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Combinatrial treatment of anti-High Mobility Group Box-1 monoclonal antibody and epothilone B improves functional recovery after spinal cord contusion injury

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ABSTRACT

Spinal cord injury (SCI) causes motor and sensory deficits and is currently considered an incurable disease. We have previously reported that administration of anti-High Mobility Group Box-1 monoclonal antibody (anti-HMGB1 mAb) preserved lesion area and improved locomotion recovery in mouse model of SCI. In order to further enhance the recovery, we here examined combinatorial treatment of anti-HMGB1 mAb and epothilone B (Epo B), which has been reported to promote axon regeneration. This combinatorial treatment significantly increased hindlimb movement compared with anti-HMGB1 mAb alone, although Epo B alone failed to increase functional recovery. These results are in agreement with that anti-HMGB1 mAb alone was able to decrease the lesion area spreading and increase the surviving neuron numbers around the lesion, whereas Epo B facilitated axon outgrowth only in combination with anti-HMGB1 mAb, suggesting that anti-HMGB1 mAb-dependent tissue preservation is necessary for Epo B to exhibit its therapeutic effect. Taken together, the combinatorial treatment can be considered as a novel and clinically applicable strategy for SCI.

1. Introduction

Spinal cord injury (SCI) disrupts neural networks and causes motor and sensory functional loss, bringing physical and mental suffering on patients. Following the direct damage to the spine which may results from earthquake, traffic accident, fall or violence, local immune response-induced secondary damage triggers a sequential process including inflammation, necrosis and apoptosis, leading to further damage to the spinal cord (Aidemise, 2011). For this reason, reducing secondary damage is considered as a promising therapeutic approach for SCI.

High Mobility Group Box-1 (HMGB1) is one of the damage-associated molecular patterns and has been reported to play an important role in the secondary injury following central nervous system (CNS) injuries: once released from damaged cells, it can bind to cell surface receptors, such as the receptor for advanced glycation end products, toll-like receptor (TLR) 2 and TLR4, which induce blood brain barrier (BBB) disruption and inflammatory response (Hayakawa et al., 2010; Lotze and Tracey, 2005; Zhang et al., 2011). We have previously shown that administration of anti-HMGB1 monoclonal antibody (mAb) after SCI in the early acute phase attenuate secondary damage and promoted motor functional recovery in mouse model (Nakajo et al., 2019; Uezono et al., 2018). Compared with untreated mice, those received anti-HMGB1 mAb showed an increase in neurite sprouting from spared axons, reduced-spinal cord swelling and -neuronal apoptosis, indicating decreased blood-spinal cord barrier (BSCB) disruption.

Since mice treated with anti-HMGB1 mAb showed only partial recovery of motor function after SCI, aiming for a further improvement, we previously transplanted human induced pluripotent stem cells-derived neural stem cells (hiPSC-NSCs) into mouse spinal cord

Abbreviations: SCI, spinal cord injury; HMGB1, High Mobility Group Box-1; mAb, monoclonal antibody; Epo B, epothilone B; CNS, central nervous system; TLR, toll-like receptor; BBB, blood brain barrier; BSCB, blood-spinal cord barrier; RRID, Research Resource Identifiers; hiPSC-NSCs, human induced pluripotent stem cells-derived neural stem cells; BW, body weight; BMS, Basso Mouse Scale; PBS, phosphate buffered saline; GFAP, glial fibrillary acidic protein; 5-HT, 5-hydroxytryptamine; RST, raphespinal tract; i.p., intraperitoneal; CST, corticospinal tract; ChAT, Choline acetyltransferase; TNF-α, tumor necrosis factor α.

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after anti-HMGB1 mAb administration (Uezono et al., 2018). As a result, neurons differentiated from hiPSC-NSCs integrated into host neural circuit and further improved the anti-HMGB1 mAb-derived functional recovery after SCI. However, the use of iPSC-derived cells still retains concerns such as teratoma formation, cell preparation and financial burden, which makes it unsuitable for current clinical application (Gutierrez-Aranda et al., 2010; Huang et al., 2019; Seki and Fukuda, 2015).

In right of these facts, we attempted to find a simple, economical and clinically applicable method that can enhance the therapeutic effect of anti-HMGB1 mAb for SCI. We thus focused, in this study, on epothilone B (Epo B), which has been known as an anticancer drug approved by U.S. Food and Drug Administration and has been shown to be effective in rat model of SCI (Forli, 2014; Ruschel et al., 2015). Epo B binds to β-tubulin subunits in microtubules and increase microtubule stability, affecting the microtubule-based functions such as cell division, cell migration and neurite outgrowth (Pagano et al., 2012; Ruschel et al., 2015). Previous study has reported that administration of low doses of Epo B promoted axon elongation and improved motor function in a rat model of spinal cord dorsal hemisection (Ruschel et al., 2015).

In the present study, we examined the therapeutic effect of a combination of anti-HMGB1 mAb and Epo B treatments in contusion SCI mouse model. In contrast to hemisection SCI model (Pagano et al., 2012; Ruschel et al., 2015), we found no effect of Epo B treatment alone on sprouting of remaining axons and locomotion recovery after contusion SCI. However, the combination therapy of anti-HMGB1 mAb and Epo B significantly improved the axonal outgrowth and motor functional recovery compared with either treatment alone, suggesting that anti-HMGB1 mAb-mediated suppression of contusion injury-induced secondary damage is prerequisite for Epo B to reveal the therapeutic effect. Therefore, it is conceivable that both anti-HMGB1 mAb-mediated preservation of lesion area and Epo B-induced axonal sprouting are necessary for efficient functional recovery after contusion SCI.

2. Experimental procedure

2.1. Animals

This study was exploratory and all mouse experiments were conducted in accordance with guidelines of the Kyushu University Center for Animal Resources and Development. A total of 55 female C57BL/6J mice (aged 8–10 weeks, weight = 18 g–22 g, Japan SLC, Research Resource Identifiers (RRID): IMSR_JAX:000664) were used in this study. All mice were arbitrarily assigned to experimental groups and no randomization was performed. For dose determination analysis, 9 mice were randomly divided into three groups; for behavioral analysis, 40 mice were randomly divided into four groups; for NMDA injection experiment, 6 mice were randomly divided into two groups. Mice were housed under a 12-h light-dark cycle in a specific pathogen-free facility with controlled temperature and humidity, and allowed free access to food and water.

2.2. Contusion SCI model

Contusive SCI was performed as described previously (Fujimoto et al., 2012). We used a moderate SCI model (Yokota et al., 2015). Briefly, Mice anesthetized with a mixture of 4 mg/kg midazolam, 0.3 mg/kg medetomidine, and 5 mg/kg butorphanol received partial laminectomies and laminectomies at the ninth and tenth thoracic spinal vertebrae, respectively. The dorsal surface of the dura mater was exposed and injury was induced at the Th9−10 level using an SCI device (70 kilodyne to induce SCI, Infinite Horizon Impactor; Precision Systems & Instrumentation). The muscle and skin were closed in layers.

2.3. anti-HMGB1 mAb and Epo B administration

8 mg/kg body weight (BW) of anti-HMGB1 mAb or control IgG2a mAb was intraperitoneally administrated to mice at 5 min after SCI (Nakajo et al., 2019; Uezono et al., 2018). 3 mg/kg BW of Epo B (A10360, Adooq, Irvine, CA) (dissolved in 1:1 mixture of DMSO and saline) or vehicle was intraperitoneally administrated to mice at day 1 and day 15 after SCI. 4 mg/kg of Epo B was intraperitoneally administrated to mice at day 1 after SCI.

2.4. Behavioral analysis

Two people, blinded to the treatment of the mice, examined motor function in an open field using the Basso Mouse Scale (BMS) locomotor rating scale once a week. (Basso et al., 2006). Hindlimb movements of the mice were captured using a high-definition digital camcorder. We edited these movies and export movie files using editing software. Footprint analysis and grid walking test were performed 56 days after SCI. For footprint analysis (Uezono et al., 2018), the forelimbs and hindlimbs of the mice were dipped in black and red dye, respectively. A narrow runway (80 cm length and 4 cm width) was lined with white paper for the animal to walk across. Paw rotation was defined as the angle between axes of the two back paws. Measurements were taken on each side for three consecutive steps and were averaged. Mice that badly dragged their hindlimbs were excluded from these measurements. Grid walking test was performed to evaluate the ability of the mice to locomote over a wire mesh grid (2.5 × 2.5 cm^2 grid spaces, 35 × 35 cm^2 total area, 7 cm height). As control and Epo B treatment groups showed poor plantar stepping, only anti-HMGB1 mAb and combination groups were tested on the grid. Before testing, mice were trained to walk over the mesh for 3 min each day for 3 days. Each mouse was videotaped for 3 min while on the grid. A mis-step was counted when a hindlimb paw protruded entirely through the grid with all toes and heel extended below the wire surface. Total walking steps without a mis-step were calculated and expressed as a percentage.

