Histological and electrophysiological analysis of the corticospinal pathway to forelimb motoneurons in common marmosets

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Abbreviations

BDA Biotinylated dextran amin

CM Corticomotoneuronal

CST Corticospinal tract

CV Conduction velocity

DC Dorsal column

DLF Dorsolateral funiculus

DR Deep radial

EPSP Excitatory postsynaptic potential

ICMS Intracortical microstimulation

LFP Local field potentials

M Median

M1 Primary motor cortex

RST Reticulospinal tract

U ulnar

VMF Ventrolateral funiculus

Abstract

Using histological and electrophysiological methods, we identified the neuroanatomical properties of the common marmoset corticospinal tract (CST), which underlies hand/arm motor control. Biotinylated dextran amine (BDA) was injected into the primary motor cortex to anterogradely label CST axons in the cervical segments, revealing that most CST axons descend in the contralateral dorsolateral funiculus (DLF; 85.0%), and some in the ipsilateral DLF (10.7%).

47 Terminal buttons were mainly found in the contralateral lamina VII of the gray matter, but
48 projection to lamina IX, where forelimb motoneurons are located, was rare. Bilateral projections
49 were more abundant than found in the rat CST, resembling the CST organization of other primates.
50 Intracellular recordings were made from 57 forelimb motoneurons on the contralateral side to
51 stimulation, which revealed no monosynaptic excitatory postsynaptic potentials (EPSPs), but di- or
52 polysynaptic EPSPs and inhibitory synaptic potentials were commonly found. Local field potentials
53 showed monosynaptic excitation mainly in laminae VII, where abundant BDA-labeled CST
54 terminals were observed. These results suggest that direct corticomotoneuronal projection is absent
55 in common marmosets but di- or oligosynaptic effects would be mediated by spinal interneurons.
56
57 Keywords: corticospinal tract, common marmoset, non-human primate, manual dexterity,
58 motoneuron, segmental interneuron
59

60 **1. Introduction**

61 The common marmoset (*Callithrix jacchus*) has been attracting much attention in the field of
62 neuroscience because of its relative ease in handling owing to its small size, high reproductive
63 efficiency, and advantages in its unique behavioral and cognitive characteristics (Okano et al., 2012).
64 The anatomy of the marmoset cerebrum has been reported to be similar to those of macaque
65 monkeys and humans; for example, although compact brain size and no central sulcus, they have
66 well developed frontal cortex and clear separation of the primary motor cortex from the
67 somatosensory cortex (Burish et al., 2008) and separation of the caudate nucleus from the putamen
68 by the bundles of corticofugal fibers (Emborg, 2007). Owing to these advantages and the recent
69 development of transgenic animals from this species (Sasaki et al. 2009; Kishi et al., 2014:

70 Belmonte et al. 2015), common marmosets have started to be treated as animal models for various
71 neurological disorders, including Parkinson's disease (Ando et al., 2012; Hikishima et al., 2015),
72 sensorimotor disorders due to stroke (Bihel et al., 2010; Puentes et al. 2015) or spinal cord injury
73 (Iwanami et al., 2005).

74 Descending pathways, such as the corticospinal tract, play pivotal roles in sensorimotor
75 control, since a substantial portion of volitional motor commands are transmitted to spinal
76 motoneurons through these tracts. The corticospinal tract exists in all mammals, but there are
77 important species differences in its origin, patterns of termination, and functions (Lemon and
78 Griffiths, 2005; Isa et al. 2007). In primates, some corticospinal fibers are known to make direct
79 corticomotoneuronal (CM) connections, and such CM connections are particularly well developed
80 in Old World monkeys and humans (Heffner and Masterton, 1983; Palmer and Ashby, 1992). A
81 large amount of evidence suggests that the direct CM connection plays a major role in the control of
82 dexterous hand movements, such as relatively independent finger movements, as represented by a
83 precision grip (Schieber and Santello, 2004; Lemon, 2008). However, little is known about whether
84 common marmosets have these direct CM connections.

85 To characterize the marmoset's CST in terms of both anatomical and physiological aspects,
86 we: 1) performed histological tracing of the CST fibers from the primary motor cortex (M1) in one
87 hemisphere to terminals in the spinal gray matter at the cervical level; and 2) tested the postsynaptic
88 effects on the cervical spinal cord electrophysiologically by recording local field potentials (LFPs)
89 over a wide area in the spinal gray matter, and intracellular membrane potentials of isolated spinal
90 motoneurons. For the former purpose, the number of descending axons in the white matter, the
91 number and horizontal distribution of synaptic terminals labeled with biotinylated dextran amine
92 (BDA) was analyzed at the cervical level. For the latter purpose, excitatory and/or inhibitory
93 synaptic responses evoked by electrical stimulation at the pyramid were recorded from spinal gray

matter (field potentials) and motoneurons (intracellular recordings) at the lower cervical level. Bilateral projections from one hemisphere have been reported in other primate species (Rosenzweig et al., 2009; Yoshino-Saito et al., 2010; Morecraft et al., 2013), in which they might provide neural substrate for some recovery after spinal cord injury (Rosenzweig et al., 2010). Therefore, we also characterized the distribution of CST axons and synaptic responses of CST stimulation at different laminae on both sides of the spinal cord.

