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EXPERIMENTAL ARTICLES

Intrathecal Infusion of Diosgenin during the Chronic Phase of Spinal Cord Injury Ameliorates Motor Function and Axonal Density

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Abstract—The chronic phase of spinal cord injury (SCI) is the most challenging to treat. The extension of disrupted axons is necessary for prominent motor function recovery. Our previous studies demonstrated that diosgenin had remarkable potential for axonal growth activity. This study investigated the effects of an intrathecal infusion of diosgenin in spinal cord-injured mice to evaluate motor function and axonal density during the chronic phase. Female ddY mice received an L1 spinal cord injury (SCI). Thirty-one days after the injury, the chronic intrathecal infusion was started. The infusion was sustained for 59 days, and motor function was evaluated using the Basso Mouse Scale, Toyama Mouse Score, and vertical cage scale through the dosing period and endpoint. 5-HT-positive raphespinal tracts were quantified in the lesion center, rostral and caudal positions 2 mm away from the center. The intrathecal infusion of diosgenin significantly improved hind limb motor function and density of the raphespinal tracts in the lesion center. The glial scar size did not differ between the vehicle-treated and diosgenin-treated groups. Cultured medulla oblongata neurons and spinal cord neurons on CSPG-coating extended axons by treatment with diosgenin. Diosgenin is a potential candidate as a therapeutic drug for chronic SCI.

Keywords: raphespinal tract, medulla oblongata neurons, spinal cord neuron, chronic spinal cord injury, motor function, diosgenin, intrathecal infusion, axon

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INTRODUCTION

The chronic phase of spinal cord injury (SCI) is the most challenging to treat. As time after injury progresses, astrocytes and fibroblasts accumulate in the lesion center and form a glial scar. This process leads to axonal regeneration failure due to the secretion of chondroitin sulfate proteoglycans (CSPGs) [1] and myelin-derived inhibitory molecules [2]. Treatment with chondroitinase ABC at the chronic phase slightly ameliorates motor function only when combined with rehabilitation [3] or neural progenitor cell transplantation [4]. Mono-treatment with chondroitinase ABC or inhibitors for myelin-derived inhibitory molecules has not been reported as effective for the chronic phase of SCI. Although the extension of disrupted axons is necessary for prominent motor function recovery, blocking these axonal inhibitory molecules is insufficient, and active enhancement of axonal growth is required.

Diosgenin is a yam-derived compound with remarkable potential for axonal growth activity [5–7]. Diosgenin has a low molecular weight (MW = 414.62) and is a hydrophobic compound. In an Alzheimer's disease mouse model, axonal repair and synaptogenesis occurred upon diosgenin treatment in the brain [5]. In normal mice, diosgenin administration increased axonal density and excitatory synaptic connections in the brain [6]. These previous studies suggest the effectiveness of diosgenin in SCI. This study aimed to investigate whether diosgenin administration might enhance axonal formation in the spinal cord and improve motor function. Diosgenin was chronically infused intrathecally to ensure delivery in close proximity to the lesion area,

MATERIALS AND METHODS

Materials. Diosgenin (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in ethanol and diluted with artificial cerebrospinal fluid (ACSF; Figs. 1 and 2) or sterile distilled water (Fig. 3). The final concentration of ethanol was 0.1%. Diosgenin concentration in the cerebrospinal fluid was set at 0.1 μM.

