

**Treatment with the neutralizing antibody against repulsive guidance
molecule-a promotes recovery from impaired manual dexterity in a
primate model of spinal cord injury**

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22 **Running title:** RGMA is a potential molecular target for SCI.

23

24 **Keywords**

25 manual dexterity; corticospinal tract; repulsive guidance molecule-a; primates; spinal

26 cord injury

27

Abstract

Axons in the mature mammalian central nervous system have only a limited capacity to grow/regenerate after injury, and, therefore, spontaneous recovery of motor functions is not greatly expected in spinal cord injury (SCI). To promote functional recovery after SCI, it is critical that corticospinal tract (CST) fibers reconnect properly with target spinal neurons through enhanced axonal growth/regeneration. Here, we applied antibody treatment against repulsive guidance molecule-a (RGMa) to a monkey model of SCI. We found that inhibition of upregulated RGMa around the lesioned site in the cervical cord resulted in recovery from impaired manual dexterity by accentuated penetration of CST fibers into laminae VII and IX where spinal interneurons and motoneurons are located, respectively. Furthermore, pharmacological inactivation following intracortical microstimulation revealed that the contralesional, but not the ipsilesional, primary motor cortex was crucially involved in functional recovery at a late stage in our SCI model. The present data indicate that treatment with the neutralizing antibody against RGMa after SCI is a potential target for achieving restored manual dexterity in primates.

Introduction

Manual dexterity as represented by a precision grip is skilled motor behavior characteristic of higher primates. It is generally accepted that manual dexterity is closely related to direct connectivity of the corticospinal tract (CST) arising from the motor cortex to motoneurons of the cervical cord that control the distal hand muscles (Lemon, 1993). The spinal cord injury (SCI) disrupts the CST and often causes an ever-lasting disability with sensorimotor dysfunctions. Therefore, the CST has been focused as an essential target for SCI treatment (GrandPré et al., 2002; Oudega and Perez, 2012). However, the mature mammalian central nervous system (CNS) has only a limited capacity to grow/regenerate once injured because of a number of inhibitory factors, including axon guidance molecules (Yiu and He, 2006).

Repulsive guidance molecule-a (RGMa) is among the axon guidance molecules. This molecule was originally identified in the visual system (Stahl et al., 1990; Monnier et al., 2002), and has been implicated in neuronal survival, proliferation and differentiation (Matsunaga et al., 2004; Matsunaga et al., 2006; Lah and Key, 2012). RGMa was upregulated around the lesioned site after SCI in rodents (Schwab et al., 2005), and treatment with the neutralizing antibody against RGMa has been shown to accelerate axonal regrowth and sprouting from the CST following thoracic cord lesions

(Hata et al., 2006). On the other hand, to promote functional recovery by enhanced sprouting of CST fibers after CNS insults, such fibers are required to extend toward an appropriate locus and reconnect properly with target spinal neurons (Wahl et al., 2014). It has recently been reported in macaque monkeys that a number of sprouting CST fibers derived from the contralesional primary motor cortex (MI) after SCI penetrate, below the lesioned site, into laminae VII and IX where spinal interneurons and motoneurons are located, respectively. Moreover, the ratio of the axonal distribution was significantly increased in lamina IX (Nakagawa et al., 2015). By contrast, the sprouting CST fibers chiefly terminate within the medial gray matter in rodents (Hata et al., 2006; Vavrek et al., 2006). These results suggest that the mechanisms underlying axonal remodeling that leads to the recovery of motor functions after SCI may differ in higher primates and rodents. Therefore, it is important to understand axonal remodeling specific to higher primates for restoration of manual dexterity after SCI. It is also crucial to confirm whether RGMa suppression promotes the recovery from impaired manual dexterity for applying to human patients with SCI.

The present study aimed at establishing the antibody treatment against RGMa in higher primates. Our results indicate that treatment with the neutralizing antibody against RGMa is critical to achieve restored manual dexterity after SCI.

Materials and Methods

Animals

Twelve rhesus monkeys (*Macaca mulatta*, 3–5 years old, 3.8–5.4 kg) of either sex were used in the present study: three lesioned monkeys for treatment with control antibody (Ctrl-A to Ctrl-C), four monkeys for treatment with anti-RGMA antibody (RGMA-A to RGMA-D), and five normal monkeys for molecular biological (western blotting) and histological (histochemical, immunohistochemical, and *in situ* hybridization) analyses. The experimental protocols were approved by the Animal Welfare and Animal Care Committee of the Primate Research Institute, Kyoto University, Japan. All experiments were conducted in accordance with the Guide for Care and Use of Laboratory Primates (Ver. 3, 2010) by the Primate Research Institute, Kyoto University, Japan.

Anti-RGMA antibody

We used an anti-human RGMA antibody (mouse IgG; IBL) as a neutralizing antibody against RGMA in the present study. The antibody was raised against the C-terminal sequences of human RGMA, LYERTRDLPGR~~AA~~AGL. The protein sequence of human RGMA fully corresponds with that for macaque monkeys. It has

previously been reported that the antibody has an inhibitory effect on human RGMa obtained from peripheral blood mononuclear cells of human patients with multiple sclerosis (Muramatsu et al., 2011).

Western blotting

Following sedation with ketamine hydrochloride (10 mg/kg, i.m.) and xylazine hydrochloride (1 mg/kg, i.m.), an intact monkey was anesthetized deeply with sodium pentobarbital (50 mg/kg, i.v.) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4). The MI (digit region) and the spinal cord (C7 to Th1 segments) were harvested and homogenized in a lysis buffer, comprising 50 mM Tris-HCl (pH 7.8) with 150 mM NaCl, 1 mM EDTA, 2 mM Na₃VO₄, 1% NP-40, and protease inhibitor cocktail. After centrifugation at 13,000 g for 20 min at 4°C, it was lysed by using equal amounts of 2 x sample buffer, comprising 250 mM Tris-HCl, 4% sodium dodecyl sulphate, 20% glycerol, 0.02% bromophenol blue, and 5% β-mercaptoethanol, and then proteins were boiled for 5 min at 95°C. The mixed proteins were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 5% skim milk in 0.01 M PBS containing 0.05% Tween-20 and then

incubated with anti-RGMA antibody (IBL). After several washes, the membrane was incubated with horseradish peroxidase-linked anti-mouse IgG antibody (1:5,000; Cell Signaling Technology). The detection was carried out with an ECL chemiluminescence system (GE Healthcare). As control samples, human- and mouse-recombinant RGMA proteins (R & D systems) were used.

Experimental time-course

The experimental time-course was summarized in Figure 1N. The monkeys were trained with the two behavioral tasks, a reaching/grasping task and a modified Brinkman board test (Freund et al., 2006, 2009) for 2–3 months prior to SCI. To reduce the inter-animal variability in the modified Brinkman board test, pairs of monkeys who exhibited similar baselines before SCI (i.e., Ctrl-A and RGMA-A, Ctrl-B and RGMA-B, and Ctrl-C and RGMA-C) usually underwent simultaneous behavioral analyses. After SCI, the two behavioral tasks were performed to assess the extent of recovered forelimb movements over 14 weeks. Following all behavioral analyses, the digit regions of the contralesional and ipsilesional MI were identified in the anti-RGMA antibody-treated monkeys using intracortical microstimulation (ICMS), and then muscimol was injected into the identified digit regions to inactivate neuronal activity therein. To examine

136 sprouting CST fibers below the lesioned site, biotinylated dextran amine (BDA) was
137 injected into the contralesional MI seven weeks prior to sacrifice. To label spinal
138 motoneurons through the median nerve on the SCI side, wheat germ
139 agglutinin-conjugated horseradish peroxidase (WGA-HRP) was infused from the cut
140 end of the nerve four days prior to sacrifice. One of the four monkeys (RGMa-B) who
141 were treated with the anti-RGMa antibody was excluded from the
142 electrophysiological/pharmacological and histological analyses, because this monkey
143 was dead by unknown accident shortly after the behavioral analyses.