2.5. Immunohistochemistry

Anesthetized mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Spinal cords were dissected and postfixed overnight in the same fixative at 4 °C. The fixed tissues were cryoprotected successively in 10 % and 20 % sucrose in PBS overnight at 4 °C, embedded in OCT compound, and sectioned axially or sagittally at 50 μm or 20 μm on a cryostat, respectively. Tissue sections were stained with primary antibodies to glial fibrillary acidic protein (GFAP) (1:500, Z0334, DAKO, Carpinteria, CA, RRID: AB_10013382), Laminin (1:500, L9393, Sigma, St. Louis, MO, RRID: AB_477163), 5-hydroxytryptamine (5-HT) (1:200, 1338, ImmunoStar, Hudson, WI, RRID: AB_572263), NeuN (1:500, MAB377, Millipore, Billerica, MA, RRID: AB_2298772), ChAT (1:500, AB144 P, Sigma, St. Louis, MO, RRID: AB_2079751), β-tubulin III (1:500, T3952, Sigma, St. Louis, MO, RRID: AB_1841226). The sections were then incubated with Alexa Fluor-conjugated secondary antibodies (1:500, Biotium, Fremont, CA). Nuclei were stained with Hoechst (bisbenzimide H33258 fluorochrome trihydrochloride, Nacalai Tesque).

2.6. Image acquisition and quantitative analysis

All images were obtained using a fluorescence microscope scanning laser confocal imaging system (LSM 800, Zeiss, Jena, Germany). To evaluate glial scar and fibrotic scar area, both cross and sagittal

sections including the epicenter were selected and the GFAP-negative area around the lesion site and Laminin-positive area in each section were measured by ImageJ. To evaluate survived host neuron number, three sagittal sections (20 μm thick, 180 μm apart; the section including the epicenter was defined as the second section) in each mouse were selected and the average number of NeuN-positive cells was counted in the area 500 μm^2 x 500 μm^2 photographed at the areas of 500 μm rostral and caudal to the epicenter. To evaluate elongation of raphespinal tract (RST) fibers, serial cross sections (50 μm thick, 450 μm apart) from rostral to caudal were selected and the 5-HT-positive area in each section was measured by ImageJ. Each value was expressed as a percentage of that in the area 500 μm rostral to epicenter. To evaluate synaptic formation, three sagittal sections (20 μm thick, 180 μm apart; the section including the epicenter was defined as the second section) in each mouse were selected and the average number of βIII-tubulin, NeuN-positive neurons surrounded by 5-HT-positive fibers were counted in an area 500 μm^2 x 500 μm^2 photographed at the areas of 500 μm caudal to the scar border.

2.7. Ablation of host neurons

Endogenous neuron ablation experiments were performed as described previously (Abematsu et al., 2010; Uezono et al., 2018). Seven weeks after injury, we stereotaxically injected the excitotoxic glutamate receptor agonist N-methyl-D-aspartic acid (NMDA) (1 μL of 10 mM in saline per site) into three sites of the spinal cord gray matter at 1 mm rostral, the epicenter, and 1 mm caudal to the lesion site (tenth thoracic level)

2.8. Statistical analysis

No statistical methods were used to determine the sample numbers in advance. Statistical Analysis was not blind and performed using Prism 8 (GraphPad Software Inc.). Shapiro–Wilk (S-W) normality test was carried out to assess the normality of all data. Values greater than the mean + 3 standard deviations (SD) or less than the mean – 3 SD were considered outliers and excluded. An unpaired two-tailed student's *t*-test was used for single comparisons of normally distributed data and a Mann-Whitney test was used for single comparisons of non-normally distributed data. A two-way analysis of variance (ANOVA) was used to assess the effect of anti-HMGB1 mAb and Epo B and Fisher's LSD post hoc analysis was used for multiple comparisons. $p < 0.05$ was considered significant.

3. Results

3.1. Combinationatrial treatment of anti-HMGB1 mAb and Epo B improve locomotion recovery after SCI

Before we investigate the effect of the combinatorial administration of anti-HMGB1 mAb and Epo B, we first examined whether intraperitoneal (i.p.) injection of Epo B has any adverse effects. In a previous study using rat SCI model, Ruschel et al. adopted i.p. injection with 0.75 mg/kg BW of Epo B at day 1 and 15 after SCI (Ruschel et al., 2015). Since the concentration of Epo B in rat spinal cord is 40 ng/g 1 week after i.p injected at 0.75 mg/kg BW (Ruschel et al., 2015), while that in mouse spinal cord is 10 ng/g when ip injected at 1 mg/kg BW (Wang et al., 2018), we administered mice with 3 and 4 mg/kg BW of Epo B at day 1 and 15 after SCI (Figure S1 A). In contrast to 4 mg/kg BW, 3 mg/kg of Epo B did not reduce survival rate (Figure S1 B), and only caused a transient BW loss at 1 week after injury compared with untreated control (Figure S1 C). We thus decided to employ the dose of 3 mg/kg Epo B in the following experiments.

Next, we investigated whether administration of Epo B affects the hindlimb motor functional recovery after contusion SCI. We found that the administration of Epo B alone had no effect on locomotion improvement (Fig. 1 A-C and Supplementary Video), in contrast to the result observed in transection models (Ruschel et al., 2015).

We then examine whether Epo B treatment enhances this anti-HMGB1 mAb-improved locomotion after SCI. Since we have previously shown that treatment with anti-HMGB1 mAb at 5 min and 3 h after SCI are equally effective to improve hindlimb motor function (Nakajo et al., 2019), we administered mice with the antibody 5 min after SCI, and i.p. injected Epo B at day 1 and 15 post injury (Fig. 1A). Basso Mouse Scale (BMS) open-field motor score was used to evaluate hindlimb recovery (Basso et al., 2006; Uezono et al., 2018). Consistent with previous reports, administration of anti-HMGB1 mAb alone significantly improved functional recovery compared with control (Fig. 1B and C, and Supplementary Video). Moreover, combination of anti-HMGB1 mAb and Epo B treatment dramatically improved functional recovery, even compared with anti-HMGB1 mAb alone groups (Fig. 1B and C, and Supplementary Video). Furthermore, combination and anti-HMGB1 mAb alone groups exhibited less toe dragging compared with control and Epo B alone groups in a footprint analysis (Cheng et al., 1997; Kunkel-Bagden et al., 1993) at 8 weeks after SCI (Fig. 1D). Since paw positioning is a reliable index for evaluating motor function of mice, we measured the angle of paw rotation, defined as the angle between the axes of the two hind paws, although we could not quantify paw rotation and excluded control and Epo B alone groups because these mice badly dragged their hindlimbs (Fig. 1D). The quantitative analysis showed that combination groups have smaller paw rotation angle compared with anti-HMGB1 mAb alone groups, indicating the enhanced improvement in the combinatorial treatment group (Fig. 1E). We next performed grid walking test to evaluate whether fine motor skills are improved by the combinatorial treatment (Ma et al., 2001). As a result, no difference was observed between anti-HMGB1 mAb alone and combination groups (Fig. 1F). Since fine motor skills are mainly controlled by the corticospinal tract (CST) (Liu et al., 2017), this result suggests that combination treatment has very little effect, if any, on the regeneration of CST. In aggregate, our results suggest that administration of Epo B, in concert with anti-HMGB1 mAb, has a therapeutic effect to improve locomotion recovery of SCI mice.

3.2. Epo B does not affect anti-HMGB1 mAb-iduced preservation of lesion site

Our previous study has already demonstrated that anti-HMGB1 mAb can protect BSCB from disruption and suppress inflammation in the early acute phase, leading to decreased lesion area surrounded by GFAP-positive astrocytes and increased survival of neurons in the spinal cord (Nakajo et al., 2019; Uezono et al., 2018). To examine the underlying mechanism of combination treatment-derived functional recovery, we next investigated whether administration of Epo B further enhanced these effects of anti-HMGB1 mAb.

Immunostaining analysis indicated that at 8 weeks after injury, the lesion area surrounded by GFAP-positive astrocyte was reduced by anti-HMGB1 mAb, but not by Epo B treatment (Fig. 2). No statistically significant difference was observed between combination and anti-HMGB1 mAb alone groups (Fig. 2). Neuronal survival in the spinal cord was examined by evaluating the number of NeuN-positive cells in the area 500 μm rostral and caudal to the lesion site. Compared with control and Epo B groups, the survived neuron numbers in anti-HMGB1 mAb groups had an increased tendency in the rostral area, and were significantly higher in the caudal area (Fig. 3), consistent with our previous results (Uezono et al., 2018).