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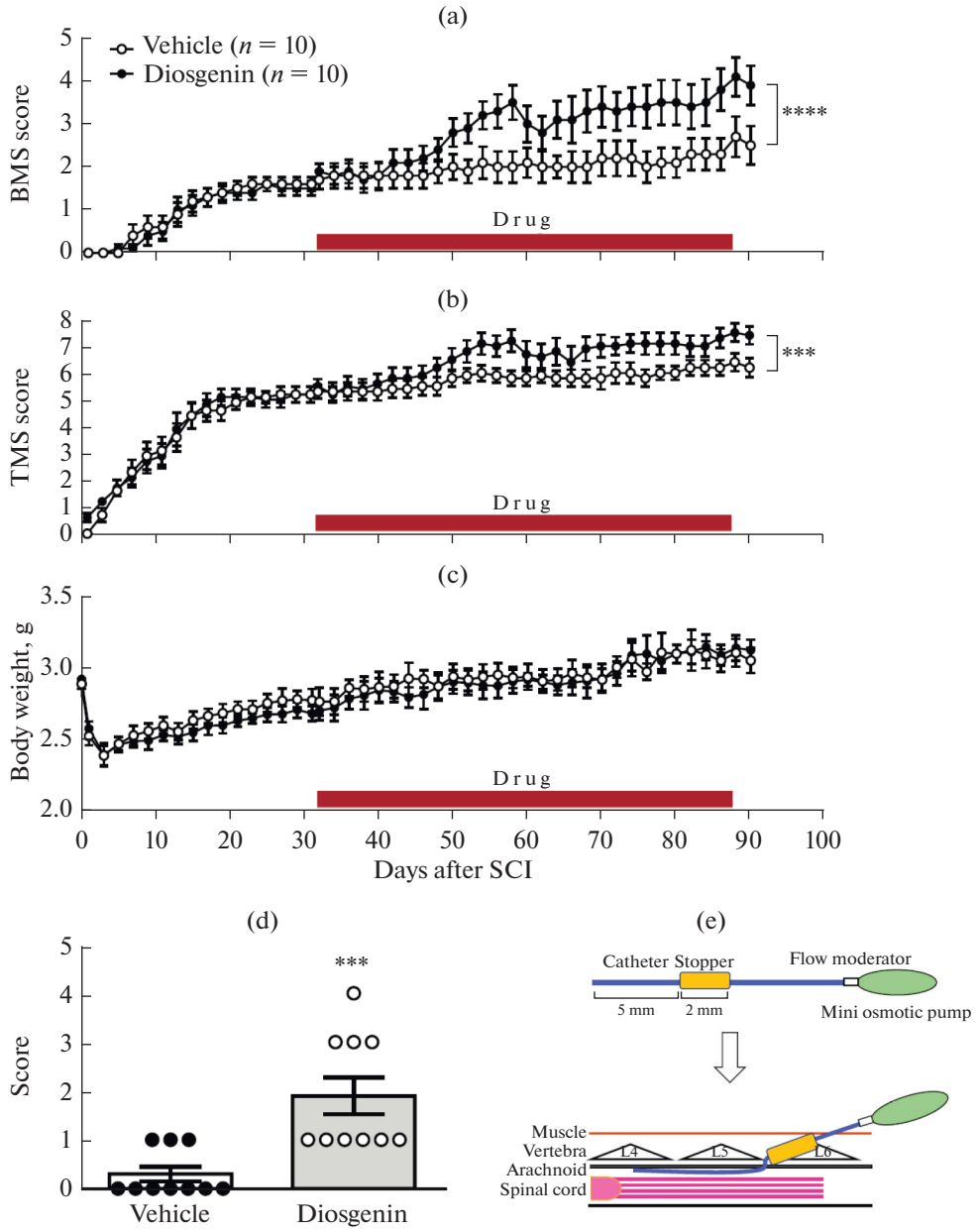

Fig. 1. Effects of intrathecal diosgenin infusion on the locomotor function of mice with contusive spinal cord injury (SCI). Thirty-one days after SCI, diosgenin (final concentration in CSF; 0.1 μM) or vehicle solution was intrathecally injected using a mouse intrathecal catheter and mini osmotic pump for 59 days. The hind limb motor function of mice with SCI was evaluated using the Basso Mouse Scale (BMS; a), Toyama Mouse Score (TMS; b), and vertical cage scale (d). A method of intrathecal injection was shown (e). Vehicle group: $n = 5$ mice, $n = 10$ hind limbs, diosgenin group: $n = 5$ mice; $n = 10$ hind limbs. In (a and b), $^{***}p < 0.001$, $^{****}p < 0.0001$, drug × day interaction, diosgenin vs. vehicle, repeated measures two-way analysis of variance (ANOVA), and *post hoc* Bonferroni's test. (c) The mice body weights were measured. Repeated measures two-way ANOVA. (d) Vertical cage scale scores at 59 days after infusion initiation. $^{***}p < 0.001$ vs. vehicle-infused group, Mann–Whitney U test.

SCI surgical operation and continuous intrathecal administration of diosgenin. Eight-week-old female ddY mice (SLC, Japan) were used for the SCI experiments. All mice were housed with access to food and water *ad libitum* and kept in a stable environment (22 ± 2°C, 50 ± 5% humidity, 12 h light cycle starting at 07:00). The mice were anesthetized with butorphanol tartrate (5 mg/kg, i.p., Meiji Seika Pharma Co., Ltd., Tokyo, Japan), medetomidine hydrochloride (0.75 mg/kg, i.p., Zenyaku Kogyo Co., Ltd., Tokyo, Japan), and midazolam (4 mg/kg, i.p., Fuji Pharma Co., Ltd., Tokyo, Japan). After laminectomy, a contusion injury was induced by dropping a 6.5 g weight from a height of 3.5 cm onto the exposed L1 spinal cord at the level of T11 vertebra using a stereotaxic instrument (Narishige, Tokyo, Japan), as described previously [8].