145 **Behavioral analyses**

146 We investigated whether RGMa inhibition with the antibody might promote the
147 recovery from impaired manual dexterity after SCI. To assess the extent to which the
148 skilled motor behavior was restored in our SCI model, the two behavioral tasks, a
149 reaching/grasping task and a modified Brinkman board test were performed. These tasks
150 were designed to take out pellets from vertical and horizontal slots. Each behavioral
151 analysis was carried out on the 3rd, 5th, 7th, and 10th day after SCI, and then twice a
152 week until the 14th week.

Reaching/grasping task

This task was performed to assess quantitatively the extent of recovery of dexterous manual movements as previously described (Nakagawa et al., 2015). Briefly, the monkeys were seated in a primate chair, and an acrylic board which consisted of three vertical or horizontal slots was placed in front of the chair (Fig. 4A,B). Each slot was filled with a small pellet (diameter, 9 mm). The monkeys reached for the vertical or horizontal slots and attempted to pick up three pellets within 10 sec in each trial. We analyzed how many pellets the monkeys successfully collected in each session (7 trials). Data were expressed as the ratio of the number of collected pellets to the total number per session (21 pellets; 3 pellets x 7 trials) in each of the vertical-slot and horizontal-slot tasks.

Modified Brinkman board test

The Brinkman board test was originally created to assess manual dexterity after SCI at the cervical cord level in macaque monkeys (Rouiller et al., 1998). Later, Freund et al. (2006, 2009) have made a minor modification for analyzing the extent of functional recovery from SCI, and named it “a modified Brinkman board test”. In the present study, we applied this modified version. The monkeys were seated in a primate

chair, and the Brinkman board was placed on the chair. The Brinkman board (10 cm x 20 cm) was made of an acrylic board where a total of 50 slots (15 mm long x 8 mm wide x 6 mm deep), consisting of 25 vertical and 25 horizontal slots, were randomly located on the board (Fig. 4E). Each slot was filled with a food pellet (94 mg, banana or very berry flavor; Bioserve). In our modified Brinkman board test, each session consisted of 50 trials (25 trials for each of the vertical- and horizontal-slot tasks) to take out a total of 50 pellets, and a trial was considered to be successful when the monkey grasped a pellet and conveyed it to the mouth within 30 sec. On the other hand, a trial was counted as an error when the monkey failed to grasp a pellet or dropped it on the way to the mouth. The baseline value prior to SCI was represented as the mean of successful trials (maximum = 25) per session in each of the vertical- and horizontal-slot tasks that were performed 7 times (5 sessions per day). In each monkey, the analyzed data were evaluated as the number of successful trials per session in a daily test.

Surgical procedures for SCI

Surgical procedures for SCI were performed as previously described with minor modifications (Nakagawa et al., 2015). Briefly, the monkeys were sedated with a combination of ketamine hydrochloride (10 mg/kg, i.m.), xylazine hydrochloride (1

190 mg/kg, i.m.), and atropine (0.05 mg/kg, i.m.), and then anesthetized with sodium
191 pentobarbital (25 mg/kg, i.v.). The monkeys who were monitored with percutaneous
192 oxygen saturation, respiration rate, heart rate, blood pressure, body temperature, and
193 electrocardiogram were stabilized in a stereotaxic frame. The skin and axial muscles
194 were dissected at the level of the C2 to T1 segments under aseptic conditions.
195 Subsequently, laminectomy of the C3 to C7 segments was performed, and the dura
196 mater was cut unilaterally. After identification of the dorsal roots at the C6 and C7
197 levels, a border between the C6 and the C7 segment was lesioned with a surgical blade
198 (No. 11) and a special needle (27G). The anti-RGMA antibody or control antibody (IgG
199 from mouse serum; Sigma-Aldrich) was continuously delivered, via an osmotic pump
200 (2ML4, Alzet) installed under the skin of the back, to the periphery of the lesioned site
201 over four weeks immediately after SCI. Both the anti-RGMA and the control antibodies
202 purified as IgGs were concentrated to 50 µg/kg/day in PBS. To estimate the extent of
203 antibody infusion in the spinal cord, a 1% solution of Fast blue (Polysciences) was used
204 instead of the antibody. To fix properly a catheter (11 cm long) attached to the osmotic
205 pump, the top of the catheter was placed 5 mm above the lesioned site under the dura
206 mater (intrathecal administration) following SCI, and the catheter was connected to the
207 dura mater and surrounding muscles with surgical suture to keep the position during the

delivery of the antibody (Fig. 1K). Subsequently, spongel (Astellas) was located on the dura mater for hemostasis, and then the muscles and skin were sutured. Following the surgery, the monkeys were given an analgesic (Lepetan; Otsuka; i.m.; Indomethacin; Isei; oral, one week) and an antibiotic (Viccillin; Meiji Seika; i.m., 3–4 days). Four weeks later, the osmotic pump was removed from the back under general anesthesia (see above) and, then, checked to ensure that the whole amount of the antibody was certainly delivered up.

Intracortical microstimulation (ICMS)

Under general anesthesia with sodium pentobarbital (25 mg/kg, i.v.), two pipes made of polyether ether ketone were mounted in parallel over the frontal and occipital lobes for head fixation. After partial removal of the skull over the central sulcus under aseptic conditions, two rectangular plastic chambers (for the contralesional MI, 37 mm long x 42 mm wide x 15 mm deep; for the ipsilesional MI, 28 mm long x 32 mm wide x 20 mm deep) were attached to the exposed skull. Several days later, each monkey was seated in a primate chair with the head fixed in a stereotaxic frame attached to the chair. A glass-coated tungsten microelectrode (0.5–1.5 M Ω at 1 kHz; Alpha Omega) was penetrated perpendicularly into the contralesional or ipsilesional MI to identify the digit

region. Parameters of electrical stimulation were as follows: trains of 11 and 44 cathodal pulses, 200- μ sec duration at 333 Hz, current of less than 65 μ A. Basically, movements of the digits on the MI stimulation are easily elicited by short trains in a normal control (Miyachi et al., 2005). However, as we could assume that the movement threshold for ICMS might become higher after SCI, we used a 44-pulse train in addition to a 11-pulse train. Evoked movements of the digits were carefully monitored by visual inspection and muscle palpation.

Muscimol injections

To confirm whether compensatory pathways arising from the contralesional MI might be involved in recovery from impaired manual dexterity in our SCI model, the gamma-aminobutyric acid type A (GABA_A) receptor agonist, muscimol (1 μ g/ μ l in physiological saline, Sigma-Aldrich), was injected into the electrophysiologically-identified digit region of the contralesional or ipsilesional MI through a 10- μ l Hamilton microsyringe following ICMS. At each of four loci, 3 μ l of muscimol was very slowly infused (Fig. 6A). Saline was injected into the same sites using the same parameters as a control. After the muscimol or saline injections into the MI digit region, skilled forelimb movements were assessed with the reaching/grasping

task (for horizontal slots). Data were expressed as the ratio of successfully collected pellets to the total per session.

Anterograde tract-tracing of CST fibers

CST fibers below the lesioned site arising from the contralesional MI were anterogradely labeled with biotinylated dextran amine (BDA; Invitrogen; 10,000 MW). The BDA injections into the contralesional MI were performed seven weeks prior to sacrifice as previously described (Nakagawa et al., 2015). Briefly, under general anesthesia (see above), a 10% solution of BDA in PBS was extensively injected into multiple loci along the central sulcus where regions representing not only the forelimb, but also the trunk and hindlimb were located, through a 5- μ l Hamilton microsyringe (150 nl each penetration) (Fig. 1B).