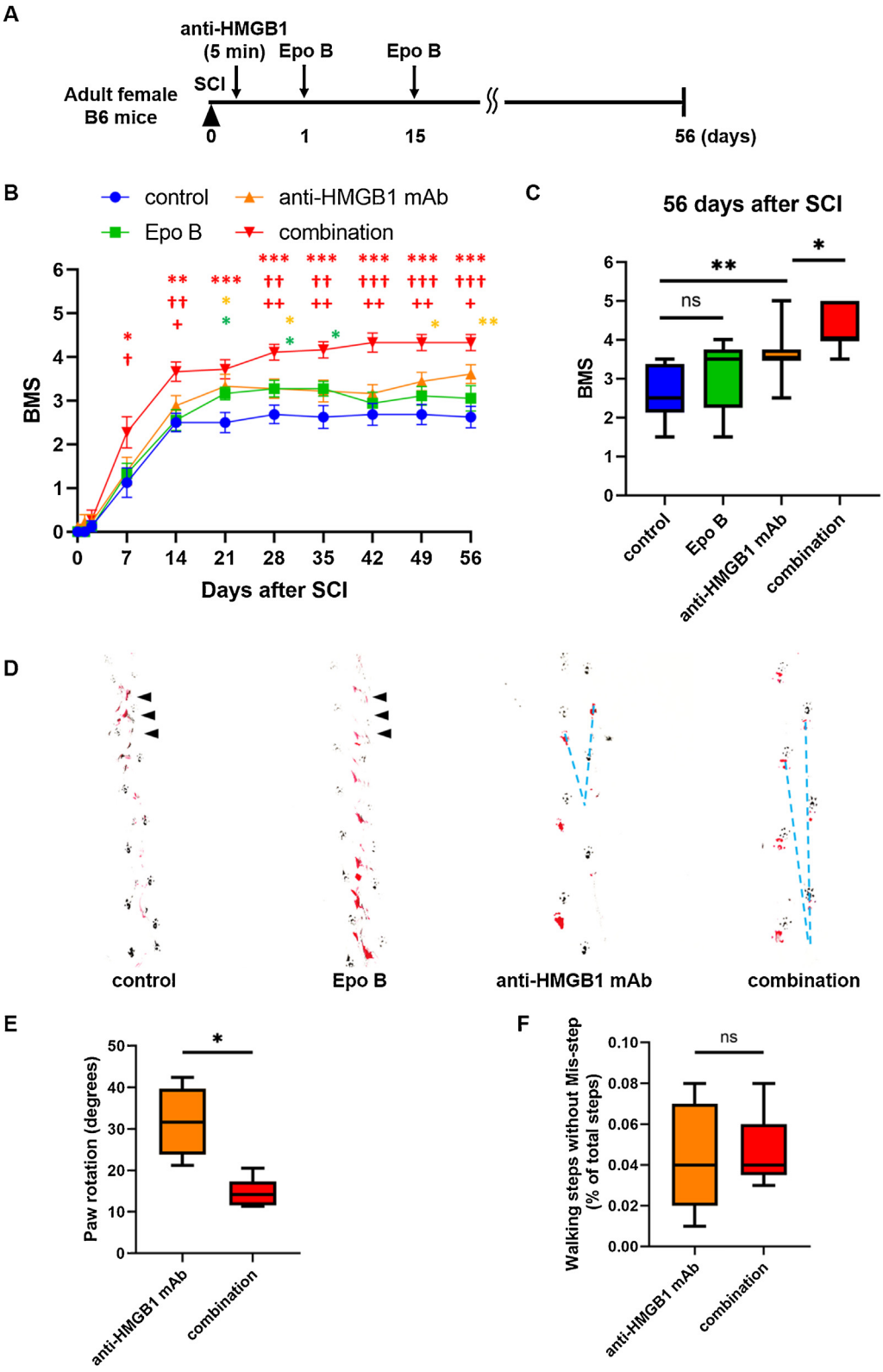

Fig. 1. Combinatrial treatment of anti-HMGB1 mAb and Epo B facilitated locomotion recovery after SCI. (A) Experimental scheme of the combination treatment. (B) Time course of hindlimbs functional recovery assessed by BMS score after SCI. n = 8 mice in control group, n = 9 mice in anti-HMGB1 mAb alone, Epo B alone groups and combination group. Data represent mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control group; †, p < 0.05; ††, p < 0.01; †††, p < 0.001 versus Epo B alone group; +, p < 0.05; ++, p < 0.01; +++, p < 0.001 versus anti-HMGB1 mAb alone group (two-way ANOVA with Fisher's LSD post hoc analysis). (C) BMS score at 56 days after SCI. n = 8 mice in control group, n = 9 mice in anti-HMGB1 mAb alone, Epo B alone groups and combination group. ns, not significant; *, p < 0.05; **, p < 0.01 versus indicated groups. (anti-HMGB1 mAb effect $F_{1,31}$ = 22.38, n = 35, p < 0.0001; Epo B effect $F_{1,31}$ = 5.804, n = 35, p = 0.0221; interaction $F_{1,31}$ = 0.3715, n = 35, p = 0.5466; two-way ANOVA with Fisher's LSD post hoc analysis). (D) Representative pictures of footprint analysis of mice in four groups at 8 weeks after SCI. Forepaws and hind paws are indicated by black and red footprints, respectively. Control and Epo B alone groups were excluded from the analysis because of toe dragging (indicated by arrowheads). Dotted lines show axis lines of hind paws. (E) Quantification of paw rotation in footprint analysis. n = 4 mice in anti-HMGB1 mAb alone group, and n = 5 mice in combination group. *, p < 0.05 versus

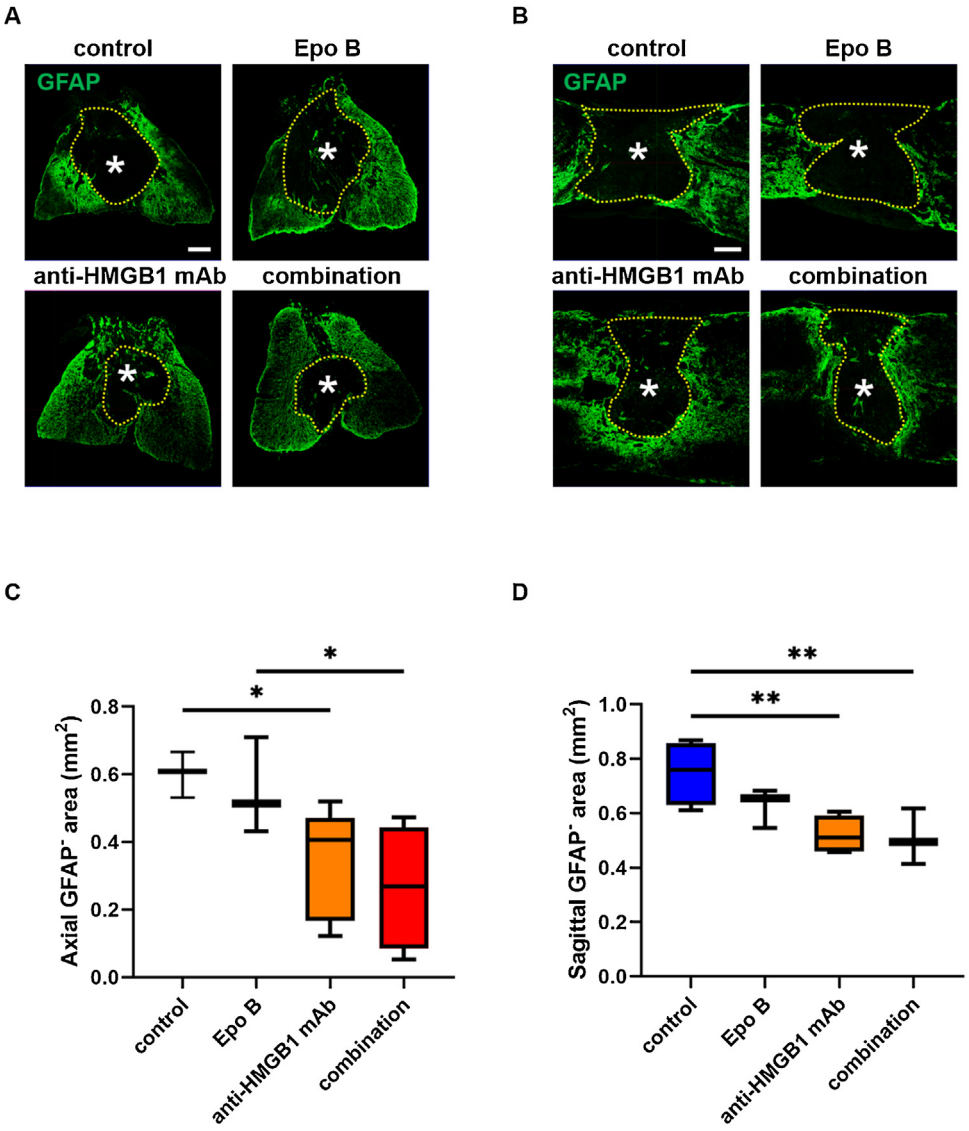

Fig. 2. Glial scar formation is inhibited by anti-HMGB1 mAb treatment. (A, B) Representative pictures of cross sections (A) and sagittal sections (B) stained for GFAP at 8 weeks after SCI. The epicenter is indicated as (*). Glial scar is defined as the area surrounded by GFAP-positive area (outlined by dotted lines). Scale bar in both cross and sagittal sections, 200 μm. (C, D) Quantification of glial scar area in (A) and (B) are indicated in (C) and (D) respectively. n = 3 mice in control and Epo B alone group, n = 5 mice in anti-HMGB1 mAb alone group, n = 4 mice in combination group (C). n = 4 mice in control and anti-HMGB1 mAb alone group, n = 3 mice in Epo B alone and combination group (D). *, $p < 0.05$; **, $p < 0.01$ versus indicated group ((C) anti-HMGB1 mAb effect $F_{1,11} = 11.46$, n = 15, p = 0.0061; Epo B effect $F_{1,11} = 0.5547$, n = 15, p = 0.472; interaction $F_{1,11} = 0.01587$, n = 15, p = 0.902; (D) anti-HMGB1 mAb effect $F_{1,10} = 11.47$, n = 14, p = 0.0069; Epo B effect $F_{1,10} = 1.69$, n = 14, p = 0.2228; interaction $F_{1,10} = 1.148$, n = 14, p = 0.3091; two-way ANOVA with Fisher's LSD post hoc analysis).
Abbreviations: GFAP, Glial fibrillary acidic protein; SCI, spinal cord injury; Epo B, epothilone B; HMGB1, High mobility group box-1; mAb, monoclonal antibody.