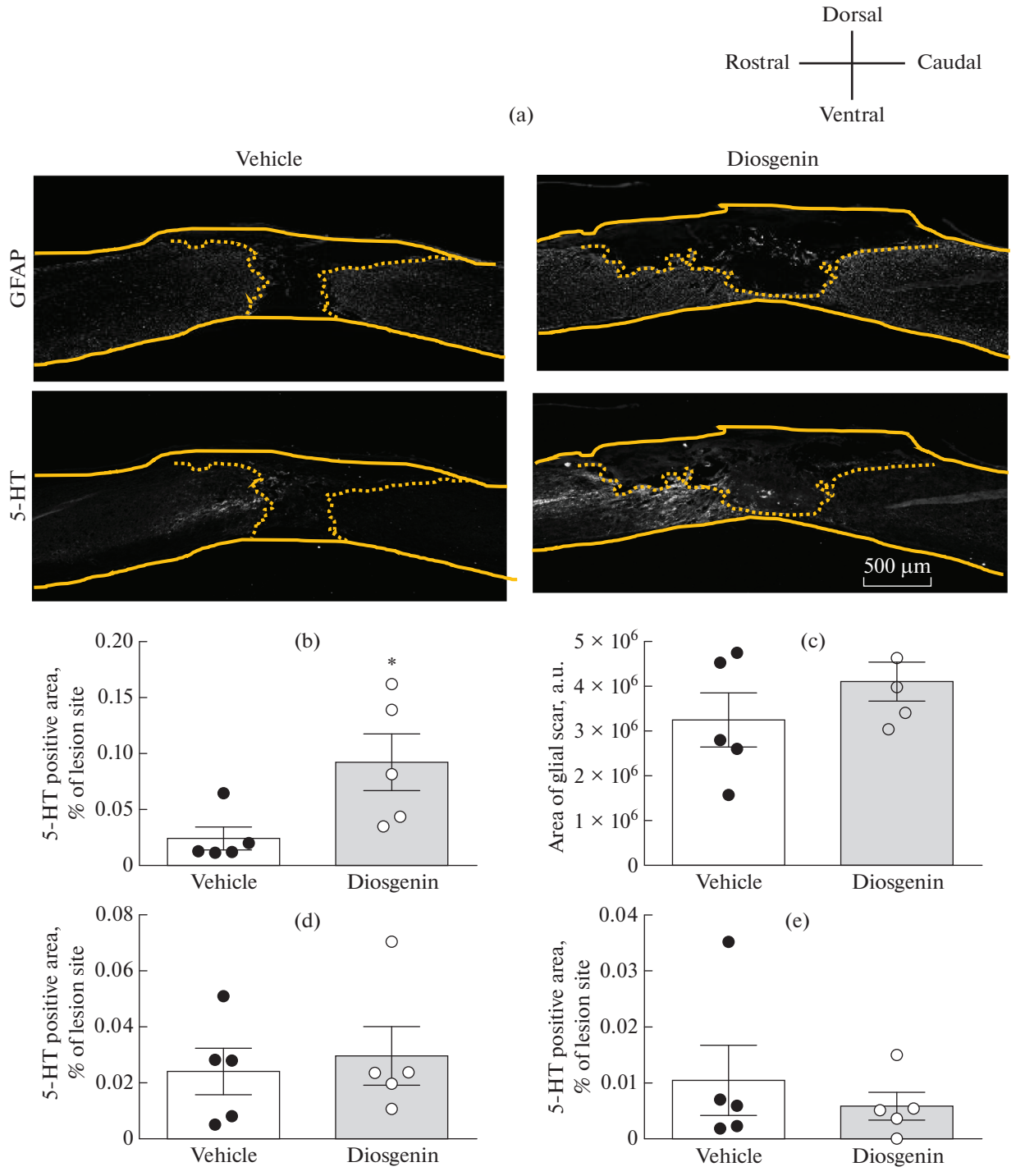

Fig. 2. Effects of the intrathecal diosgenin infusion on the increase in axons at the lesion center of mice with chronic spinal cord injury (SCI). Thirty-one days after SCI, diosgenin (final concentration in CSF; 0.1 μM) or a vehicle solution was intrathecally injected using a mouse intrathecal catheter and a mini osmotic pump for 59 days. Fluorescent immunostaining was performed in the spinal cord slices using 5-hydroxytryptamine (5-HT) antibody. 5-HT-positive regions in the gray matter were quantified at the lesion center. The lesion center was specified as the inside region of the GFAP-positive glial scar. (a) Representative images of GFAP-positive and 5-HT-positive immunostaining in the sagittal sections. The yellow lines represent the borders of the spinal cord slices, and the yellow dotted lines are the borders of the quantified regions (inside GFAP-positive glial scar). Scale bar indicates 500 μm. (b) Quantified densities of 5-HT-positive axons in the lesion center are shown as percentages of the measured lesion area. (c) Quantified glial scar area surrounded by GFAP-positive astrocytes. (d) Quantified densities of 5-HT-positive axons in the rostral position 2 mm away from the center are shown as percentages of the measured lesion area. (e) Quantified densities of 5-HT-positive axons in the caudal position 2 mm away from the center are shown as percentages of the measured lesion area. *$p <$ 0.05, two-tailed unpaired *t*-test vehicle group: $n = 5$ mice, diosgenin group: $n = 5$ mice.