Motoneuron labeling through the median nerve

To label spinal motoneurons through the median nerve on the SCI side, a 5% solution of WGA-HRP in physiological saline (Sigma-Aldrich) was applied to the cut end of the nerve four days prior to sacrifice. Under deep-anesthesia conditions (see above), the skin and intermuscular septum of the arm were dissected, and the median

nerve was exposed. The nerve was fully transected approximately 3.5 cm distal to the elbow joint. A total of 10 μ l WGA-HRP was put inside a special tube, the cut end of the nerve was immersed in the tracer solution for 10 min, and then, the muscles and skin were sutured. Following the surgery, the monkeys were given the analgesic and antibiotic.

Histochemical and immunohistochemical procedures

Following sedation with ketamine hydrochloride (10 mg/kg, i.m.) and xylazine hydrochloride (1 mg/kg, i.m.), the monkeys were anesthetized deeply with sodium pentobarbital (50 mg/kg, i.v.) and perfused transcardially with 10% formalin. The fixed brain and spinal cord were removed, immersed in PBS containing 30% sucrose until they sank, and then cut serially into 40- μ m thick transverse sections (for the spinal cord) or 50- μ m thick frontal sections (for the brain) on a freezing microtome. Procedures for histochemical visualization of injected and transported BDA with DAB-nickel and for immunohistochemistry with the Vector NovaRed substrate kit (Vector laboratories) were done as described elsewhere (Nakagawa et al., 2015). For immunofluorescence histochemical procedures, floating sections were blocked with 2% normal goat or donkey serum and 3% bovine serum albumin in PBS containing 0.1% Triton X-100 for

280 60 min. The sections were then incubated with primary antibodies for 120 min at room
281 temperature (RT), followed by two overnights at 4°C. Primary antibodies used in the
282 present study were as follows: mouse anti-RGMA (IBL), rabbit anti-Iba1 (Wako), goat
283 anti-ChAT (Millipore), mouse anti-NeuN (Millipore), goat anti-WGA (Vector
284 laboratories), rabbit anti-WGA (Sigma-Aldrich), guinea pig anti-VGluT1 (Millipore),
285 and sheep anti-Chx10 (Abcam) antibodies. The anti-WGA antibody was pre-absorbed
286 with powder of the monkey's brain and spinal cord for prevention of cross-reaction with
287 unknown endogenous molecules. The goat anti-WGA antibody was used for all analyses
288 except double immunostaining with the rabbit anti-WGA antibody in combination with
289 the goat anti-ChAT antibody. BDA immunofluorescence histochemistry was carried out
290 with Alexa Fluor 488-conjugated streptavidin two overnights at 4°C. Subsequently, the
291 sections were incubated with secondary antibodies for 120 min at RT in the dark.
292 Secondary antibodies used in this study were as follows: goat and donkey anti-mouse,
293 rabbit, goat, guinea pig, and sheep IgG Alexa Fluor 647, 568, and 488 (Invitrogen). To
294 reduce endogenous autofluorescence, the sections were immersed in a TrueBlack
295 (Biotium) solution diluted by 70% ethanol for 30 sec. All histochemical and
296 immunohistochemical images were acquired with a microscope (Biorevo BZ-9000,
297 Keyence) or a confocal laser-scanning microscope (Fluo View FV1000, Olympus and

298 LSM 800, Zeiss).

299

300 ***In situ* hybridization procedures**

301 Under deep anesthesia (see above), the monkeys underwent perfusion-fixation
302 with 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer (PB; pH 7.4).
303 The brain was removed and immersed in a 30% sucrose solution containing 4% PFA at
304 4°C overnight. *In situ* hybridization was performed as previously described with minor
305 modifications (Watakabe et al., 2007). Briefly, 40-μm-thick frontal sections were placed
306 onto glass slides (Thermo Fisher Scientific) and fixed with 4% PFA in PB for 15 min at
307 RT. The sections were treated sequentially with proteinase K (7.2 μg/ml) for 30 min at
308 37°C, 4% PFA for 15 min at RT, 0.25% acetic anhydride in 0.1 M triethanolamine for 10
309 min at RT, 0.3% Triton X-100 in PB for 20 min at RT. Subsequently, the sections were
310 hybridized with a Digoxigenin (DIG)-labeled riboprobe (0.12 μg/ ml) for 16 hr at 68°C
311 in a hybridization buffer comprising 50% formamide, 5x SSC, 5x Denhard's solution
312 (Invitrogen), 250 μg/ml tRNA (Roche Diagnostics), 500 μg/ml ssDNA (Sigma-Aldrich).
313 The riboprobe for *Neogenin* (XM_015142632.1; 346–979) was purchased from Geno
314 Staff (Tokyo, Japan) and heated at 80°C for 5 min before use. After overnight, the
315 sections were washed with 0.2x SSC for 30 min x 3 at 68°C. Next, the sections were

blocked with 1% blocking reagent (Roche Diagnostics) and 10% lamb serum in Tris-buffered saline containing 0.05% Tween 20 (TBS) for 1 hr at RT and then incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:5,000 dilution, Roche Diagnostics) in TBS containing 1% blocking reagent and 1% lamb serum for 2 hr at RT. To visualize hybridization signals, the sections were immersed in a coloring buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Data analyses

Quantification of BDA-labeled CST fibers below the lesioned site

BDA-labeled CST fibers in the C7, C8, and Th1 segments were quantified in laminae VII and IX of transverse sections (400 μm apart) on the SCI side. Additionally, BDA-labeled midline-crossing fibers from the ipsilateral and ventral CSTs were quantified in each segment below the lesioned site. In lamina VII, the intensity of BDA-labeled CST fibers stained by DAB reaction was measured within a rectangular area (273 μm high x 362 μm wide) that was located 800 μm lateral to the center of the central canal using an ImageJ software (National Institutes of Health, Bethesda, MD, USA). Regarding lamina VII or IX, the number of contacts of BDA-labeled CST fibers with single Chx10-labeled neuron (Immunofluorescence) or single WGA-HRP-labeled

motoneurons (DAB staining) was analyzed at a magnification of x400, respectively. The contacts were defined by the presence of button-like swellings of BDA-labeled CST fibers on somas or dendrites. The result was normalized by the mean number of BDA-labeled CST fibers in the dorsolateral area at the C5 segment (three serial sections) on the SCI side. BDA-labeled CST fibers that were observed in the gray matter within a radius of 50 μ m from the midline of the spinal cord on each of the SCI and non-SCI sides were counted as midline-crossing fibers from the ipsilateral and ventral CSTs (Fig. 5U). It should be noted here that such midline-crossing fibers may contain some of contralaterally sprouting CST fibers. The result was normalized by the mean number of BDA-labeled CST fibers in the dorsolateral area at each segment (three serial sections) on the non-SCI side.

Measurements of the extent of spinal cord lesions

The extent of spinal cord lesions was measured, with the ImageJ software described above, as the mean of the maximum lesioned area in three serial sections which were Nissl-stained with 1% Cresyl violet (see also Nakagawa et al., 2015). The lesioned area was expressed as the ratio to the total area of the hemi-transverse section on the lesioned side.

352

353 **Statistics**

354 For behavioral analyses of the control antibody-treated and anti-RGMA
355 antibody-treated monkey groups, two-way ANOVA was applied. Increases in
356 BDA-labeled CST fibers in laminae VII, IX and the midline area (i.e., midline-crossing
357 CST fibers) were analyzed by two-way ANOVA, followed by Student's *t*-test. For
358 within-group comparisons for pre- versus post-muscimol injections in the
359 reaching/grasping task and for the extent of spinal lesions, paired *t*-test was applied. All
360 data were represented as mean $\pm$ S.E.M., and statistical significance was accepted at $P <$
361 0.05.