However, there is no significant difference between anti-HMGB1 mAb alone and combination groups (Fig. 3). These results indicate that administration of anti-HMGB1 mAb in acute phase is solely able to preserve lesion area without the help of Epo B.

Since previous research has reported that Epo B reduces fibrotic scar by inhibiting fibroblast migration in transection models (Ruschel et al., 2015), we then investigated whether Epo B inhibits fibrosis in our contusion models. Eight weeks after injury, mice were fixed and spinal cords were removed followed by immunostaining for laminin. The fibrotic scar area is defined as the laminin positive area in both cross and sagittal sections at the lesion sites. Inconsistent with previous report, fibrotic scar in Epo B groups did not show significant difference compared with that in control (Fig. 4), likely due to the different injury severity or different origin of fibroblasts in contusion and transection injury (Beattie and Bresnahan, 2000; Soderblom et al., 2013). It has been reported that pericytes from blood vessels are the main source of fibrotic scar in contusion injury, whereas in transection injury, fibroblasts from meningeal cells also participate in fibrosis when the dura matter is opened by the injury (Dias et al., 2018; Göritz et al., 2011).

anti-HMGB1 mAb alone group ($t_7 = 4.095$, p = 0.0046; unpaired Student's *t*-test). (F) Quantification of walking steps without mis-step in a grid walking test. n = 5 mice in anti-HMGB1 mAb alone group and n = 5 mice in combination group (p = 0.873; Mann-Whitney test).
Abbreviations: SCI, spinal cord injury; Epo B, epothilone B; HMGB1, High mobility group box-1; mAb, monoclonal antibody; BMS, Basso Mouse Scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

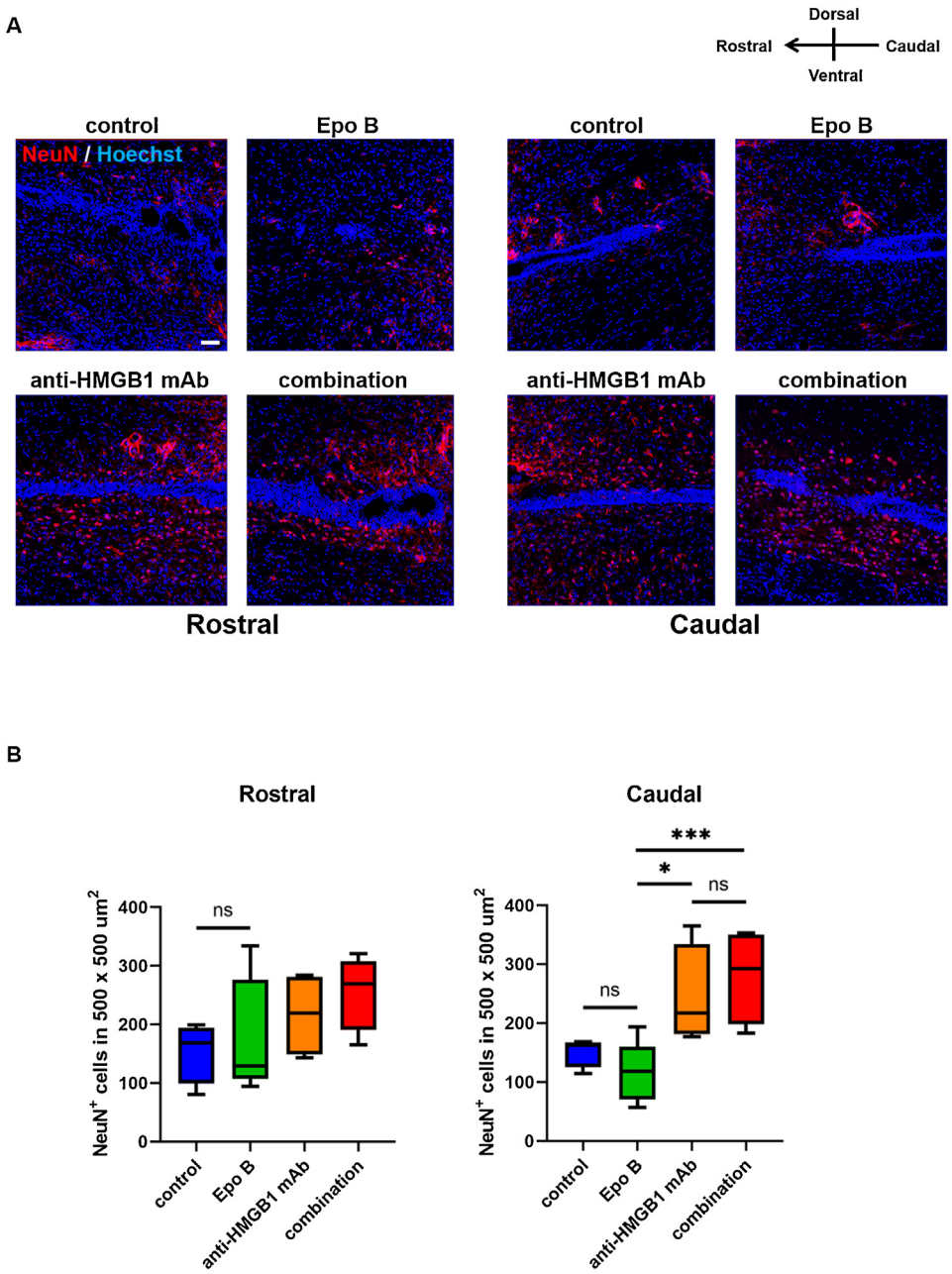

Fig. 3. Intraspinal neuron survival is promoted with anti-HMGB1 mAb treatment. (A) Representative pictures of NeuN-positive intraspinal neurons in 500 μm x 500 μm area at the site 500 μm rostral (left) and caudal (right) to the epicenter. Scale bar, 50 μm. (B) Quantification of survived neuron number in (A). n = 4 mice in control, anti-HMGB1 mAb alone and combination group. n = 5 mice in Epo B alone group. ns, not significant; *, $p < 0.05$; ***, $p < 0.001$ versus indicated group ((rostral) anti-HMGB1 mAb effect $F_{1,13} = 3.525$, n = 17, p = 0.0831; Epo B effect $F_{1,13} = 0.7514$, n = 17, p = 0.4018; interaction $F_{1,13} = 0.0373$, n = 17, p = 0.8498; (caudal) anti-HMGB1 mAb effect $F_{1,13} = 16.75$, n = 17, p = 0.0013; Epo B effect $F_{1,13} = 3.412e\text{-}005$, n = 17, p = 0.9954; interaction $F_{1,13} = 1.308$, n = 17, p = 0.2734; two-way ANOVA with Fisher's LSD post hoc analysis). Abbreviations: SCI, spinal cord injury; Epo B, epothilone B; HMGB1, High mobility group box-1; mAb, monoclonal antibody.

Although the lesion area surrounded by GFAP-positive astrocytes were reduced in anti-HMGB1 mAb and combination groups, no significant difference in the fibrotic scar area could be observed between these two groups and control groups (Fig. 4), suggesting that the tissue-protective effect of anti-HMGB1 mAb is insufficient to prevent fibrosis.