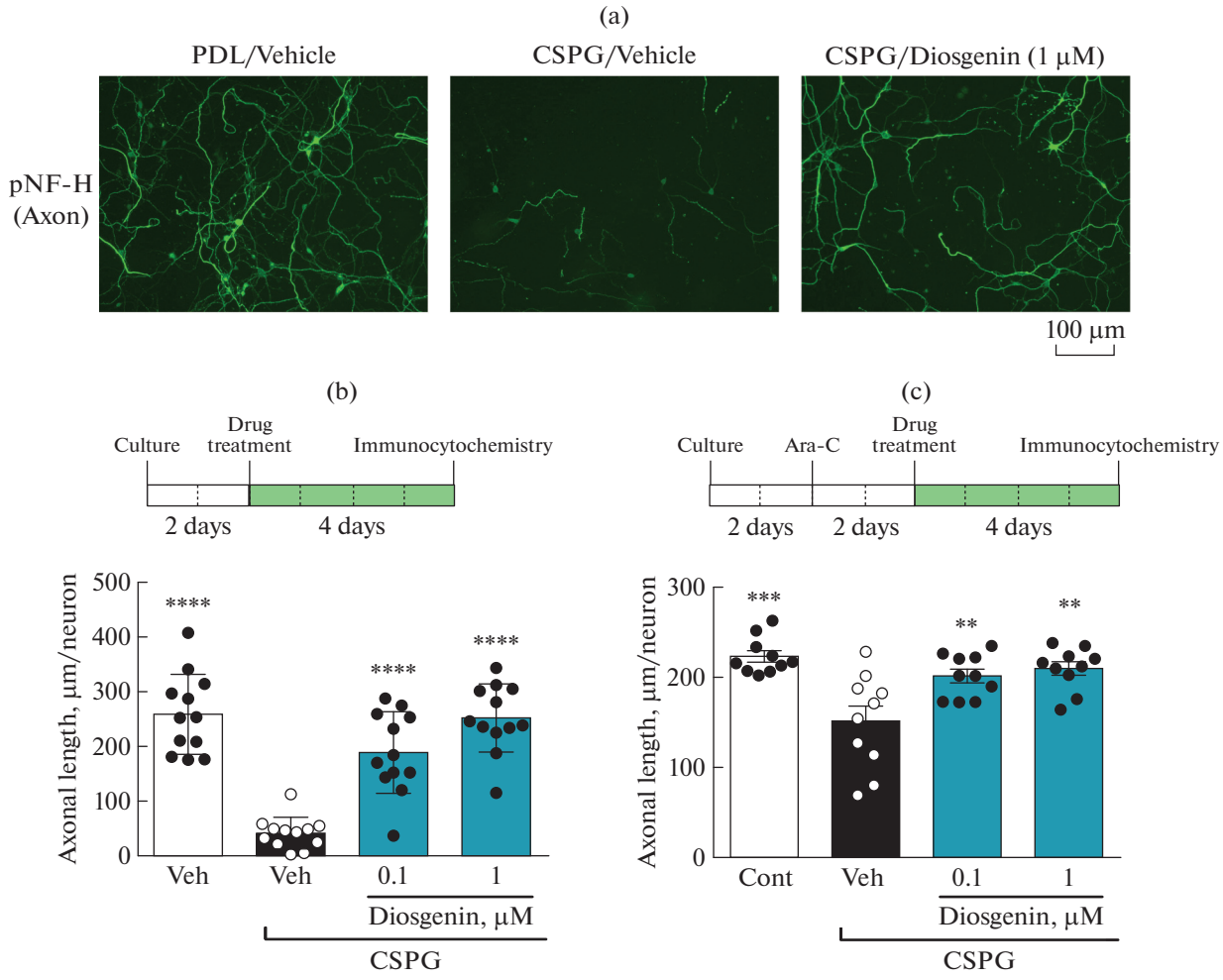

Fig. 3. Effects of diosgenin on the axonal extension in the medulla oblongata and spinal cord neurons. (a) (b) Medulla oblongata neurons were cultured with or without diosgenin on CSPG-coating. The cells were fixed and double-immunostained for pNF-H and MAP2. (a) Representative photos of the pNF-H-positive axons are shown. Scale bar indicates 50 μm. (b) The density of the pNF-H-positive axons was quantified for each treatment in the medulla oblongata neurons. (c) The density of the pNF-H-positive axons was quantified for each treatment in spinal cord neurons. $^{**}p$ the < 0.01, $^{***}p < 0.001$, $^{****}p < 0.0001$, vs. CSPG-coating/vehicle, one-way ANOVA with *post hoc* Bonferroni's test. $n = 12$ (b), $n = 10$ (c).

During and after surgery, the mice were placed on a heating pad to maintain their body temperature. Thirty-one days after SCI surgery, the mice were divided into two groups: vehicle solution (ACSF; $n = 5$) and 0.1 μM diosgenin ($n = 5$). Under anesthesia, the waist was shaved, followed by a sagittal midline incision to expose the lumbar cord. A mouse intrathecal catheter (#0007743, Alzet Osmotic Pumps, Cupertino, CA, USA) was inserted 5 mm between L5 and L6 (Fig. 1e). The free end of the cannula was connected to a micro-osmotic pump (Alzet model 1004) via a 2 cm piece of polyvinylchloride (PVC) tubing (Alzet Osmotic Pumps). The catheter was fixed to the muscle and skin using sutures. The pump was placed into a subcutaneous pocket on the back of the mouse. The infusion rate of the micro-osmotic pump was 0.11 μL/h. For the vehicle solution, ACSF (containing 130 mM NaCl, 24 mM $NaHCO_3$, 3.5 mM KCl, 1.3 mM NaH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgCl_2 \cdot 6H_2O$, and 10 mM glucose at pH 7.4) was filled into the micro-osmotic pump and connected PVC tube. In the treatment group only, the micro-osmotic pump and tube were filled with 16.4 μM diosgenin dissolved in ACSF, considering that the pump efflux was 0.11 μL/h, and cerebrospinal fluid (CSF) was produced at a speed of 0.325 μL/min [9]. Thus, the final concentration of diosgenin was 0.1 μM when it was delivered to the CSF of the SCI mice. The dose was sufficient to induce axonal extension by diosgenin (Fig. 3). Twenty-eight days after the infusion, a new pump-filled drug was replaced for a total of 56 days of continuous drug delivery. Three days after day 56, the mice were euthanized for histological evaluation.

Behavioral evaluation. For behavioral scoring after surgery, the mice were placed in an open cage (black color, 50.0 cm × 42.5 cm × 15.0 cm) and observed