362

Results

SCI model and the extent of spinal cord lesions

In our SCI model, the spinal cord was unilaterally lesioned in macaque monkeys. The lesions were made at the border between the C6 and the C7 level to infringe upon the lateral area of the hemi-transverse section (Fig. 1A). When BDA was injected into the contralateral (contralesional) MI, laterally-situated CST fibers were found to be fully removed in all of the lesioned monkeys, and, therefore, no BDA-labeled CST fibers in any cases were observed in the dorsolateral funiculus of the spinal cord below the lesioned site (Figs. 1B-E,G-I and 2A; see also Fig. 5B,G,L). The lesioned sites in all SCI monkeys were Nissl-stained to assess the ratio of the lesioned area, and there was no marked difference between the control antibody-treated (control) monkey group and the anti-RGMA antibody-treated (RGMA antibody-treated) monkey group (Fig. 2A,B). Part of the medial gray matter was kept intact to retain a route of sprouting CST fibers in our SCI model, and anterograde tract-tracing with BDA from the contralesional MI resulted in CST fiber labeling in this area (Figs. 1C,D,F,H,J and 2A). To confirm cross-reaction of the anti-RGMA antibody to the MI and the spinal cord of rhesus monkeys, western blotting was performed. We observed double and triple bands in the monkey MI and spinal cord (Fig. 1M). It has been reported that the RGMA protein is a tethered

membrane-bound molecule, and that proteolytic processing amplifies RGMA diversity by creating soluble versions, the full-length and proteolytic cleavage RGMA, with long-range effects (Tassew et al., 2009, 2012). The antibody was delivered continuously to the lesioned site via the osmotic pump for four weeks immediately after SCI (Fig. 1K,L,N).

Upregulation of RGMA around the lesioned site after SCI

First, the expression patterns of RGMA and *Neogenin* (a receptor for RGMs) were examined by immunohistochemistry or *in situ* hybridization in the cervical cord or MI, respectively, in our SCI model (one intact and two lesioned monkeys; Fig. 3). Longitudinal sections in the cervical cord ten days after SCI were immunostained with the anti-RGMA antibody, and then the RGMA expression was compared with a region remote from the lesioned site in the same section and with the same region in an intact (uninjured) section (Fig. 3A–C,F). Our immunohistochemical analyses revealed that the RGMA expression was obviously increased around the lesioned site after SCI. Next, we characterized RGMA-expressing cells by double immunofluorescence histochemistry and found that RGMA was present in Iba1-positive cells (Fig. 3D–F). Thus, RGMA was expressed in microglia/macrophages and upregulated specifically around the lesioned

site. In addition, *Neogenin* was expressed in layer 5 (identified in the adjacent Nissl-stained sections) of the contralesional and ipsilesional MI (Fig. 3G–J).

Effects of anti-RGMa antibody on impaired manual dexterity after SCI

In the reaching/grasping task, the motor performance through both the vertical-slot and the horizontal-slot tasks was greatly recovered in the RGMa antibody-treated monkey group, compared with the control monkey group, to reach as highly as the pre-SCI level (Fig. 4C,D, Video S1). Similar results were obtained in the modified Brinkman board test, especially when the RGMa antibody-treated monkey group took out pellets from vertical slots (Fig. 4F,G, Video S2). In the case where skilled forelimb movements through the horizontal-slot task were attempted, the RGMa antibody-treated monkey group did not so prominently exhibit functional recovery, although motor behavior was improved in comparison with the control monkey group in which no such movements were restored at all (Fig. 4H, Video S2). Moreover, our behavioral analyses revealed that in both the reaching/grasping task and the modified Brinkman board test, the antibody treatment against RGMa not only promoted the recovery of motor functions, but also advanced the start of recovery following SCI (Fig. 4C,D,F–H).

Increase in sprouting CST fibers with the neutralizing antibody against RGMa

To begin with addressing the reorganization of CST fibers below the lesioned site, we analyzed the distribution pattern of BDA-labeled CST fibers in the C7, C8, and Th1 segments. We observed no BDA-labeled CST fibers in the dorsolateral funiculus in both the control and the RGMa antibody-treated monkey groups (Fig. 5A,B,F,G,K,L). Approximately 30–50% of the BDA-labeled CST fibers, relative to an intact monkey, was found in lamina VII of the RGMa antibody-treated monkey group, whereas less than 10% of them were seen in lamina VII of the control monkey group (Fig. 5A,C,F,H,K,M,P). In lamina IX, all WGA-HRP-labeled neurons corresponded with ChAT-positive motoneurons (Fig. 5D'), indicating that all of them are motoneurons for the median nerve. In the RGMa antibody-treated monkey group, the number of contacts of sprouting CST fibers with single WGA-positive neurons was increased in lamina IX below the lesioned site, compared with the control monkey group (Fig. 5A,D,E,F,I,J,K,N,O,Q,E'). At least part of the sprouting CST fibers seemed to be in contact with spinal interneurons immunolabeled for Chx10. Similarly, we observed that the sprouting CST fibers in the RGMa antibody-treated monkey group appeared to make contact with single Chx10-positive neurons more frequently than in the control monkey

group (Fig. 5W-B',C'). We found a certain difference in the reinnervation pattern of sprouting CST fibers in each segment. In lamina VII, the intensity of BDA-labeled CST fibers and the number of contacts of these CST fibers with single Chx10-positive neurons were gradually decreased as the distance from the lesioned site became larger (C7 < C8 < Th1) in the RGMa antibody-treated monkey group. By contrast, the number of contacts of sprouting CST fibers with single WGA-positive neurons was increased in the same group, which was similar to the findings in an intact monkey (Fig. 5P,Q,C'). The number of midline-crossing fibers was also increased by the neutralizing antibody against RGMa (Fig. 5R-V). It should be noted here that the midline-crossing fibers observed may involve some extended fibers from the injured CST.

Impairments in recovered manual dexterity by muscimol inactivation in the contralesional MI

Following all behavioral analyses, ICMS was performed in the contralesional and ipsilesional MI of both the control and the RGMa antibody-treated monkey groups whether the contralesional MI might contribute to the functional recovery from impaired manual dexterity due to SCI (Fig. 1N). The movements of the digits ipsilateral to SCI were successfully elicited by intracortical microstimulation in four loci of the

453 contralesional MI, but not of the ipsilesional MI in the RGMa antibody-treated SCI
454 monkeys (Fig. 6A). Subsequent injections of muscimol into all of the
455 electrophysiologically-identified digit region of the contralesional MI, but not of the
456 ipsilesional MI, induced reversible impairments in skilled forelimb movements on the
457 SCI side (Fig. 6C,D). The reaching/grasping task (for horizontal slots) was employed to
458 assess the motor function. About 20 min after the muscimol injections, manual dexterity
459 was gradually deteriorated and severely impaired within one hour. Thereafter, impaired
460 forelimb movements were continually seen for a few hours. Although all RGMa
461 antibody-treated monkeys could move the digits individually on the next day, manual
462 dexterity was not so sufficiently restored, as compared to that before the muscimol
463 injections into the contralesional MI. When we carried out the ICMS experiment in the
464 control monkey group, no movements of the digits were evoked in the contralesional MI
465 (Fig. 6B). In general, the digit region of the MI was surrounded by the regions
466 representing the wrist, elbow, and face in macaque monkeys (Sessle and Wiesendanger,
467 1982). However, a sector surrounded by the wrist, elbow, and face regions exhibited no
468 response to ICMS (Fig. 6B). As we could not identify the digit region in the
469 contralesional MI of the control monkey group, we injected muscimol only into the
470 ipsilesional MI. Then, the muscimol injections into the digit region of the ipsilesional MI

471 induced reversible impairments in skilled movements of the SCI-unimpaired forelimb
472 only on the uninjured side (Fig. 6E), just like the RGMa antibody-treated monkey group
473 (Fig. 6D).

474

Discussion

Owing to the low capacity of growth/regeneration in the mature CNS, various therapeutic approaches to CNS injuries have been attempted against extrinsic and intrinsic inhibiting factors using *in vivo* and *in vitro* experiments with rodents (Sandvig et al., 2004; Hata et al., 2006; Mar et al., 2014). According to previous works (Yiu and He, 2006; Popovich and Longbrake, 2008; Rolls et al., 2009) glial cells expressing growth-inhibiting factors are rich in the lesioned area after SCI in rodents, and immune and inflammatory cells are also accumulated in the same area. It has repeatedly been demonstrated that RGMA is expressed in these cells (Schwab et al., 2005; Mirakaj et al., 2010; Muramatsu et al., 2011). The present study revealed that RGMA was specifically upregulated around the lesioned site with increases in microglia/macrophages after SCI (see Fig. 3D,E).

