# Zonisamide ameliorates progression of cervical spondylotic myelopathy in a rat model

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**Supplementary Information**

## Materials and Methods

### Isolation of primary astrocytes

Primary cultures of spinal astrocytes were prepared as described previously [1]. Five female or male pups (0- to 2-day-old) of Wistar rats were immediately decapitated and the isolated spinal cord was minced in divalent cation-free Hanks' balanced salt solution (Nacalai). The minced spinal cords were harvested by grinding on a cell strainer (40 μm) (Falcon, 352340) with instillation of cation-free Hanks' balanced salt solution [HBSS(-), Nacalai tesque, 1746-15]. The cells were centrifuged for 5 min at 270 x *g* to remove debris in the supernatant. The cells were suspended with HBSS(-) including papain (10 U/ml, WAKO, 164-00172), and were left at room temperature for 10 min. Then, the solution was added with DNase (0.1 mg/ml, Sigma, DN25), and was left at room temperature for 10 min. After the enzymatic dissociation, the tissues were mechanically dissociated with a Pasteur pipette in culture medium. Samples were centrifuged for 5 min at 270 x *g*, and the culture medium was added after dumping the supernatants. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, 172012) and 1% of Pen Strep (10,000 U/ml, Gibco, 151400-122). Cells were seeded and maintained for 2 weeks in culture medium in a T-75 poly-L-lysine-coated culture flask (Sigma-Aldrich) at 37°C in 5% $CO_2$. Culture medium was changed three times a week. When cells reached 80-90% confluency, the flask was shaken vigorously at 250 rpm for at least 12 h to remove non-astrocytic cells. The remaining astrocytes were seeded into a new poly-L-lysine-coated plate.

**Culture of NSC34 cells**

NSC34 cells (mouse neuroblastoma-spinal cord hybrid cells displaying a multipolar motor neuron-like phenotype) that stably expressed the doxycycline-induced green fluorescent protein (GFP) construct (NSC34-pTetR12-TO / GFP) [2] were kindly provided by Dr. Shinsuke Ishigaki, Department of Neurology, Nagoya University Graduate School of Medicine, and were cultured as previously described [3]. Briefly, NSC34 cells were cultured in a humidified atmosphere with 95% air-5% $CO_2$ in a 37°C incubator in DMEM supplemented with 10% FBS (Sigma-Aldrich, 172012) for proliferation.

**References**


1. Eguchi, R., et al., Different mechanisms of extracellular adenosine accumulation by reduction of the external Ca(2+) concentration and inhibition of adenosine metabolism in spinal astrocytes. J Pharmacol Sci, 2015. **128**(1): p. 47-53.
2. Asanuma, M., et al., Neuroprotective effects of zonisamide target astrocyte. Ann Neurol, 2010. **67**(2): p. 239-49.
3. Wang, X.F. and M.S. Cynader, Astrocytes provide cysteine to neurons by releasing glutathione. J Neurochem, 2000. **74**(4): p. 1434-42.

**Supplemental Table S1. Primer sequences for qRT-PCR**

| Rat gene | Primer sequence |
|---|---|
| *Slc7a11* (xCT) | For. 5' CATTGTATGGGACAAGAAACC 3' |
| | Rev. 5' GGCACTAGACTCAAGAACTGT 3' |
| *Mt2a* (metallothionein 2A) | For. 5' CACAGATGGATCCTGCTCCT 3' |
| | Rev. 5' GAGAACCGGTCAGGGTTGTA 3' |
| *Pcna* (PCNA) | For. 5' TAAGTTGTCCCAGACAAGCA 3' |
| | Rev. 5' GCGATCGTCAAAGGTTTAGT 3' |
| *Actb* (β actin) | For. 5' TCTACAATGAGCTGCGTGTG 3' |
| | Rev. 5' TACATGGCTGGGGTGTTGAA 3' |