while moving free for 3 min. The motor function of the hind limbs was evaluated using the Basso Mouse Scale (BMS) [10], Toyama Mouse Score (TMS) [11], and vertical cage test [8] under 500-lux illumination. The vertical cage scale wire netting (29.0 cm × 61.8 cm, grid width; 1.4 cm × 1.4 cm) was placed at an 85° angle. A mouse was placed at the bottom of the net, and the climbing performance was observed and evaluated with appropriate scales independently for the left and right hindlimbs (Table S1). The left and right hind limb movements were evaluated independently. Behavioral observations were performed once every seven days during the pre-injection period (31 days) and once every two days during the injection period (59 days).

Immunohistochemistry for 5-hydroxytryptamine (5-HT)-positive axonal tracts [8]. After the behavioral observations, the tissue from the mice was isolated by perfusing 4% paraformaldehyde in phosphate-buffered saline under anesthesia by administering a mix of three anesthetics by intraperitoneal injection. The spinal cords were dissected at the T13-L3 level, soaked in 30% sucrose, embedded with cryomold 3 (Sakura Finetech Japan, Tokyo, Japan), and stored at −30°C until use. Sagittal sections of the spinal cords were cut into 14 μm slices using a cryostat (CM 3050S; Leica Microsystems, Wetzlar, Germany). After post-fixation in 4% paraformaldehyde, the sections were immunostained for 24 h at 4°C with the rabbit polyclonal anti-serotonin (5-HT; 1 : 1000, Sigma-Aldrich) and mouse monoclonal anti-glial fibrillary acidic protein (GFAP; 1 : 1000, Sigma-Aldrich) antibodies. Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1 : 400, Life Technologies) and Alexa Fluor 594-conjugated goat anti-mouse plus IgG_1 antibody (1 : 400, Life Technologies) were used as secondary antibodies. Nuclei were stained with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI). Images were captured using a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan) and quantified using Image J (National Institutes of Health, Bethesda, MD, USA). The injured area was defined as the inside surrounded by the GFAP-positive area, where the glial scar formed. Quantification was performed on the three most center slides of the serial sections. Areas of 5-HT-positive axons in the lesion area, the rostral position 2 mm away from the center and the caudal position 2 mm away from the center were quantified. The region of interest (ROI) was selected as the quantified area, and a 5-HT-positive area was detected in the ROI. The percentage of 5-HT-positive areas to the ROI was calculated.

