Okano H. Cell transplantation therapies for spinal cord injury focusing on induced pluripotent stem cells. Cell Res. 2013; 23(1):70–80. doi: 10.1038/cr.2012.171 PMID: 23229514; PubMed Central PMCID: PMC3541652.

14. Ramón-Cueto A, Cordero MI, Santos-Benito FF, Avila J. Functional Recovery of Paraplegic Rats and Motor Axon Regeneration in Their Spinal Cords by Olfactory Ensheathing Glia. Neuron. 2000; 25 (2):425–35. doi: 10.1016/s0896-6273(00)80905-8 PMID: 10719896

15. Wright KT, Uchida K, Bara JJ, Roberts S, El Masri W, Johnson WE. Spinal motor neurite outgrowth over glial scar inhibitors is enhanced by coculture with bone marrow stromal cells. Spine J. 2014; 14 (8):1722–33. Epub 2014/01/28. doi: 10.1016/j.spinee.2014.01.021 PMID: 24462452.

16. Witheford M, Westendorf K, Roskams AJ. Olfactory ensheathing cells promote corticospinal axonal outgrowth by a L1 CAM-dependent mechanism. Glia. 2013; 61(11):1873–89. Epub 2013/09/17. doi: 10.1002/glia.22564 PMID: 24038549.

17. Spejo AB, Carvalho JL, Goes AM, Oliveira AL. Neuroprotective effects of mesenchymal stem cells on spinal motoneurons following ventral root axotomy: synapse stability and axonal regeneration. Neuroscience. 2013; 250:715–32. Epub 2013/07/31. doi: 10.1016/j.neuroscience.2013.07.043 PMID: 23896572.

18. Kurimoto S, Kato S, Nakano T, Yamamoto M, Takanobu N, Hirata H. Transplantation of embryonic motor neurons into peripheral nerve combined with functional electrical stimulation restores functional muscle activity in the rat sciatic nerve transection model. J Tissue Eng Regen Med. 2013. Epub 2014/03/29. doi: 10.1002/term.1844 PMID: 24668934.

19. Guo Y, Liu S, Wang P, Zhao S, Wang F, Bing L, et al. Expression profile of embryonic stem cell-associated genes Oct4, Sox2 and Nanog in human gliomas. Histopathology. 2011; 59(4):763–75. Epub 2011/10/22. doi: 10.1111/j.1365-2559.2011.03993.x PMID: 22014056.

20. Fu Q, Hue J, Li S. Nonsteroidal anti-inflammatory drugs promote axon regeneration via RhoA inhibition. J Neurosci. 2007; 27(15):4154–64. Epub 2007/04/13. doi: 10.1523/JNEUROSCI.4353-06.2007 PMID: 17428993.

21. Cui SS, Yang CP, Bowen RC, Bai O, Li XM, Jiang W, et al. Valproic acid enhances axonal regeneration and recovery of motor function after sciatic nerve axotomy in adult rats. Brain Res. 2003; 975(1–2):229–36. doi: 10.1016/s0006-8993(03)02699-4 PMID: 12763612.

22. Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L. Rho signaling pathway targeted to promote spinal cord repair. J Neurosci. 2002; 22(15):6570–7. Epub 2002/08/02. doi: 20026637. PMID: 12151536.

23. Padhy BM, Gupta YK. Drug repositioning: re-investigating existing drugs for new therapeutic indications. J Postgrad Med. 2011; 57(2):153–60. doi: 10.4103/0022-3859.81870 PMID: 21654146.

24. Ashburn TT, Thor KB. Drug repositioning: identifying and developing new uses for existing drugs. Nat Rev Drug Discov. 2004; 3(8):673–83. doi: 10.1038/nrd1468 PMID: 15286734.

25. Cashman NR, Durham HD, Blusztajn JK, Oda K, Tabira T, Shaw IT, et al. Neuroblastoma x spinal cord (NSC) hybrid cell lines resemble developing motor neurons. Dev Dyn. 1992; 194(3):209–21. Epub 1992/07/01. doi: 10.1002/aja.1001940306 PMID: 1467557.

26. Jungnickel J, Haase K, Konitzer J, Timmer M, Grothe C. Faster nerve regeneration after sciatic nerve injury in mice over-expressing basic fibroblast growth factor. J Neurobiol. 2006; 66(9):940–8. doi: 10.1002/neu.20265 PMID: 16758491.