For., forward primer; Rev., reverse primer

**Supplemental Figure legends**

**Supplemental Figure S1. Water-absorbing polyurethane blade for CSM operation in rats**

**(A)** Volumetric measurement of expansion of a blade-shaped water-absorbing polyurethane elastomer (Aquaprene Dx) implanted under the rat skin. The blade gradually expanded and reached a volume of 150% in 24 h. Mean and SD are indicated ($n$ = 3). **(B)** Frontal/lateral views and schematic presentation of the blade before implantation. A slanted edge was made at the corner not to damage the spinal cord during implantation. A thread was added to the blade to smoothly place the blade underneath the C5 and C6 laminae. **(C)** Experimental protocol of gait and histological analyses of both sham-operated and CSM rats. Water-absorbing polyurethane blade was implanted in CSM rats at 12 weeks of age. **(D)** Dorsal view of a representative intraoperative image. Following the resection of ligamentum flavum at C5–6 and C6–7, the blade passed through the resected C6-7 space and epidurally slipped underneath the C5 and C6 laminae without laminectomy. **(E)** Gait analysis with CatWalk to measure the distance ("print position") between the forepaw step and the hindpaw step (yellow double-headed arrow in a representative CatWalk image). Print positions extended from 5 weeks after surgery in CSM rats. Mean and SE are indicated ($n$ = 6 rats in each group).

**Supplemental Figure S2. Effects of Zonisamide (ZNS) on gene expressions of *Pcna* encoding proliferating cell nuclear antigen (PCNA), *Slc7a11* encoding cystine/glutamate exchange transporter (xCT), and *Mt2a* encoding metallothionein**

**2A in primary astrocytes and in NSC34 neuronal cells.**

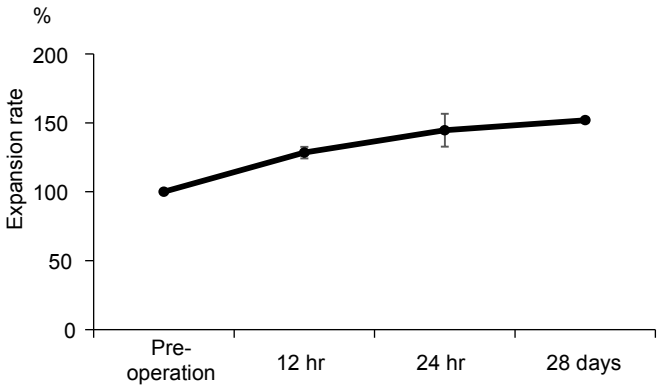
Primary astrocytes isolated from the rat spinal cord around C5-6 on postnatal day 1 were cultured with growth media. NSC34 cells were cultured with differentiation medium for 3 days. Primary astrocytes and NSC34 cells were treated with 10 μM ZNS. After 1 h, cells were added with 100 μM $H_2O_2$ for 24 h. Gene expressions of *Pcna* encoding proliferating cell nuclear antigen **(A)**, *Slc7a11* encoding cystine/glutamate exchange transporter (xCT, **B**), and *Mt2a* encoding metallothionein 2A **(C)** were normalized for the expression of *Actb* encoding β-actin. Mean and SD are indicated ($n$ = 3 culture dishes in each group). *$p < 0.05$ and **$p < 0.01$ by Kruskal-Wallis test followed by Dunn-Bonferroni HSD.

**Supplemental Figure S3. Effects of Zonisamide (ZNS) on immunostaining for glutathione (GSH) in ChAT-positive spinal motor neurons in CSM rats.**

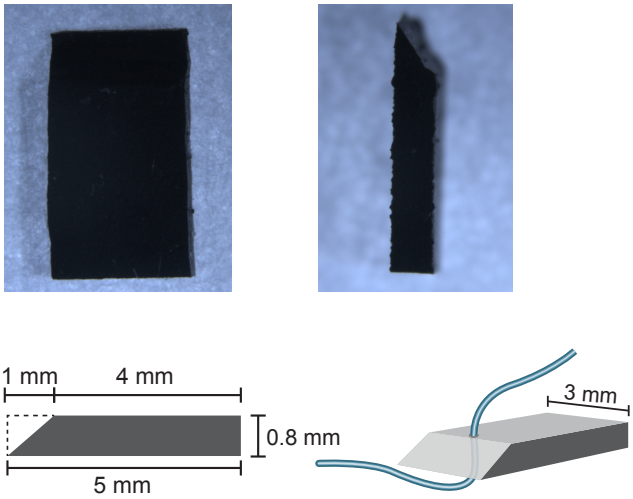
Low- and high-magnification images for Fig. 5B of GSH (green)- and ChAT (red)-positive cells in the anterior horn (AH) region of the spinal cord at the C5-6 disc level in CSM rats with [ZNS (+), B] or without [ZNS (-), A] ZNS administration. White arrowhead indicate ChAT-positive motor neurons in the AH region. Bars = 50 μm