Primary culture. The primary culture was performed as previously described [5]. Pregnant mice and embryos of ddY mice (Japan SLC, Shizuoka, Japan) were anesthetized with isoflurane (FUJIFILM Wako Pure Chemical, Osaka, Japan). Embryos were removed 14 days after gestation. The spinal cord or medulla oblongata was dissected, and the dura mater was removed. The tissues were minced, dissociated, and grown in cultures with neurobasal medium (Invitrogen, Grand Island, NY, USA) that included 12% B-27 supplement (Invitrogen), 0.6% D-glucose, and 2 mM L-glutamine in eight well chamber slides (Falcon, Franklin Lakes, NJ, USA) coated with 5 μg/mL poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified incubator with 10% CO_2. For CSPG-coating, culture dishes were coated with 5 μg/mL poly-D-lysine (PDL) and 2 μg/mL aggrecan (Sigma-Aldrich) in phosphate-buffered saline overnight at 37°C. The spinal cord culture was treated with 2 μM cytosine β-D-arabinofuranoside (Ara-C, Sigma-Aldrich) for two days to increase the purity of the neurons (% of neurons is 90.2%). The seeding cell density was 4.4×10^4 cells/cm^2.

Measurement of the axonal length. Evaluation of the axonal length was performed as previously described [5]. Two days after the culture, the cells were treated with or without diosgenin or vehicle solution (distilled water containing 0.1% ethanol) for four days. The neurons were fixed with 4% paraformaldehyde for 30 min and immunostained with a polyclonal antibody against microtubule-associated protein 2 (MAP2, 1 : 1000, Abcam, Cambridge, UK) as a neuron marker. A monoclonal antibody against phosphorylated neurofilament-H (1 : 250, SMI-35, Covance, Dedham, MA, USA) was used as an axonal marker. Alexa Fluor 594-conjugated goat anti-rabbit IgG (1 : 400) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1 : 400) were used as secondary antibodies (Molecular Probes, Eugene, OR, USA). Nuclear counterstaining was performed using DAPI (1 μg/mL, Sigma-Aldrich). The fluorescence images were captured with a 10× objective lens using a fluorescence microscope system (Cell Observer, Carl Zeiss, Tokyo, Japan). In total, 11 to 15 images were captured per treatment. The lengths of the pNF-H-positive axons were measured using a MetaMorph analyzer (Molecular Devices, Sunnyvale, CA, USA), which automatically traced and measured the neurite length without measuring the cell bodies. The numbers of MAP2-positive and DAPI-positive cells were counted using MetaMorph. The sum of the axon lengths was divided by the number of MAP2-positive neurons.

Detection of $1,25D_3$-membrane-associated rapid response steroid-binding receptor ($1,25D_3$-MARRS). The neurons were fixed with 4% paraformaldehyde for 30 min and immunostained with a polyclonal antibody against $1,25D_3$-MARRS (1 : 200, Ab099 clone, gifted by Dr. Ilka Nemere). A monoclonal antibody against MAP2a, 2b (1 : 150, Thermo Fisher Scientific, Waltham, MA, USA) was used as a neuron marker (Fig. 4). Alexa Fluor 568-conjugated goat anti-rabbit IgG (1 : 400) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1 : 400) were used as secondary antibodies (Molecular Probes). Nuclear counterstaining was performed using DAPI (1 μg/mL, Sigma-