The spinal cord repair strategies through growing/regenerating axons include a series of events, consisting of the initiation of axonal growth, the maintenance of axonal elongation, the connectivity with appropriate target neurons, and the reorganization of neural circuitry (Bradbury and McMahon, 2006). In rodent SCI models, a reaching/grasping behavior was promoted by enhanced sprouting CST fibers, and many of these fibers were extended through the medial part of the spinal cord (Hollis et al.,

2016). Thus, the enhanced sprouting CST fibers would make a synaptic connection with spinal interneurons as the target for restoration of forelimb functions in rodents. For a precision grip in higher primates, corticomotoneuronal pathway neurons in the MI are specifically activated (Muir and Lemon, 1983). Moreover, during the monkey's performance of a pinching task, motor commands descending to spinal motoneurons are mediated at least partly by the spinal interneurons (Takei and Seki, 2010, 2013). Taken together, it is most likely that the reorganization of both the direct and the indirect (via the spinal interneurons) corticomotoneuronal pathways is required to promote recovery from impaired manual dexterity through enhanced sprouting of CST fibers in our SCI model.

In our behavioral tasks, to take out pellets skillfully from vertical and horizontal slots, cortically-derived compensatory input would be transmitted to the motoneurons and/or interneurons in the C7, C8, and Th1 segments that are situated below the SCI site. Particularly when taking out a pellet from the horizontal slot in the modified Brinkman board test, the monkey is required to move the wrist to the ulnar direction for achieving digit flexion with ulnar deviation. It has been reported that the wrist angle in the ulnar direction is largely reduced after SCI when the monkey takes out a pellet from the horizontal slot (Hoogewoud et al., 2013). In macaque monkeys, spinal motoneurons

511 innervating the extensor carpi ulnaris muscle responsible for digit flexion with ulnar
512 deviation are mainly distributed in the C8 and Th1 segments (Jenny and Inukai, 1983;
513 Schieber, 1995).

514 We demonstrated that sprouting CST fibers originating from the contralesional
515 MI in RGMa antibody-treated monkeys penetrated more densely, compared with the
516 control monkey group, into laminae VII and IX where spinal interneurons and
517 motoneurons were located, respectively, below the lesioned site (see Fig. 5P,Q). In our
518 experiments, the motoneurons in the C7, C8, and Th1 segments were labeled through
519 the median nerve that predominantly governs the distal forelimb muscles, activity of
520 which is essential for execution of manual dexterity with a precision grip (Dun et al.,
521 2007). In addition, a large number of motoneurons for hand (digits) muscles in primates
522 are located in the Th1 segment (Jenny and Inukai, 1983). With respect to the pattern of
523 reinnervation of the enhanced corticomotoneuronal pathway after SCI with the
524 anti-RGMa antibody treatment, sprouting CST fibers might lead to appropriate target
525 motoneurons with guidance factors to promote the recovery of motor functions
526 effectively and efficiently.

527 At least part of sprouting CST fibers seemed to be in contact with the
528 interneurons immunolabeled for Chx10. Chx10 was originally identified as one of the

529 markers for glutamatergic interneurons in rodents (Ericson et al., 1997), and
530 Chx10-positive neurons are reportedly distributed throughout the hindbrain and the
531 spinal cord in mammals and regulate motor functions, such as breathing and locomotion
532 (Ai-Mosawie et al., 2007; Crone et al., 2008, 2012; Dougherty and Kiehn, 2010; Azim
533 et al., 2014). According to a recent work (Liu et al., 2015), there is a correlation between
534 the number of Chx10-positive neurons in contact with sprouting CST fibers and
535 functional recovery of the forelimb with unilateral cervical cord injury in mice. Together
536 with the direct corticomotoneuronal pathway, the compensatory indirect pathway via
537 spinal interneurons may also be crucial to the recovery of a series of forelimb
538 movements, reaching for slots and taking out pellets from slots using a precision grip, in
539 our SCI model.

540 Interestingly, according to our behavioral analyses, the antibody treatment
541 against RGMa not only promoted the recovery of motor functions, i.e., the ability to
542 move the digits individually, but also advanced the start of recovery following SCI. It
543 has been well documented that treatment with the RGMa-neutralizing antibody in a
544 rodent model of multiple sclerosis promotes functional recovery by preventing
545 neurodegeneration (Muramatsu et al., 2011; Tanabe and Yamashita, 2014; Demicheva et
546 al., 2015). Recently, it has been reported that the RGMa antibody treatment promoted

neuronal survival around the lesioned site with improvement of functional recovery in a rodent SCI model (Mothe et al., 2017). Moreover, RGMa has been shown to reduce leukocyte trafficking and retard inflammation (Mirakaj et al., 2010). Thus, RGMa suppression has a certain impact on neuroprotection as well as on neurite outgrowth, and, therefore, the present results might be ascribed, at least partly, to the neuroprotective effect of the RGMa antibody.

The spinal cord lesions in some cases (i.e., Ctrl-B and RGMa-A monkeys) infringed, to some extent, upon uncrossed ventral CST region, though it almost unaffected recovery of manual dexterity, especially in the RGMa antibody-treated (RGMa-A) monkey (data not shown). It has previously been reported that the ventral CST distributes the fibers to proximal muscles, such as the neck, trunk, and proximal upper extremities (Nyberg-Hansen, 1963; Davidoff, 1990; Canedo, 1997). The motor tasks that we used in the present work (i.e., reaching/grasping task and the modified Brinkman board test) are required for movements of the proximal upper limb, but the spinal region responsible for the proximal upper limb remains intact in our SCI model.

By carrying out a series of pharmacological experiments following ICMS, we have demonstrated that the contralesional MI is greatly involved in recovery from impaired manual dexterity in our SCI model with the anti-RGMa antibody treatment. In

565 the control monkey group, on the other hand, forelimb movements once impaired by
566 SCI could not be evoked at all by our ICMS protocol (44-pulse train, 65- μ A current).
567 This may depend on an increase in sprouting CST fibers from the contralesional MI to
568 the cervical cord on the injured side. Additionally, it should be noted here that other
569 descending pathways, including the cortico-brainstem and cortico-propriospinal
570 pathways, might contribute to the functional recovery (Isa et al., 2013). In a similar
571 primate model of SCI (C7/C8 lesion model), reorganization of CST fibers originating
572 from the contralesional MI with the somatotopic map altered is crucial to recovery of
573 motor functions (Schmidlin et al., 2004, 2005). Part of uncrossed CST fibers derived
574 from the ipsilesional MI was disrupted in our SCI model, and, also, *Neogenin* was
575 expressed in layer 5 of the ipsilesional MI as well as of the contralesional MI (see Fig.
576 3I,J). RGMa has been shown to evoke the strong inhibition of axonal outgrowth and
577 regrowth through the transmembrane receptor, Neogenin (Tassew et al., 2014). Hence, it
578 can be considered that injured CST fibers extend beyond the lesioned site from the
579 ipsilesional MI by the aid of the anti-RGMa antibody. However, the CST fibers arising
580 from the ipsilesional MI may not work as a functional wiring at a late stage after SCI. In
581 view of the fact that the ipsilesional MI is activated at an early, but not a late stage of
582 recovery in a different primate model of SCI (Nishimura et al., 2007), the ipsilesional

583 MI might participate in functional restoration only transiently.

584 The present study defines that RGMa is a critical target molecule to promote
585 recovery from impaired manual dexterity after SCI. The antibody treatment against
586 RGMa may probably be applied not only to SCI, but also to other CNS insults, such as
587 traumatic brain injuries, stroke, and multiple sclerosis.