Supplemental Figure 1

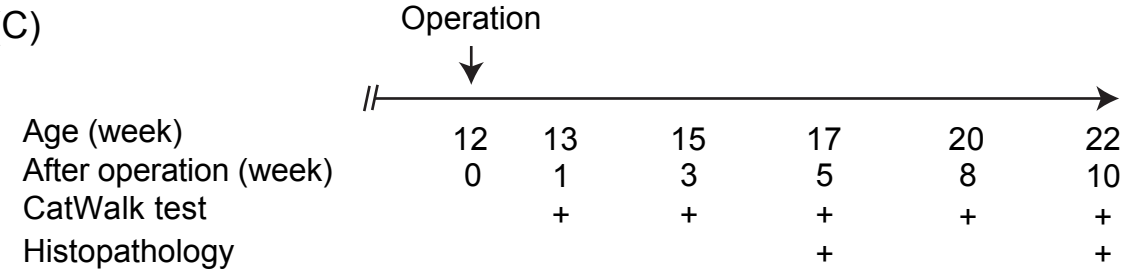
(A)



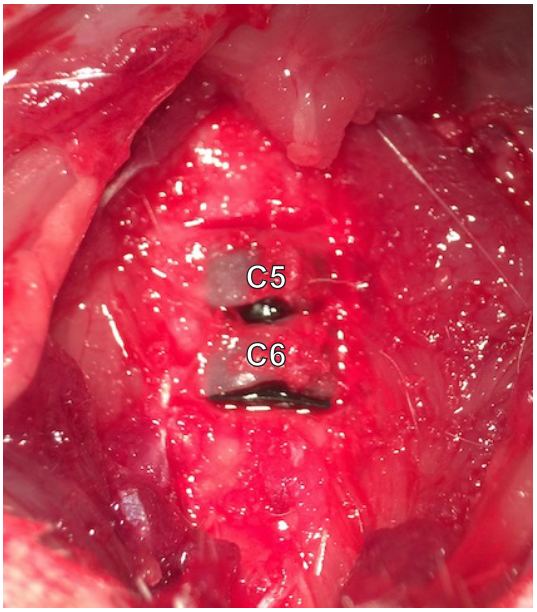

(B)




(C)




(D)