3.3. *Epo B enhances axon elongation in combination with anti-HMGB1 mAb*

Descending serotonergic raphespinal tract (RST) originating from the raphe nuclei of the brainstem play an important role in the regulation of locomotion (Ghosh and Pearse, 2015). Following SCI, RST exhibit higher regenerative capacity compared with CST (Fink and Cafferty, 2016). We have previously reported that anti-HMGB1 mAb treatment facilitated RST elongation after SCI (Uezono et al., 2018). As Epo B is also suggested to promote RST regeneration through its microtubule stabilizing effect (Ruschel et al., 2015), we next investigated whether the combination treatment-derived functional recovery resulted from enhanced RST elongation. Spinal cord cross-sections at 8 weeks after injury were stained for serotonin (5-HT) and arranged from rostral to caudal as shown in (Fig. 5A). 5-HT-positive area in each section was calculated and divided by the 5-HT-positive area in the section at 500 μm rostral to the epicenter (Fig. 5B). Consistent with our previous data, proportion of 5-HT-positive area in the caudal site of anti-HMGB1 mAb

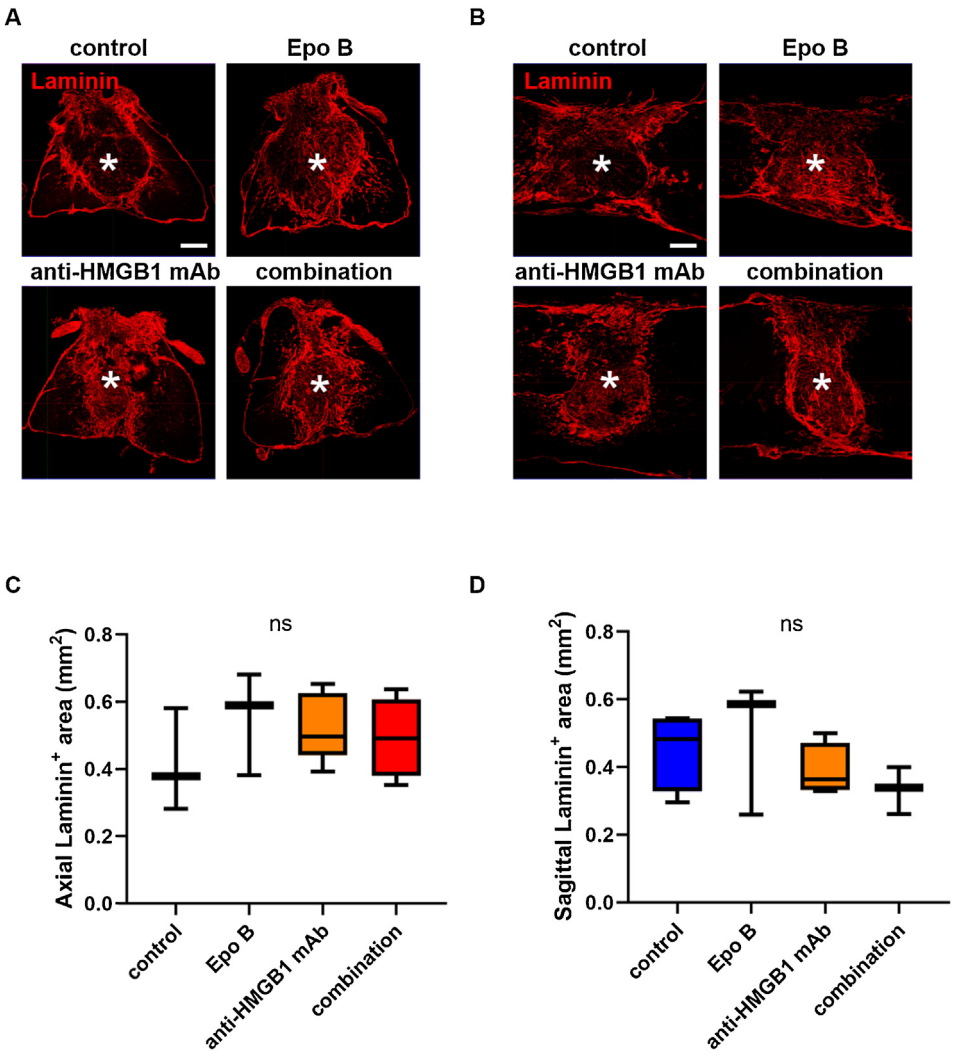

Fig. 4. Fibrotic scar formation is not changed with either or combinatorial treatment of anti-HMGB1 mAb and Epo B. (A, B) Representative pictures of cross sections and sagittal sections stained for laminin at 8 weeks after SCI. The epicenter is indicated as (*). Fibrotic scar is defined as the Laminin-positive area. Scale bar in both cross and sagittal sections, 200 μm. (C, D) Quantification of fibrotic scar area in (A) and (B) is depicted in (C) and (D), respectively. n = 3 mice in control and Epo B alone group, n = 5 mice in anti-HMGB1 mAb alone group, n = 4 mice in combination group (C). n = 4 mice in control and anti-HMGB1 mAb alone group, n = 3 mice in Epo B alone and combination group (D). ns, not significant ((C) anti-HMGB1 mAb effect $F_{1,11} = 0.1629$, n = 15, p = 0.6942; Epo B effect $F_{1,11} = 0.6025$, n = 15, p = 0.454; interaction $F_{1,11} = 1.614$, n = 15, p = 0.2301; (D) anti-HMGB1 mAb effect $F_{1,10} = 2.73$, n = 14, p = 0.1295; Epo B effect $F_{1,10} = 0.01796$, n = 14, p = 0.896; interaction $F_{1,10} = 0.4965$, n = 14, p = 0.4971; two-way ANOVA with Fisher's LSD post hoc analysis).
Abbreviations: SCI, spinal cord injury; Epo B, epothilone B; HMGB1, High mobility group box-1; mAb, monoclonal antibody.

groups is higher than that of control groups, indicating increased regenerative capacity of RST by anti-HMGB1 mAb (Fig. 5B and C). However, no significant difference could be detected between control and Epo B groups, which suggests that Epo B alone is not sufficient to promote RST elongation in our contusion model. By contrast, combination groups showed higher proportion of 5-HT-positive area in the caudal site, even compared with anti-HMGB1 mAb alone group (Fig. 5B and C, and Figure S2). Microtubule stabilizing agents such as Epo B or taxol have been reported to promote axon extension both *in vitro* and *in vivo* (Hellal et al., 2011; Ruschel et al., 2015; Sengottuvel et al., 2011), whereas we could not detect the effect of Epo B alone on RST elongation in our SCI setting *in vivo* and this is likely attributable to more severe environment induced by contusion than transection. However, Epo B was able to exert RST elongation function when the severity of the lesion area was reduced by anti-HMGB1 mAb treatment, although it is difficult to determine whether increased RST fibers are formed by the elongation of more rostrally existing fibers or sprouting of spared fibers at the site. Nevertheless, our results suggest that Epo B increased neurites from injured RST in combination with anti-HMGB1 mAb, contributing to locomotion improvement.

3.4. Ablation of neurons around lesion site abolishes functional recovery-induced by the combinatorial treatment

In intact mice, RST connect directly to motor neurons expressing serotonin receptors or indirectly to motor neurons through neurons in the spinal cord (Ghosh and Pearse, 2015). Considering that even mice without any treatment showed spontaneous recovery (Fig. 1B), we firstly checked whether direct connection between RST and motor neurons exist after SCI. Immunostaining revealed a motor neuron marker ChAT-positive neurons surrounded by 5-HT signal at the lumbar segment in both untreated control and combination groups (Fig. 6A and B), suggesting the existence of direct connection between RSTs and motor neurons, which may contribute to the motor function after SCI.

After injury, spontaneous neuronal circuit reorganization occurs and spared RST sprout onto host neurons which relay informa-