27. Bain JR, Mackinnon SE, Hunter DA. Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. Plast Reconstr Surg. 1989; 83(1):129–38. PMID: 2909054.

28. Jones G, Herczeg A, Ruegg MA, Lichtsteiner M, Kroger S, Brenner HR. Substrate-bound agrin induces expression of acetylcholine receptor epsilon-subunit gene in cultured mammalian muscle cells. Proc Natl Acad Sci U S A. 1996; 93(12):5985–90. PMID: 8650206; PubMed Central PMCID: PMCPMC39175.

29. Trinkaus M, Pregelj P, Trkov S, Sketelj J. Neural regulation of acetylcholinesterase-associated collagen Q in rat skeletal muscles. J Neurochem. 2008; 105(6):2535–44. doi: 10.1111/j.1471-4159.2008.05328.x PMID: 18373559.

30. Brockhausen J, Cole RN, Gervasio OL, Ngo ST, Noakes PG, Phillips WD. Neural agrin increases post-synaptic ACh receptor packing by elevating rapsyn protein at the mouse neuromuscular synapse. Dev Neurobiol. 2008; 68(9):1153–69. doi: 10.1002/dneu.20654 PMID: 18506821.

31. Murata M. Novel therapeutic effects of the anti-convulsant, zonisamide, on Parkinson's disease. Curr Pharm Des. 2004; 10(6):687–93. Epub 2004/02/18. PMID: 14965331.

32. Schauf CL. Zonisamide enhances slow sodium inactivation in Myxicola. Brain Res. 1987; 413(1):185–8. Epub 1987/06/09. PMID: 2439177.

33. Kito M, Maehara M, Watanabe K. Mechanisms of T-type calcium channel blockade by zonisamide. Seizure. 1996; 5(2):115–9. Epub 1996/06/01. PMID: 8795126.

34. Suzuki S, Kawakami K, Nishimura S, Watanabe Y, Yagi K, Seino M, et al. Zonisamide blocks T-type calcium channel in cultured neurons of rat cerebral cortex. Epilepsy Res. 1992; 12(1):21–7. Epub 1992/06/01. PMID: 1326433.

35. Yoshida S, Okada M, Zhu G, Kaneko S. Effects of zonisamide on neurotransmitter exocytosis associated with ryanodine receptors. Epilepsy Res. 2005; 67(3):153–62. Epub 2005/11/18. doi: 10.1016/j.eplepsyres.2005.10.001 PMID: 16289509.

36. Mimaki T, Suzuki Y, Tagawa T, Karasawa T, Yabuuchi H. Interaction of zonisamide with benzodiazepine and GABA receptors in rat brain. Med J Osaka Univ. 1990; 39(1–4):13–7. Epub 1990/03/01. PMID: 1369646.

37. Yamamura S, Ohoyama K, Nagase H, Okada M. Zonisamide enhances delta receptor-associated neurotransmitter release in striato-pallidal pathway. Neuropharmacology. 2009; 57(3):322–31. Epub 2009/06/02. doi: 10.1016/j.neuropharm.2009.05.005 PMID: 19482038.

38. Asanuma M, Miyazaki I, Diaz-Corrales FJ, Kimoto N, Kikkawa Y, Takeshima M, et al. Neuroprotective effects of zonisamide target astrocyte. Ann Neurol. 2010; 67(2):239–49. Epub 2010/03/13. doi: 10.1002/ana.21885 PMID: 20225289.

39. Minato H, Kikuta C, Fujitani B, Masuda Y. Protective effect of zonisamide, an antiepileptic drug, against transient focal cerebral ischemia with middle cerebral artery occlusion-reperfusion in rats. Epilepsia. 1997; 38(9):975–80. Epub 1998/05/14. PMID: 9579935.

40. Komatsu M, Hiramatsu M, Willmore LJ. Zonisamide reduces the increase in 8-hydroxy-2'-deoxyguanosine levels formed during iron-induced epileptogenesis in the brains of rats. Epilepsia. 2000; 41 (9):1091–4. Epub 2000/09/22. PMID: 10999546.

41. Kawajiri S, Machida Y, Saiki S, Sato S, Hattori N. Zonisamide reduces cell death in SH-SY5Y cells via an anti-apoptotic effect and by upregulating MnSOD. Neurosci Lett. 2010; 481(2):88–91. doi: 10.1016/j. neulet.2010.06.058 PMID: 20600601.