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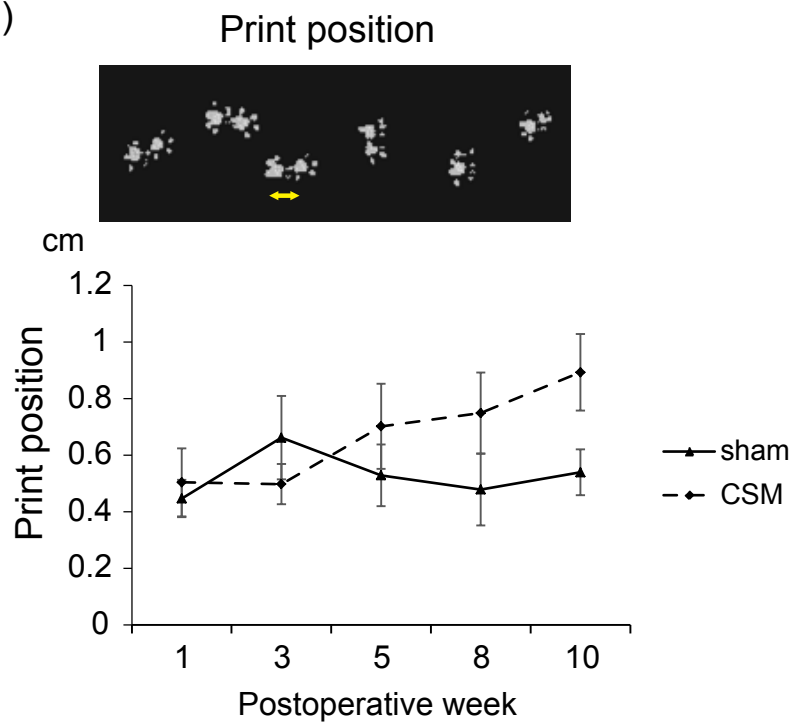
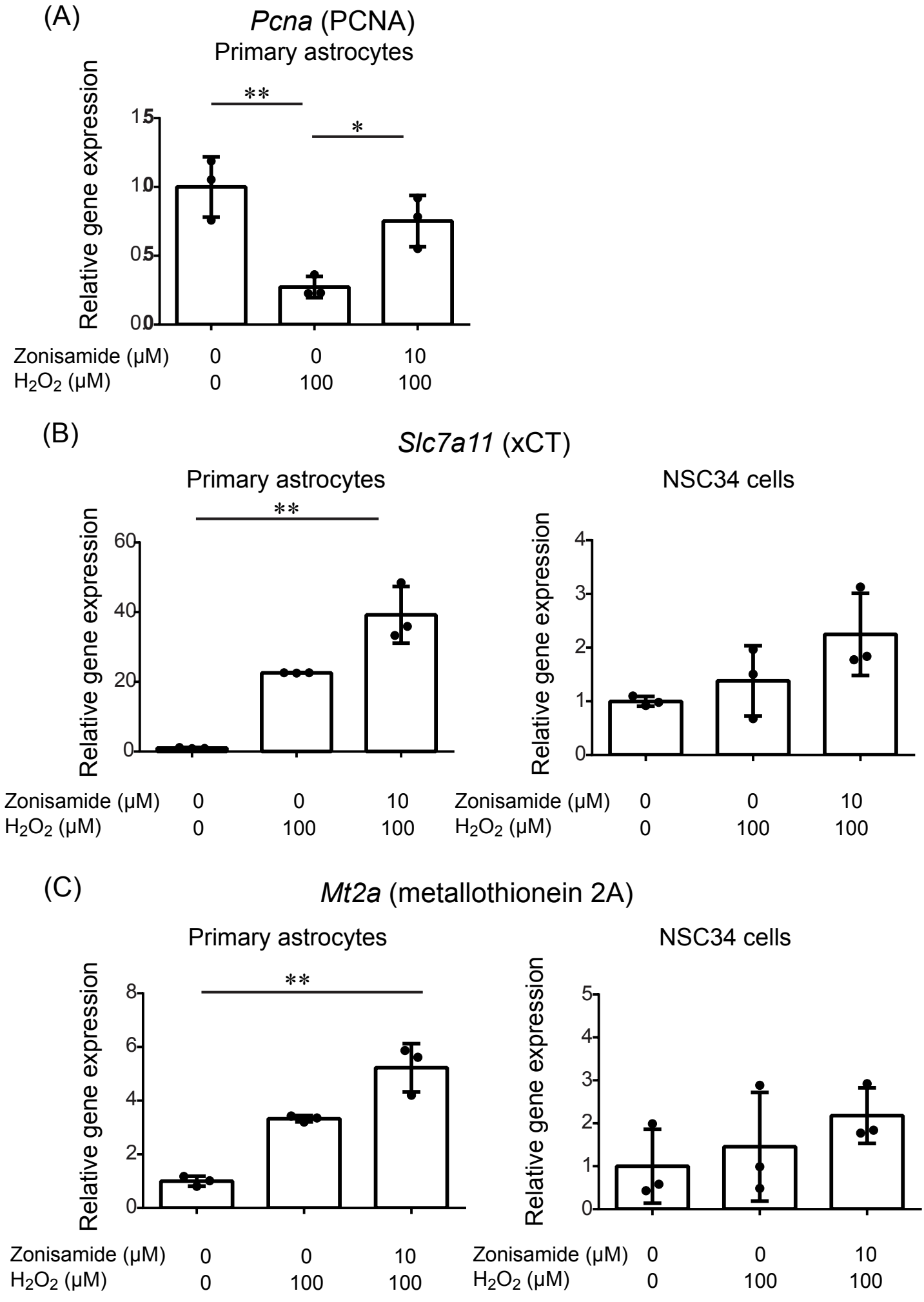


Supplemental Figure 2



Supplemetal Figure 3