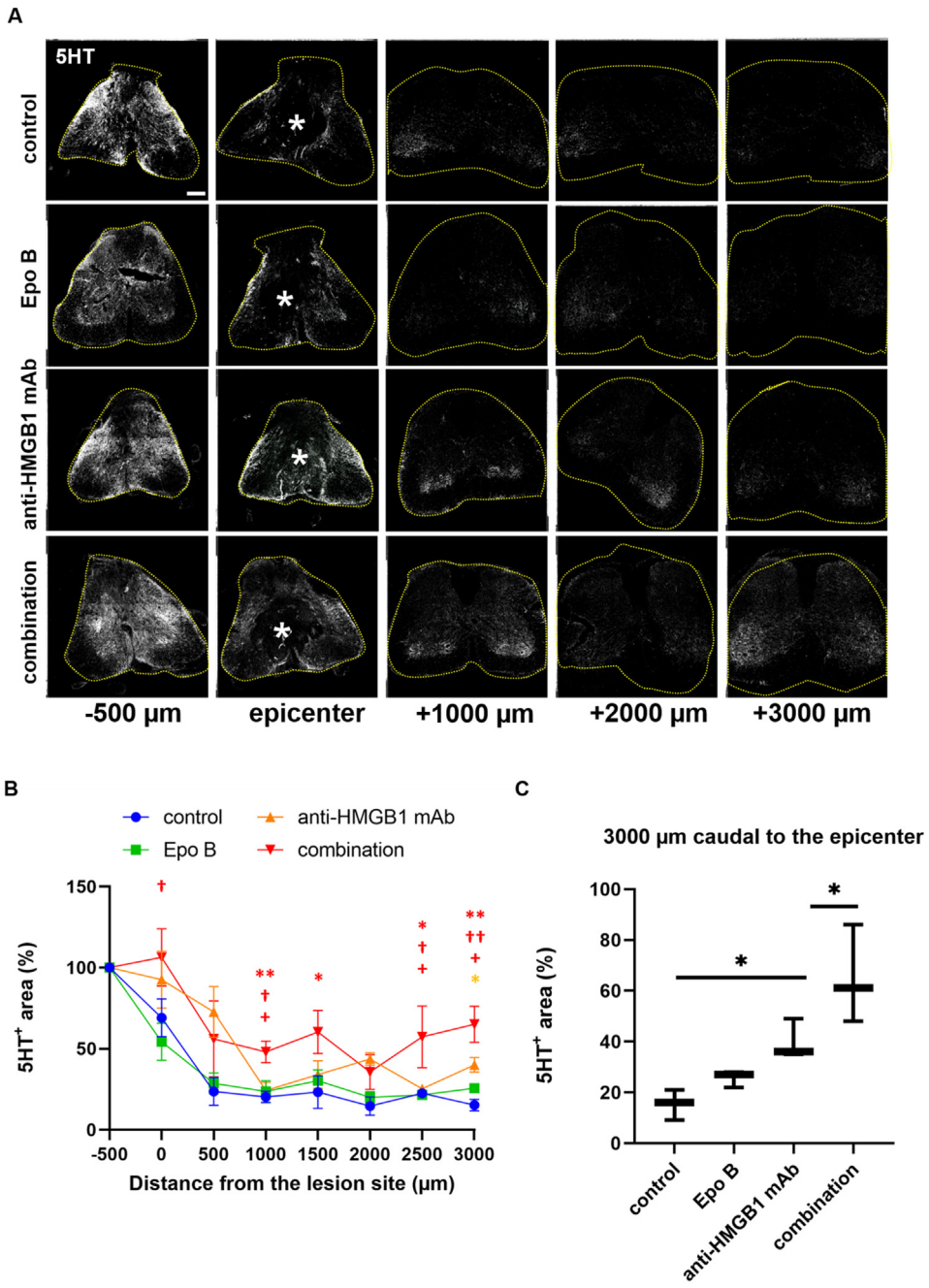

Fig. 5. 5-HT-positive neurite elongation is enhanced by the combinatorial treatment of anti-HMGB1 mAb and Epo B. (A) Representative pictures of spinal cord serial cross sections stained for 5-HT from rostral to caudal at 8 weeks after SCI. The epicenter is indicated as (*). Scale bar, 200 μm. (B) Quantification of proportion of 5-HT-positive area in (A). The x-axis indicates specific locations along the rostro-caudal axis of the spinal cord. The proportion of 5-HT-positive area in the area 500 μm rostral to the epicenter is defined as 100 %. n = 3 mice per group. Data represent mean ± SEM. ns, not significant; *, $p < 0.05$; **, $p < 0.01$ versus control group; †, $p < 0.05$; ††, $p < 0.01$ versus Epo B alone group; +, $p < 0.05$; ++, $p < 0.01$ versus anti-HMGB1 mAb alone group (two-way ANOVA with Fisher's LSD post hoc analysis). (C) Proportion of 5-HT-positive area in the site 3000 μm caudal to the epicenter. n = 3 mice per group. *, $p < 0.05$ versus indicated group. (anti-HMGB1 mAb effect $F_{1,8} = 25.56$, n = 12, p = 0.001; Epo B effect $F_{1,8} = 7.792$, n = 12, p = 0.0235; interaction $F_{1,8} = 1.343$, n = 12, p = 0.28; two-way ANOVA with Fisher's LSD post hoc analysis).
Abbreviations: RST, raphespinal fibers; SCI, spinal cord injury; Epo B, epothilone B; HMGB1, High mobility group box-1; mAb, monoclonal antibody.

tion from brain to motor neurons (Courtine and Sofroniew, 2019). Given that administration of anti-HMGB1 mAb together with Epo B increased spared neuron numbers and promoted RST elongation (Figs. 3 and 5), we next sought to investigate whether this combination treatment-derived functional recovery is attributable to the increased detour circuits. Quantitative analysis revealed that more connection between intraspinal neurons and RST fibers existed in the site 500 μm caudal to the epicenter in combination groups compared with that in control groups (Fig. 6C–E), probably because more neurons survived in the region after SCI in combination groups (Fig. 3). These results assert that elongated RST established connections with survived intraspinal neurons which could relay signals to motor neurons.

To further investigate whether the newly formed neuronal circuits are critical for combination treatment-derived functional recovery, we performed ablation experiment as previously conducted (Uezono et al., 2018). Seven weeks after SCI, axon-sparing excitotoxin NMDA (Agrawal and Fehlings, 1997; Yu et al., 1999) was

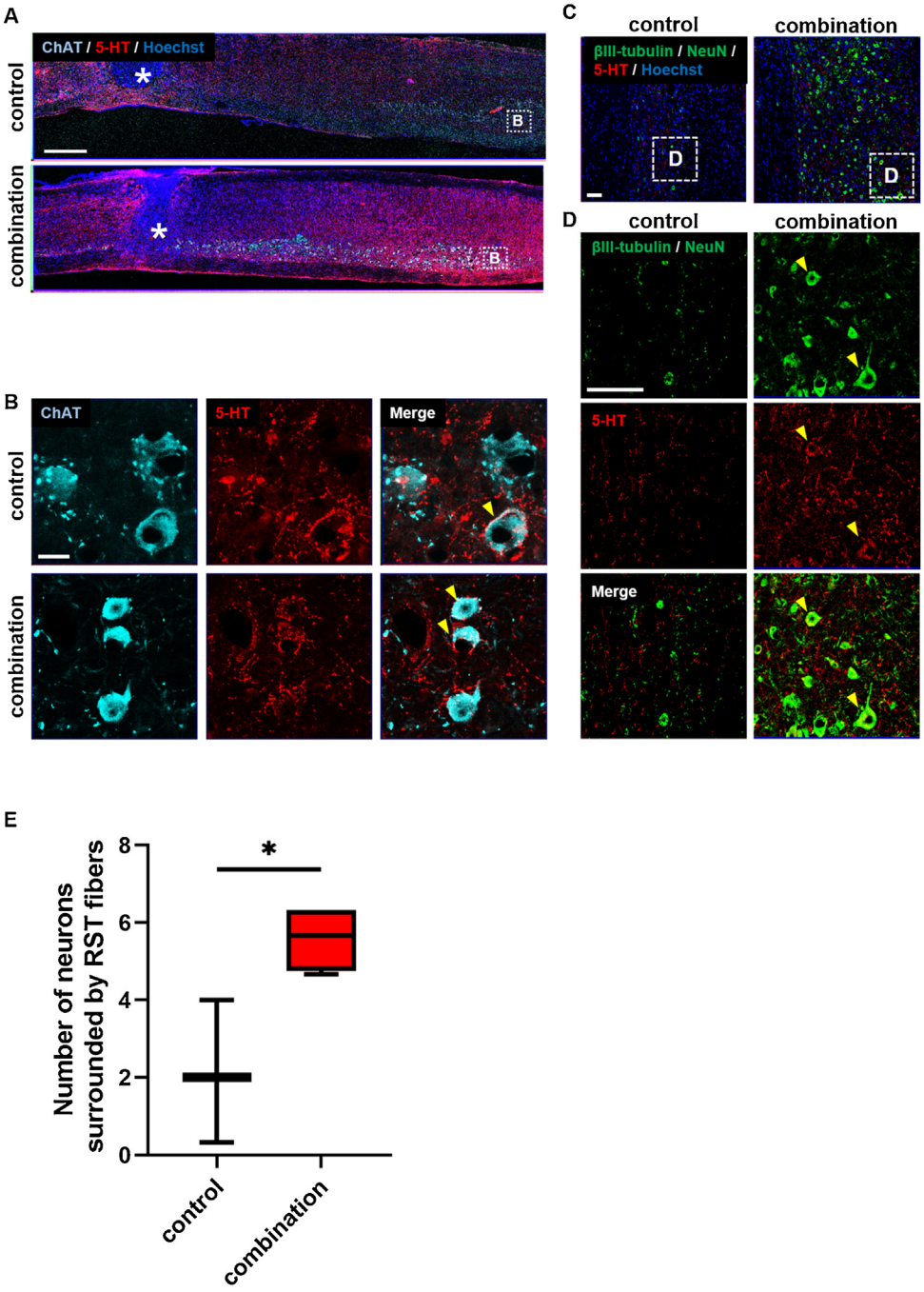

Fig. 6. Connection of RSTs with motor neurons and survived intraspinal relay neurons. (A) Representative pictures of spinal cord sagittal section stained with 5-HT (red), ChAT (cyan) and Hoechst (blue) at 8 weeks after SCI. The epicenter is indicated as (*). Scale bar, 500 μm. (B) Higher-magnification views of the boxed areas in (A). Yellow arrowheads indicate ChAT-positive motor neuron surrounded by 5-HT signals. Scale bar, 20 μm. (C) Representative pictures of βIII-tubulin, NeuN-positive intraspinal neurons and 5-HT-positive RST fibers in 500 μm x 500 μm area at the site 500 μm caudal to the epicenter. Scale bar, 50 μm. (D) Boxed areas in (C). Yellow arrowheads indicate intraspinal neuron surrounded by RST. Scale bar, 50 μm. (E) Quantification of cell number of intraspinal neurons surrounded by RST. n = 3 mice in control group, n = 4 mice in combination group. *, $p < 0.05$ versus combination group ($t_5 = 3.38$, $p = 0.0197$; unpaired Student's t test).
Abbreviations: RST, raphespinal fibers; SCI, spinal cord injury. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

injected into the lesion epicenter in mice combinatorially treated with anti-HMGB1 mAb and Epo B (Fig. 7A). Successful ablation of interspinal neuron was confirmed by immunostaining against a mature neuronal marker NeuN (Fig. 7B and C). In addition, quantitative analysis of 5-HT-positive area at the same section indicated that NMDA injection had no effect on the local RST (Fig. 7B and D). This ablation completely reversed improved locomotion recovery attained by the combination treatment to the similar level that was observed in untreated mice (Fig. 7E). These results suggest that RST connects with motor neurons through survived intraspinal relay neurons and the newly formed neuronal circuits also play a critical role in the functional recovery induced by the combinatorial treatment.