588

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754

Figure legends

Figure 1. SCI model and experimental time-course.

(A) Schematic diagram showing our primate model of SCI. Unilateral lesions were made at a border between the C6 and the C7 segment. Asterisk indicates the lesioned site. (B) Example of the injection site in the contralesional MI. (C and G) Nissl-stained transverse sections in the injured (SCI) and uninjured spinal cords. The strongly stained area represents the lesion extent in panel C. (D and H) Transverse sections labeled with BDA injected into the contralesional or contralateral MI in the injured or uninjured spinal cord, respectively. The dotted line demarcates the lesioned area in panel D. (E and I) Higher-power magnifications of areas in the dorsolateral funiculus in panels D and H, respectively. (F and J) Higher-power magnifications of areas in the medial gray matter in panels D and H, respectively. Note that BDA-labeled CST fibers extend beyond the lesioned site through the intact medial gray matter in the injured spinal cord. (K) Schematic diagram showing the antibody delivery system using an osmotic pump. (L) Anti-RGMA antibody delivered around the lesioned site via an osmotic pump. To visualize the extent of antibody infusion, Fast blue was used. (M) Western blot analysis of the anti-RGMA antibody. The antibody crosses to the MI and the spinal cord of a rhesus

monkey. (N) Experimental time-course. Scale bars, B and C 1 mm for B to D and G, H,
for B, C, E; J 50 μ m for E, F, I and J.

Figure 2. Extent of spinal cord lesions.

(A) Extent of SCI (in blue) in a representative transverse section in each monkey. (B)
Ratio of the lesioned area to the total area in a hemisection in each of control
antibody-treated (Ctrl) and anti-RGMA antibody-treated (RGMA) monkeys. There is no
marked difference in the averaged ratio between the two monkey groups. Because of the
death by unknown accident shortly after the behavioral analyses, monkey RGMA-B was
excluded from the electrophysiological/pharmacological and histological analyses.

Figure 3. Expression patterns of RGMA and *Neogenin* in the cervical cord and MI.

(A) RGMA expression (in purple) around the lesioned site (at the C6/C7 border) 10 days
after SCI in a longitudinal section. Shown in black are CST fibers anterogradely labeled
after BDA injections into the contralesional MI. (B) RGMA expression in a region
remote from the lesioned site in the same section. (C) RGMA expression in the same
region as in panel A in a normal (uninjured) case. (D and E) Expression of RGMA in
combination with Iba1 around the lesioned site. (F) Schematic diagram representing the

approximate locations of panels A, B, D, and E. (G) *In situ* hybridization for *Neogenin* messenger RNA in layer 5 (V) of the contralesional MI 10 days after SCI in a frontal section. (H) Higher-power magnification of a square area in panel G. (I) *In situ* hybridization for *Neogenin* messenger RNA in layer 5 (V) of the ipsilesional MI 10 days after SCI in a frontal section. (J) Higher-power magnification of a square area in panel I. Scale bars, A, 50 μ m for A to C; D,G,I, 200 μ m; E,H,J, 50 μ m.

Figure 4. Recovery from impaired manual dexterity with the neutralizing antibody against RGMa.

(A and B) Reaching/grasping task. The monkey reaches for vertical (A) or horizontal (B) slots and grasps pellets. (C and D) Ratio of pellets collected through vertical (C) or horizontal (D) slots. (E) Modified Brinkman board test. The monkey takes out pellets from randomly-set vertical and horizontal slots. (F) Total number of pellets collected through vertical and horizontal slots. (G and H) Number of pellets collected through vertical (G) or horizontal (H) slots. All data are based on three control antibody-treated (Ctrl) and four anti-RGMa antibody-treated (RGMa) monkeys. Error bars denote S.E.M. (two-way ANOVA, $*P < 0.05$).

Figure 5. Sprouting of CST fibers with the neutralizing antibody against RGMa.

(A, F, and K) BDA-labeled CST fibers in the Th1 segment in an intact (Uninjured; A), a control antibody-treated (Ctrl; F) and an anti-RGMa antibody-treated (RGMa; K) monkey. (B, G, and L) Higher-power magnifications of areas of the dorsolateral funiculus in panels A, F, and K, respectively. (C, H, M, D, I, and N) Higher-power magnifications of areas of laminae VII (C, H, and M) and IX (D, I, and N) in panels A, F, and K, respectively. (E, J, and O) Higher-power magnifications of dotted zones in panels D, I, and N, respectively. (P) Intensity of BDA-labeled CST fibers in lamina VII in the intact (Uninjured; $n = 1$), control antibody-treated (Ctrl; $n = 3$), and anti-RGMa antibody-treated (RGMa; $n = 3$) monkey groups. (Q) Number of contacts of BDA-labeled CST fibers with single WGA-positive motoneurons in lamina IX in the same monkey groups as in panel P. (R, S, and T) BDA-labeled CST fibers crossing the midline of the spinal cord in an intact (Uninjured; R), a control antibody-treated (Ctrl; S), and an anti-RGMa antibody-treated (RGMa; T) monkey. BDA was injected into the contralateral or contralesional MI. (U) Schematic diagram showing the midline area examined. CC, central canal. (V) Number of midline-crossing CST fibers below the lesioned site (i.e., C7, C8, and Th1 segments). (W, Y, and A') Spinal interneurons triple-labeled for BDA, NeuN, and Chx10 in lamina VII of the Th1 segment in an intact

(Uninjured; W), a control antibody-treated (Ctrl; Y), and an anti-RGMA antibody-treated (RGMa; A') monkey. (X, Z, and B') Higher-power magnifications of dotted zones in panels W, Y, and A', respectively. BDA-labeled CST fibers in contact with single Chx10-positive neurons (specified by arrowheads). (C') Number of contacts of BDA-labeled CST fibers with single Chx10-positive neurons in lamina VII in the same monkey groups as in panels P and Q. (D') Motoneurons double-labeled for WGA-HRP through the median nerve and ChAT in lamina IX of the Th1 segment in an anti-RGMA antibody-treated monkey. All WGA-HRP-labeled neurons are ChAT-positive. The dotted line indicates the border between the gray and the white matter. (E') Triple labeling for BDA, WGA-HRP, and VGluT1 in lamina IX of the Th1 segment in an anti-RGMA antibody-treated monkey. A BDA-labeled CST fiber in contact with a single WGA-positive motoneuron (specified by arrowheads). Scale bars, A, 1 mm for A,F,K; N, 100 μ m for B to D, G to I, L to N; O, 50 μ m for E, J and O; R, 100 μ m for R, S and T; W, 200 μ m for W, Y and A'; X and D', 5 μ m for X, Z, B' and D'; C', 200 μ m. Error bars denote S.E.M. (two-way ANOVA, followed by Student's *t*-test, **P* < 0.05).

Figure 6. Effects of muscimol injections into the MI on manual dexterity.

843 (A) Sites of muscimol injections into the contralesional and ipsilesional MI in a
844 representative anti-RGMA antibody-treated monkey (RGMA-A). Filled circles indicate
845 the injection loci in all of which ICMS induced movements of the digits on the
846 contralateral side. AS, arcuate sulcus; CS, central sulcus. Scale bar, 2 mm. (B) Results of
847 ICMS mapping of the contralesional MI in a representative control antibody-treated
848 monkey (Ctrl-C). Each letter denotes the electrophysiologically-identified body part as
849 follows: E, elbow; F, face; W, wrist; X, no response. Scale bar, 2 mm. (C–E) Skilled
850 movements after muscimol injections into the digit region of the contralesional MI (C) or
851 the ipsilesional MI (D) in the anti-RGMA antibody-treated monkey group ($n = 3$), or of the
852 contralesional MI in the control antibody-treated monkey group (E; $n = 3$). Manual
853 dexterity that had been impaired by SCI and then recovered by RGMA treatment
854 (SCI-impaired forelimb) was again impaired after muscimol (but not saline) injections
855 into the contralesional MI. On the other hand, the muscimol injections into the
856 ipsilesional MI only caused impairments in skilled movements of the normal forelimb
857 (SCI-unimpaired forelimb) contralateral to the injections. Error bars indicate S.E.M.
858 (Student's t -test, $*P < 0.05$).