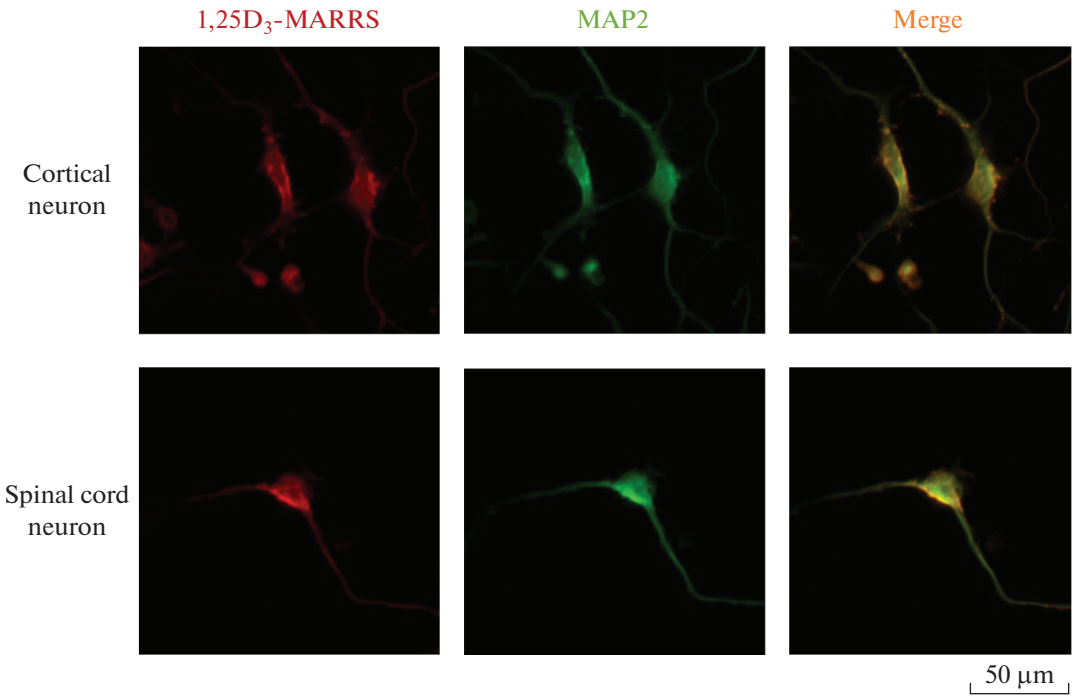

Fig. 4. Detection of $1,25D_3$-MARRS on cortical and spinal cord neurons. Cortical neurons and spinal cord neurons were cultured for 4 days. The cells were fixed and double-immunostained for $1,25D_3$-MARRS and MAP2. Scale bar indicates 50 μm.

Aldrich). The fluorescence images were captured with a 10× objective lens using a fluorescence microscope system (Cell Observer).

Statistical analysis. Data are expressed as the mean ± standard error. The statistically significant differences were determined using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA), and running one-way analysis of variance (ANOVA) *post hoc* Bonferroni's test (Figs. 3b, 3c) and repeated measures two-way ANOVA *post hoc* Bonferroni's test (Figs. 1a–1c), Mann-Whitney U test (Fig. 1d), and two-tailed unpaired *t*-test (Figs. 2b, 2c). Data are shown as the mean ± standard error. The significance level was set at 5%.

RESULTS

Intrathecal diosgenin infusion improves motor function in chronic spinal cord injured mice. In our previous investigation, intraperitoneal administration of diosgenin to the chronic phase of SCI mice gave no recovery effect on motor function. Therefore, we investigated the effect of intrathecal diosgenin administration on functional recovery in the chronic phase of SCI mice in this study. Thirty-one days after injury, continuous administration of diosgenin or vehicle solution (ACSF) was started using a micro-osmotic pump for totally 56 days (Fig. 1e). The concentration of diosgenin was maintained at approximately 0.1 μM in the CSF during the administration period, which is considered an effective dose in culture cell experiments (Fig. 3). The hind limb motor functions were evaluated using the BMS (Fig. 1a), TMS (Fig. 1b), and vertical cage scale (Fig. 1c). The BMS and TMS scores of diosgenin-treated mice were gradually and significantly elevated compared to the scores of vehicle-treated mice. In each scoring, repeated two-way ANOVA analyses showed a significant difference in time × drug interactions between the vehicle-treated group and diosgenin-treated group ($F(45, 810) = 3.764$, $p < 0.0001$ in the BMS; $F(45, 810) = 1.851$, $p < 0.001$ in the TMS). A vertical cage test was performed on the final day of observation (59 days after infusion). The scale point of diosgenin-treated mice was significantly higher than that of vehicle-treated mice (Fig. 1d). No significant body weight changes were observed between the two groups during the experimental period (Fig. 1c).