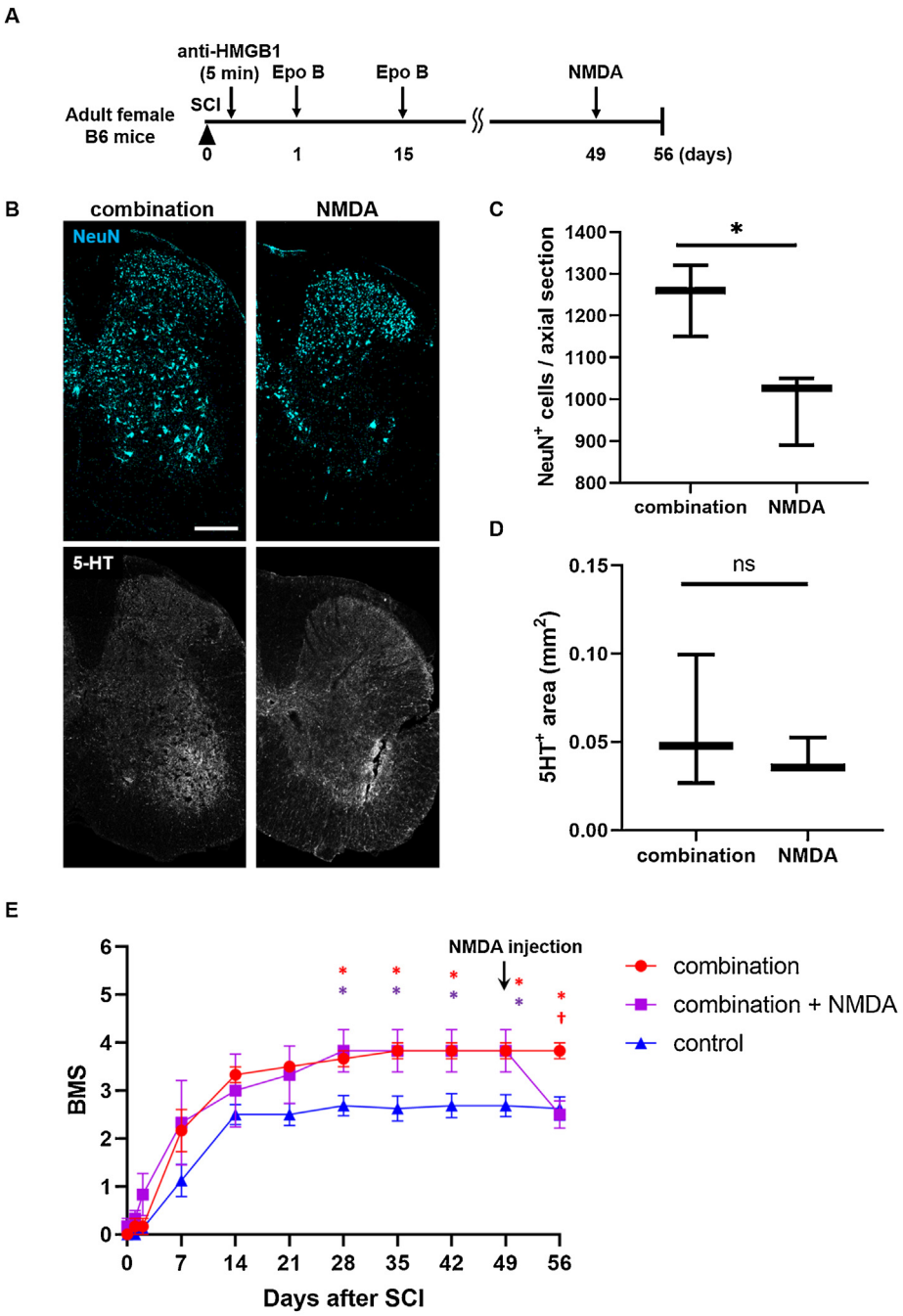

Fig. 7. Ablation of intraspinal neurons reversed the attained functional recovery by the combinatorial treatment. (A) Experimental scheme of the ablation experiment. (B) Representative pictures of spinal cord stained for NeuN (upper panel) and 5-HT (lower panel) at the site 2 mm caudal to epicenter at 8 weeks after SCI (1 week after NMDA injection). Scale bar, 200 μm. (C) Quantification of NeuN-positive host neuron number of cross sections at the site 2 mm caudal to epicenter. n = 3 mice per group. *, $p < 0.05$ versus combination group ($t_4 = 3.616$, $p = 0.0224$, unpaired Student's t test). (D) Quantification of 5-HT-positive area in (C). n = 3 mice per group. ns, not significant ($p > 0.9999$, Mann-Whitney test). (E) Time course of hindlimbs functional recovery assessed by BMS score after SCI. Purple line shows combination treatment group injected with NMDA at 7 weeks after SCI. n = 8 mice in control. n = 3 mice in combination group and NMDA-injected combination group. Data represent mean ± SEM. *, $p < 0.05$ versus control group; †, $p < 0.05$ versus NMDA group (two-way ANOVA with Fisher's LSD post hoc analysis).
Abbreviations: B6, C57BL/6J; SCI, spinal cord injury; Epo B, epothilone B; HMGB1, High mobility group box-1; mAb, monoclonal antibody; BMS, Basso Mouse Scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

In the present study, we have found that combination of Epo B and anti-HMGB1 mAb treatment improved the intraspinal neuron survival, increased axons outgrowth, and improved locomotion recovery after SCI. Administration of Epo B enhanced axon elongation only when it is combined with anti-HMGB1 mAb, which may be due to the decreased inflammatory response by anti-HMGB1 mAb. We also performed ablation experiment and have found that survived intraspinal neurons are critical for functional recovery. Correctively, this study provides a promising therapeutic option for the treatment of SCI.

Many studies to date have suggested that transplantation of iPSC-derived NSCs is efficient and one of the promising strategies for the treatment of spinal cord injury (Fujimoto et al., 2012; Nakamura and Okano, 2013; Nori et al., 2011; Salewski et al.,