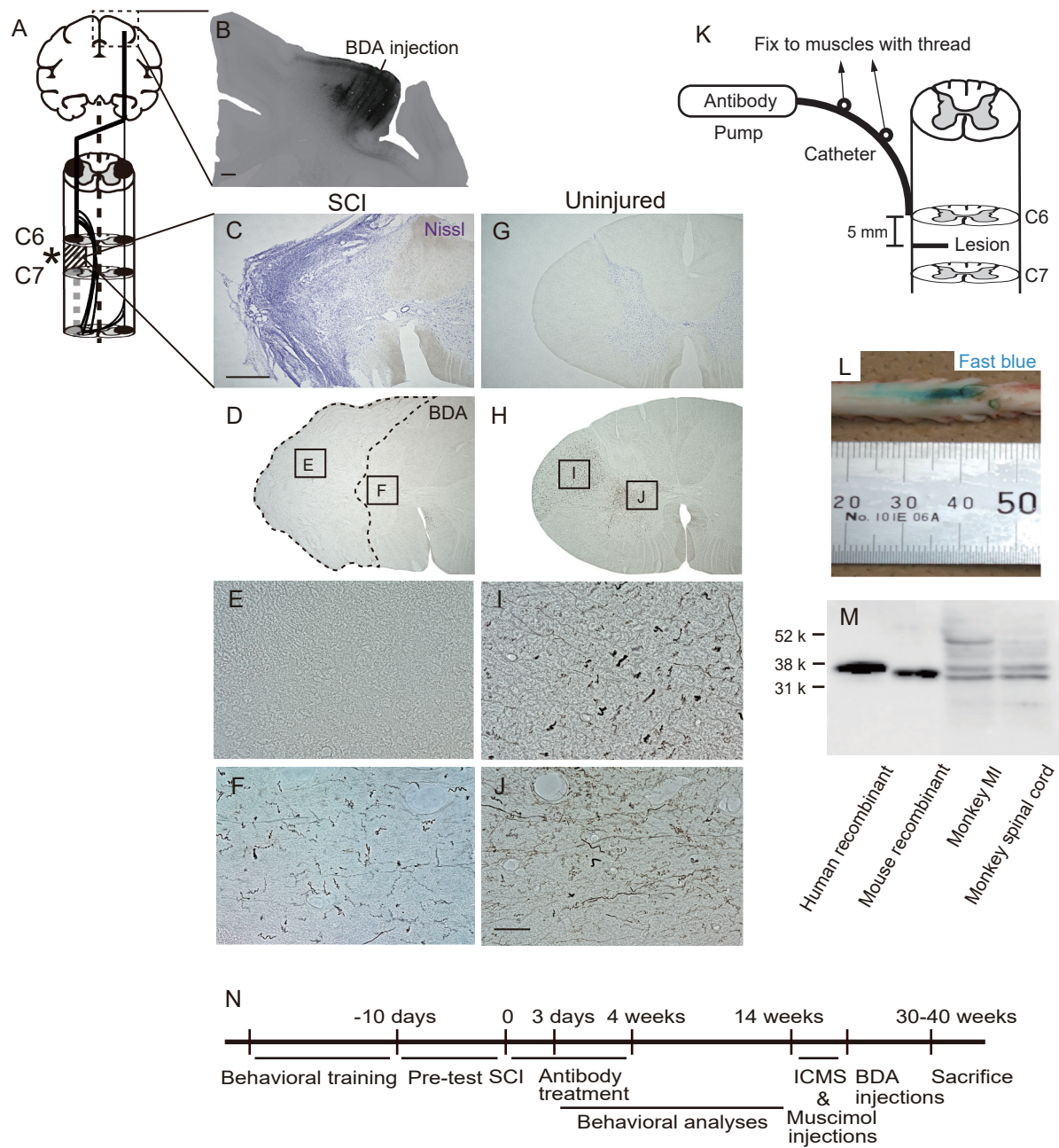


Figure 1
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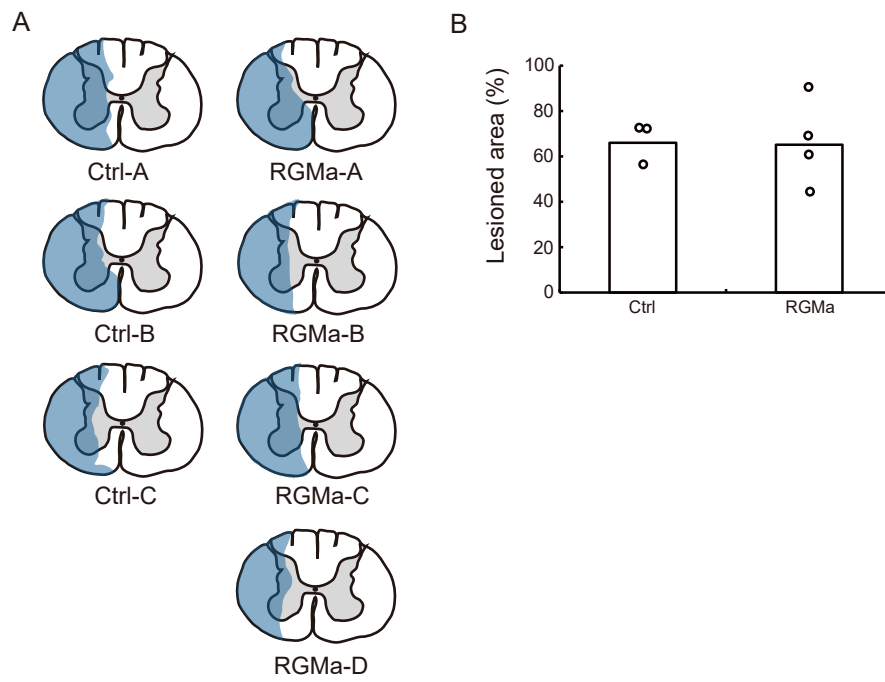


Figure 2
Nakagawa et al.

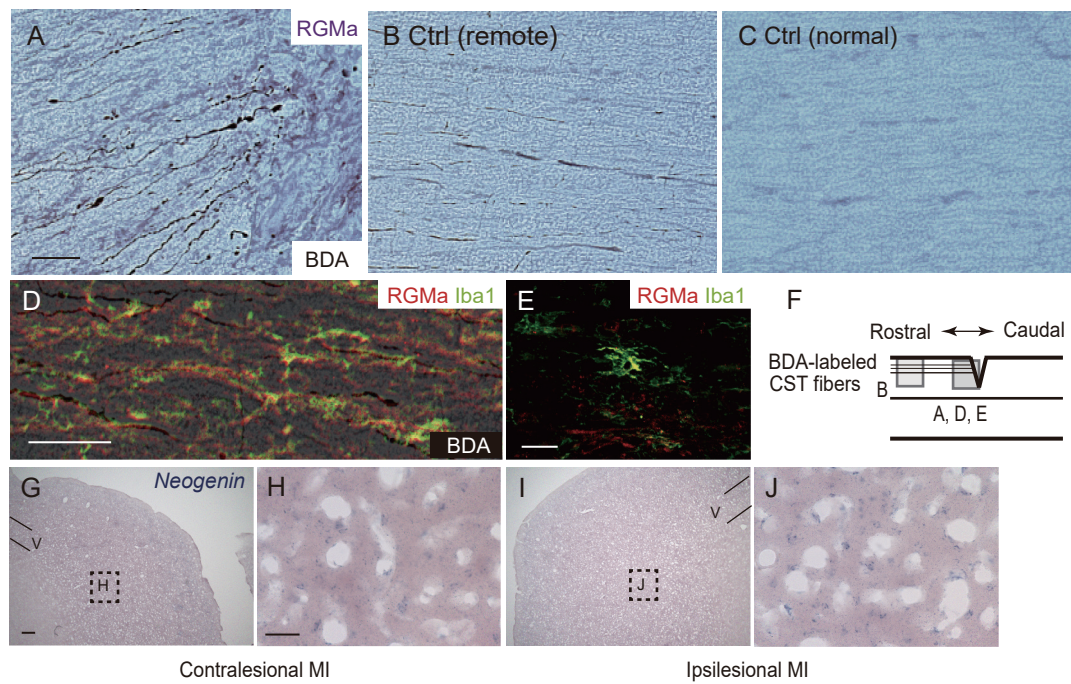


Figure 3
Nakagawa et al.