After the behavioral tests, the spinal cord was isolated. Slices of the spinal cord that included the lesion area were prepared and immunostained with the raphespinal tract marker, 5-HT (Fig. 2a). The raphespinal tract is serotonergic and is one of the major descending tracts that modulate the excitability of motor neurons. To determine the size of lesion area, the GFAP-positive glial scar area was stained. Diosgenin intrathecal infusion increased the density of 5-HT-positive axons at the lesion center in mice with chronic SCI (Fig. 2b). The areas of glial scar were not changed by diosgenin treatment (Fig. 2c). In rostral part (Fig. 2d) and caudal part (Fig. 2e) 2 mm away

from the center region, 5-HT-positive axons were not increased by diosgenin treatment.

Diosgenin induces axonal extension in spinal cord and medulla neurons. Mouse medulla (Figs. 3a, 3b) and spinal cord neurons (Fig. 3c) were seeded on CSPG-coated dishes and treated with diosgenin or vehicle solution for four days. Axonal density was significantly lower in-vehicle solution-treated neurons cultured on CSPG substrate than in those cultured without CSPG. In contrast, diosgenin significantly increased the axonal density at doses of 0.1 and 1 μM.

Our previous study identified the receptor of diosgenin was $1,25D_3$-MARRS [5–7]. Expression of $1,25D_3$-MARRS was investigated in cultured cortical and spinal cord neurons. All neurons expressed $1,25D_3$-MARRS at cell surface in spinal cord neurons as well as cortical neurons (Fig. 3d).

DISCUSSION

The diosgenin intrathecal infusion significantly improved motor function and density of the raphespinal tracts in SCI mice. The effectiveness of diosgenin in the chronic phase of SCI is critical because very few interventions have been reported as efficacious for chronic SCI. Diosgenin treatment resulted in no change in the glial scar size but increased 5-HT-positive raphespinal tract density (Fig. 2). In the in vitro experiments, we selected medulla oblongata neurons and spinal cord neurons because the 5-HT-positive raphe nucleus is located in the medulla oblongata. In the spinal cord tissue, interneurons and motor neurons are included. Figure 3 indicates that diosgenin extended the axons of both the medulla oblongata and spinal cord neurons. Among the descending tracts, the raphespinal tract is one of the major tracts regulating locomotor function and is important for the recovery of motor function after SCI. Many reports about therapies for SCI showed a positive correlation between the growth of raphespinal tracts and the recovery of motor function [12–14]. Even in chronic phase of spinal cord injury, the raphespinal tract is the main target of evaluation [15, 16]. However, other descending tracts and interneurons should also be evaluated in the future, although this study evaluated the axonal density of raphespinal tracts in vivo (Figs. 2a, 2b).

Diosgenin enhanced axonal growth, even on CSPG-coating (Fig. 3). Since diosgenin extended axons on normal PDL coating in our previous study [5] diosgenin probably demonstrates axonal extension activity independent of CSPG-elicited axonal inhibitory signaling. We revealed the diosgenin signal pathway in neurons, in which $1,25D_3$-MARRS is stimulated by diosgenin [5–7] and activates downstream protein kinases such as PKA, PKC, PI3K, and MEK [5]. As shown in Fig. 3d, $1,25D_3$-MARRS was expressed in spinal cord neurons as well as cortical neurons. Therefore, in spinal cord injury, diosgenin seems to stimulate $1,25D_3$-MARRS. The PKA pathway is associated with axonal extension in cortical neurons [17]. PKC phosphorylates GAP-43 in growth cones, resulting in axonal outgrowth [18]. The PI3K-Akt pathway is known to regulate axon regeneration [19]. PI3K also regulates Cdc42, a key regulator of cytoskeletal reorganization in axonal tips [20]. ERK signaling is related to local axon assembly [21] and local protein translation at the growth cone [22]. This study selected intrathecal infusion as a dosing route for diosgenin, as this was considered the most deliverable approach to the lesion site. In human clinical situations, the Food and Drug Administration approved baclofen as an intrathecal infusion medicine using an implantable pump [23]. Therefore, intrathecal diosgenin infusions could be used for chronic SCI in humans.

CONCLUSIONS

The intrathecal infusion of diosgenin improved the motor function and raphespinal tract density in the spinal cord when applied in the chronic phase after injury. Diosgenin can extend axons on CSPG-coating in cultured neurons. Diosgenin is a potential candidate as a therapeutic approach for chronic SCI.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no competing interests.

Ethical approval. All experiments were performed following the Guidelines for the Care and Use of Laboratory Animals of the Sugitani Campus of the University of Toyama. All protocols were approved by the Committee for Animal Care and Use of the Sugitani Campus of the University of Toyama. The approval number for animal experiments was A2016INM-3 and A2019INM-3. All efforts were made to minimize the number of animals used.

SUPPLEMENTARY INFORMATION

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