42. Choudhury ME, Sugimoto K, Kubo M, Iwaki H, Tsujii T, Kyaw WT, et al. Zonisamide up-regulated the mRNAs encoding astrocytic anti-oxidative and neurotrophic factors. Eur J Pharmacol. 2012; 689(1– 3):72–80. doi: 10.1016/j.ejphar.2012.05.012 PMID: 22659113.

43. Hirayama T, Yoshii Y, Kawabe K, Ikeda K. Zonisamide treatment delays motor neuron degeneration and astrocyte proliferation in wobbler mice. J Neurol Res. 2011; 1(4):139–44. doi: 10.4021/jnr59w

44. English AW, Liu K, Nicolini JM, Mulligan AM, Ye K. Small-molecule trkB agonists promote axon regeneration in cut peripheral nerves. Proc Natl Acad Sci U S A. 2013; 110(40):16217–22. doi: 10.1073/ pnas.1303646110 PMID: 24043773; PubMed Central PMCID: PMC3791704.

45. Tsuda Y, Kanje M, Dahlin LB. Axonal outgrowth is associated with increased ERK 1/2 activation but decreased caspase 3 linked cell death in Schwann cells after immediate nerve repair in rats. BMC Neurosci. 2011; 12:12. Epub 2011/01/22. doi: 10.1186/1471-2202-12-12 PMID: 21251262; PubMed Central PMCID: PMCPmc3031266.

46. Wang Z, Wang J, Li J, Wang X, Yao Y, Zhang X, et al. MEK/ERKs signaling is essential for lithium-induced neurite outgrowth in N2a cells. Int J Dev Neurosci. 2011; 29(4):415–22. Epub 2011/03/15. doi: 10.1016/j.ijdevneu.2011.03.001 PMID: 21397003.

47. Oliva AA Jr., Atkins CM, Copenagle L, Banker GA. Activated c-Jun N-terminal kinase is required for axon formation. J Neurosci. 2006; 26(37):9462–70. Epub 2006/09/15. doi: 10.1523/jneurosci.2625-06. 2006 PMID: 16971530.

48. Barnat M, Enslen H, Propst F, Davis RJ, Soares S, Nothias F. Distinct roles of c-Jun N-terminal kinase isoforms in neurite initiation and elongation during axonal regeneration. J Neurosci. 2010; 30 (23):7804–16. doi: 10.1523/JNEUROSCI.0372-10.2010 PMID: 20534829.

49. Guise S, Braguer D, Carles G, Delacourte A, Briand C. Hyperphosphorylation of tau is mediated by ERK activation during anticancer drug-induced apoptosis in neuroblastoma cells. J Neurosci Res. 2001; 63(3):257–67. Epub 2001/02/15. PMID: 11170175.

50. Alessandrini A, Namura S, Moskowitz MA, Bonventre JV. MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. Proc Natl Acad Sci U S A. 1999; 96(22):12866– 9. Epub 1999/10/27. PMID: 10536014; PubMed Central PMCID: PMC23136.

51. Namura S, Iihara K, Takami S, Nagata I, Kikuchi H, Matsushita K, et al. Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia. Proc Natl Acad Sci U S A. 2001; 98(20):11569–74. Epub 2001/08/16. doi: 10.1073/pnas.181213498 PMID: 11504919; PubMed Central PMCID: PMCPmc58770.

52. Satoh T, Nakatsuka D, Watanabe Y, Nagata I, Kikuchi H, Namura S. Neuroprotection by MAPK/ERK kinase inhibition with U0126 against oxidative stress in a mouse neuronal cell line and rat primary cultured cortical neurons. Neurosci Lett. 2000; 288(2):163–6. Epub 2000/07/06. PMID: 10876086.

53. Barnes DG, Dourson M. Reference dose (RfD): description and use in health risk assessments. Regul Toxicol Pharmacol. 1988; 8(4):471–86. PMID: 3222488.

54. Dourson ML, Felter SP, Robinson D. Evolution of science-based uncertainty factors in noncancer risk assessment. Regul Toxicol Pharmacol. 1996; 24(2 Pt 1):108–20. doi: 10.1006/rtph.1996.0116 PMID: 8933624.

55. Foronda NM, Fowles J, Smith N, Taylor M, Temple W, Darlington C. The use of myocardial and testicular end points as a basis for estimating a proposed tolerable daily intake for sodium monofluoroacetate (1080). Regul Toxicol Pharmacol. 2007; 47(1):29–36. doi: 10.1016/j.yrtph.2006.08.005 PMID: 17030370.