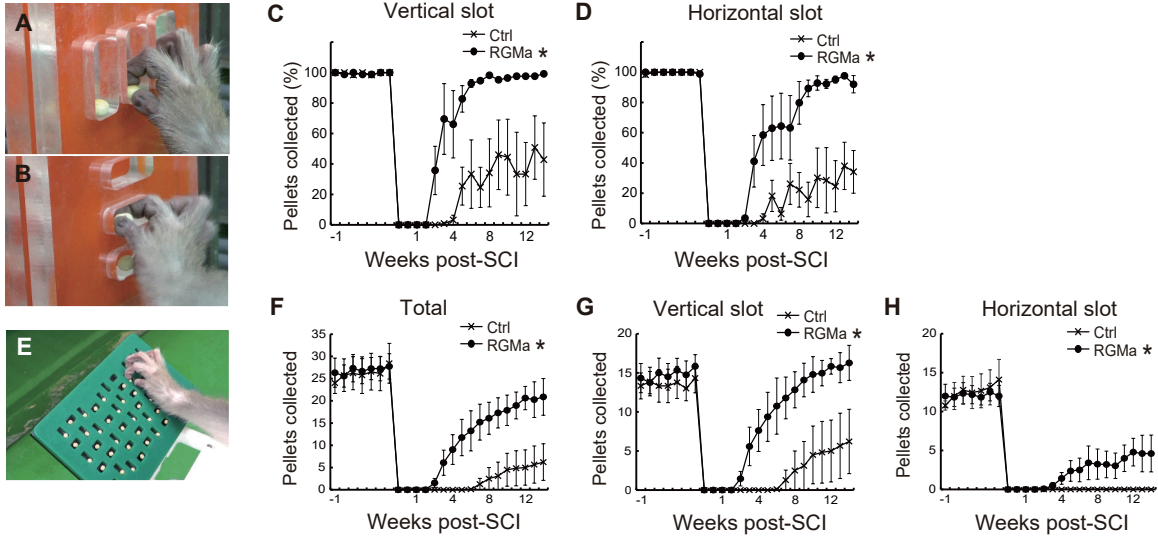


Figure 4
Nakagawa et al.

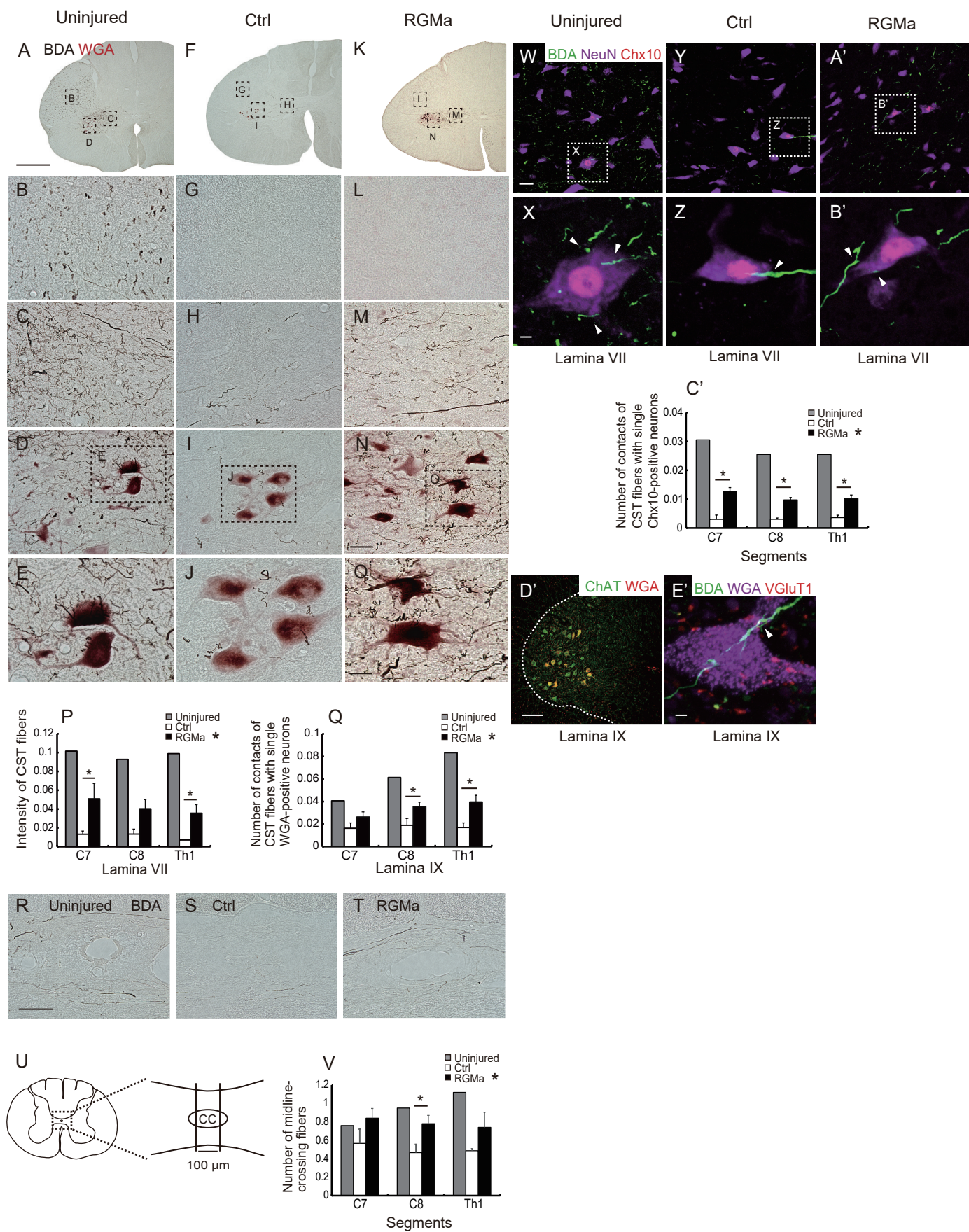


Figure 5
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1 **Supplementary material**

2 **Treatment with the neutralizing antibody against repulsive guidance molecule-a**
3 **promotes recovery from impaired manual dexterity in a primate model of spinal**
4 **cord injury**

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Video S1. Recovery process of the reaching/grasping task performance in a control antibody-treated and an anti-RGMA antibody-treated monkey. Fourteen weeks after SCI, the control antibody-treated monkey can manage to take out pellets from vertical and horizontal slots mainly using a power grip. On the other hand, the anti-RGMA antibody-treated monkey can take out at least some pellets from both types of the slots skillfully using a precision grip.

Video S2. Recovery process of the modified Brinkman board test performance in a control antibody-treated and an anti-RGMA antibody-treated monkey. This movie shows that the monkeys take out pellets before, 3 days, and 14 weeks after SCI. Before SCI, the monkeys smoothly take out pellets from both the vertical and the horizontal slots. Just after SCI, the monkeys cannot take out any pellet at all. Fourteen weeks after SCI, the control antibody-treated monkey can take out some pellets only from the vertical slots. On the other hand, the anti-RGMA antibody-treated monkey can take out pellets not only from the vertical slots, but also from the horizontal slots.