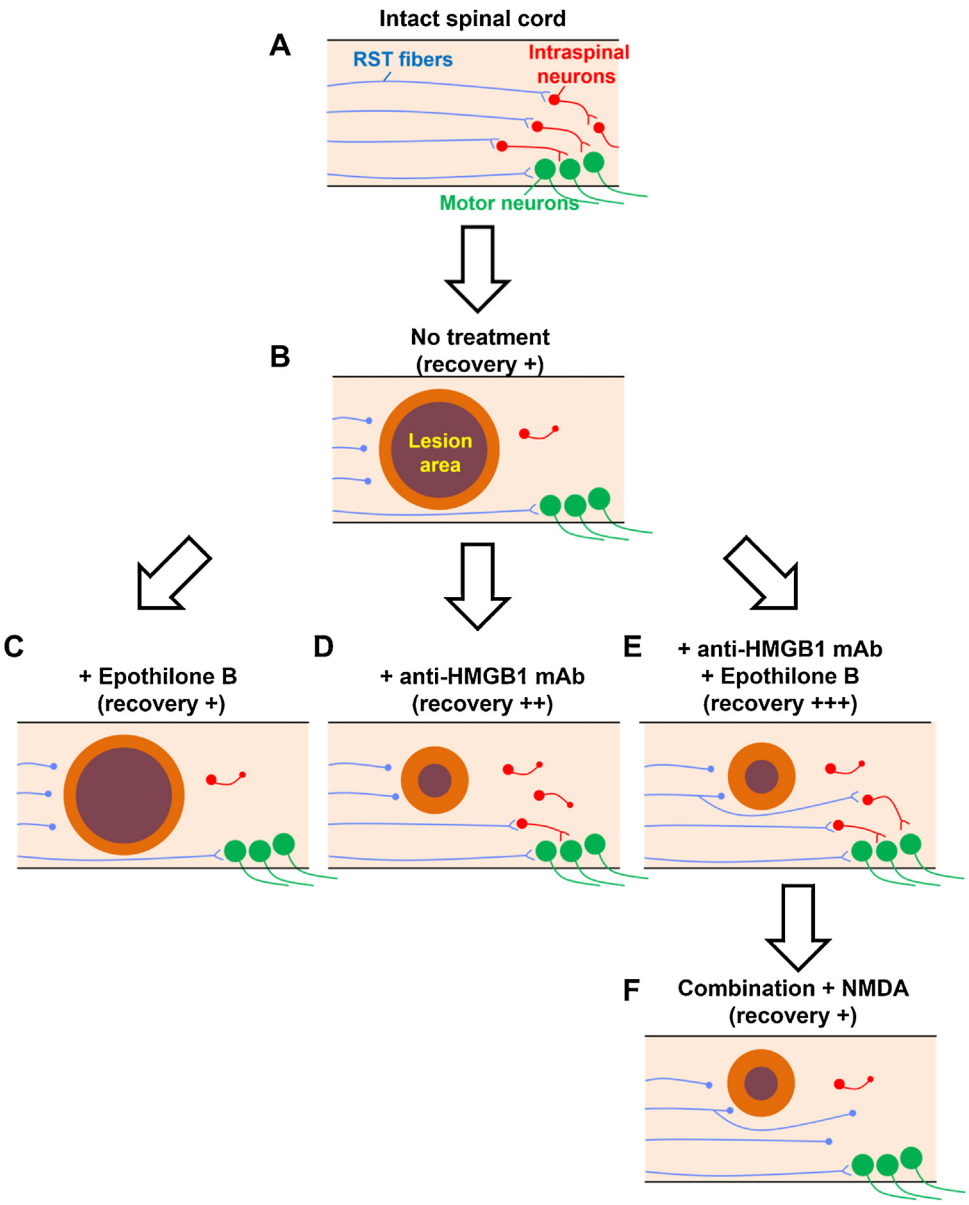

Fig. 8. Hypothetical model of combination treatment-dependent functional recovery. (A) In intact spinal cord, RST fibers connect to motor neurons directly or indirectly through intraspinal neurons. (B) Contusion injury disrupts neural networks and results in function loss. Small number of RST fibers is spared and/or spontaneously regenerate and directly connect to motor neurons, which results in limited motor function recovery. (C) Administration of Epo B alone does not improve functional recovery because injury-induced inflammatory response and scar formation may suppress its therapeutic effects. (D) Anti-HMGB1 mAb inhibits BSCB disruption and inflammation, resulting in smaller glial scar, increased neuronal survival and enhanced neural tracts elongation, which partially improved functional recovery. (E) Preserved lesion environment by anti-HMGB1 mAb makes Epo B function effectively to enhance axon elongation ability, thereby increasing detour circuits are established by elongated axons and survived neurons, leading to better functional recovery. (F) NMDA injection induces intraspinal neuron death and disrupts newly formed detour circuits, which reduces the functional recovery attained by combination treatment to the level that observed without treatment.

2015). In addition, we have indicated that the therapeutic effect of iPSC-derived NSC transplantation can be further expedited by preservation of lesion site using anti-HMGB1 mAb (Uezono et al., 2018). However, many problems relevant to clinical application of iPSCs still remain to be solved. For instance, the preparation of suitable cells for autologous transplantation takes exceedingly long time beyond the expected therapeutic time window (Nakamura et al., 2003; Takahashi et al., 2007). For SCI patients, the most appropriate time for transplantation is about 2–4 weeks after injury, whereas preparation of iPSCs-derived NSCs from patients' somatic cells requires at least 4 months. Although establishment of cell bank with iPSCs that do not induce immune rejection can partially solve this problem, generation and maintenance of such cells are costly and the quality control for clinical application is still challenging (Huang et al., 2019). Besides, contamination of undifferentiated iPSCs in transplanted cells raises a concern about risk of teratoma formation after engraftment, although several strategies to eliminate cancerous cells from iPSC-derived cells have been developed (Okubo et al., 2016; Tanosaki et al., 2020). These issues make transplantation of iPSC-derived cells not absolutely suitable for current clinical use and we thus sought to establish a new strategy for SCI treatment without using iPSC-derived cell transplantation in the present study.

Epo B is a considerable option as it is a clinically used drug and has been proven to be effective for SCI treatment in rats (Ruschel et al., 2015). After SCI, transected axon tracts form the so-called retraction bulbs which contain disorganized microtubules and lose the capacity to regenerate. The binding of Epo B to microtubule sta-

bilizes and reorganizes microtubule, promoting the formation of growth cone and axon elongation (Goodin et al., 2004). Additionally, it is reported that stabilization of microtubules by Epo B also prevented fibroblast migration, resulting in reduced fibrotic scar formation in rat transection SCI models. However, in the present study, treatment of Epo B alone neither promoted axon elongation nor inhibited scar formation compared with control, which is presumably due to the severer contusion injury than the transection injury. Unlike transection model used in the previous study, spinal cord contusion injury causes wide spread of secondary damage and more complex environment (Beattie and Bresnahan, 2000). Therefore, enhanced inflammatory and increased inhibitory factors for axon outgrowth such as chondroitin sulfate proteoglycan, Reticulon 4, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein in contusion injury may influence the therapeutic effect of Epo B (Zhu et al., 2018). Consistent with present result, Sandner et al. reported that in rat contusion SCI model, administration of epothilone D, which is an analog of Epo B, did not reduce fibrotic scar formation and promote axon elongation (Sandner et al., 2018). As contusion model mimics the typical SCI in humans, these data indicate that in human SCI condition, application of Epo B alone may not have or limited, if any, therapeutic effects as were observed in our present study with contusion SCI model (Fig. 1A–C).

In stark contrast, combinatorial administration of Epo B and anti-HMGB1 mAb shows dramatic locomotion recovery and increased outgrowth of 5-HT positive serotonergic fibers, and these effects are significant compared even with the anti-HMGB1 mAb administered group, suggesting that prior treatment of anti-HMGB1 mAb increased the therapeutic effect of Epo B and *vise versa* in the contusion model. Although it is still unclear if the increased 5-HT positive signals in the caudal site are resulted from enhanced regeneration or alleviated degeneration of serotonergic fibers by combinatorial treatment, it is possible that combinatorial treatment can at least partially reduce acute axonal degeneration as locomotion improvement were observed in a very early phase as shown in Fig. 1B. HMGB1 acts as a key mediator in the inflammatory response after tissue damage, and its expression level increases earlier than that of many proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-1β, and interleukin-6. We and others have demonstrated that anti-HMGB1 mAb administration inhibited inflammation and protected BBB disruption in CNS injury models. In mouse SCI model, administration of anti-HMGB1 mAb attenuated edema formation, reduced CD11b-postive area and inhibited *TNF-α* expression (Nakajo et al., 2019; Uezono et al., 2018). These effects contribute to the survival of intraspinal neurons and increased axon elongation. As Epo B treatment alone has no effect on axon elongation and locomotion improvement, the alleviated inflammation and preserved injury area by anti-HMGB1 mAb may account for the enhanced effect of Epo B in combination therapy (Fig. 8). More work needs to be done to evaluate whether different functional outcomes of Epo B depend on the lesion severity and its underlying mechanisms.

Regeneration of transected axon tracts after SCI have been investigated for decades. However, the effort to promote neurite outgrowth in contusion SCI model appear to have only limited effect, precluding complete function recovery (Danilov and Steward, 2015; Liu et al., 2011; Pearse et al., 2004). Recently, the neuronal circuits reorganization after SCI has been identified and the survived neurons in the spinal cord are supposed to play an important role in this process (Abematsu et al., 2010; Bareyre et al., 2004; Murray et al., 2010). These intraspinal neurons around the lesion site form new circuits, which relay information from disrupted axon tracts and their downstream targets. In the present study, ablation of survived neurons around the lesion site completely abolished the attained functional recovery, even though the 5-HT-postive serotonergic fibers elongation was improved by combination of anti-HMGB1 mAb and Epo B. These results suggest that the newly formed neuronal circuits by survived neurons are essential for functional recovery, and only when these neuronal circuits exist, can combination therapy-induced axon elongation improve locomotion recovery.

Although combination of anti-HMGB1 mAb and Epo B treatments displayed better motor functional recovery compared with anti-HMGB1 mAb treatment alone, this recovery is still far from complete, which may be partly because of the immature synaptic connection between severed axon tracts and their targets. Different from normal neuronal circuits, reconstructed circuits are formed randomly, and it is conspicuous that appropriate functional neuronal circuits require synapses connected with appropriate targets. Several studies have suggested that exercise training after SCI enhanced synaptic connection between severed nerve fibers and intraspinal host neurons, or transplant-derived neurons (Flynn et al., 2013; Zhu et al., 2018). Based on these results, we expect that application of Epo B and anti-HMGB1 mAb combined with exercise training further improve functional recovery after SCI.

Author contributions

Y.Z. and K.N.: conception/design, manuscript writing; Y.Z.: performed the experiments, data analysis; Y.Z. and T.N.: behavior analysis; D.W. and M. Nishibori: contributed to the purification of anti-HMGB1 mAb; N.U., T.Y. and M. Nakajo: experiment support; Y.Z., N.U., T.N., and K.N.: results discussion. K.N.: financial support, project supervising, and final approval of manuscript.

Disclosure

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neures.2021.04.002.

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