2. Materials and Methods

2.1. Subjects

Ten common marmosets (*Callithrix jacchus*; body weight: 300–400 g, aged 1 year and 6 months to 3 years and 4 months) were used in the present study (identified by letters A–F). For the tracer study we used marmoset (Ma) A, B, C, and D; for LFP recordings, we used Ma-B, D, and E; for intracellular recordings, we used Ma-F, G, H, I, and J. All animal experiments were approved by the ethics committee of Keio University (number: 11006) and the National Institutes of Natural Sciences, and were in accordance with the guidelines of the National Institutes of Health, and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

2.2. Tracer injection

2.2.1 Head chamber setting

Marmosets were trained to sit quietly in the marmoset chair during intracortical microstimulation (ICMS), and after training, they were anesthetized by i.m. injection of ketamine (Daiichi-Sankyo, 30 mg/kg) and xylazine (Bayer Healthcare, 2.5 mg/kg), and atropine sulfate

117 (Tanabe Pharma, 0.05 mg/kg) with ampicillin (Meiji Pharma, 50 mg/kg) and dexamethasone
118 (Banyu Pharma, 0.30 mg/kg) as premedication. During the surgery, anesthesia was maintained by
119 i.m. injection of ketamine (10 mg/kg) and xylazine (0.8 mg/kg) as needed, and electrocardiogram
120 and oxygen saturation level were monitored continuously. Marmosets were mounted on a
121 stereotaxic frame (Muromachi Kikai Inc., Tokyo, Japan), and the head chamber was attached to the
122 forehead of the skull by dental acrylic for stabilizing the head's position.

123 2.2.2 ICMS and Tracer injection

124 A few days after head implantation, the marmosets were made to sit on the stereotaxic chair
125 and sedated using isoflurane inhalation (Pfizer, New York, USA. 1-3%) with i.m. injection of
126 atropine sulfate, ampicillin and dexamethasone in order to prepare them for craniotomy. After
127 craniotomy, ICMS was performed in awake condition. A liquid reward was given to keep them calm
128 condition. Because marmosets do not have a central sulcus (Burish et al., 2008), the location of the
129 M1 area was estimated based on the observation of evoked motor responses during ICMS over the
130 frontal region (10 biphasic pulses composed of 0.2 ms cathodal and 0.2 ms anodal pulses, at 333
131 Hz). The somatotopic maps derived from ICMS in this study were consistent with those of previous
132 reports (Burish et al., 2008; Burman et al., 2008).

133 After ICMS, marmosets were anesthetized, and their vital signs were monitored in the same
134 head chamber setting. Forelimb, hindlimb, shoulder and trunk regions of the M1 were selected for
135 tracer injection to label the CST axons anterogradely (Fig. 1A). BDA solution (Molecular Probes;
136 10,000 MW, 10% dissolved in 0.1 M phosphate buffer [pH 7.3]) was injected (0.5 μ l) at each of 10–
137 12 penetration tracks and at two depths (1500 and 2500 μ m from the cortical surface). The tracer
138 was pressure-injected slowly over 7 min using a 10- μ l Hamilton microsyringe, which was attached
139 to the same manipulator as the electrode used for ICMS.

140

141 2.3. Histological processing

142 Five to 8 weeks (37–56 days) after injection of the tracer, the marmosets were anesthetized
143 deeply with an overdose of sodium pentobarbital (50 mg/kg, i.v.) and perfused transcardially with
144 0.1 M phosphate-buffered saline (PBS; pH 7.3), followed by 4% paraformaldehyde in 0.1 M PBS
145 (pH 7.3). After perfusion, both the brain and spinal cord were removed and postfixed in the same
146 fresh PBS containing 4% paraformaldehyde, followed by subsequent exposure to 10%, 20% and
147 30% sucrose. After saturation with 30% sucrose, serial 50- μ m-thick sections of the brain and
148 cervical spinal cord were obtained using a freezing microtome (Retortome REM-710, Yamato,
149 Saitama, Japan). To verify the injection sites, the cerebral cortex was sectioned in the coronal
150 (frontal) plane.

151 The sections of the spinal cord and cortical tissues were collected serially in 0.1 M PBS, pH
152 7.4. Some sections were Nissl-stained with 1% Cresyl Violet. In order to visualize the BDA labeling,
153 the remaining sections were processed using the avidin-biotin-peroxidase method (ABC Elite kit,
154 Vector, Burlingame, CA, USA) with 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) as
155 the chromogen. Sections were washed twice in 0.05 M PBS for 10 min, followed by incubation
156 with 0.6% hydrogen peroxide in methanol for 30 min to reduce the activity of endogenous
157 peroxidase. Then, the sections were washed three times in PBS, and incubated for 120 min in a
158 solution containing ABC, which was diluted 1:50 in 0.05 M PBS plus 0.4% Triton X-100. The
159 sections were rinsed twice in PBS and twice in 0.05 M Tris-HCl-buffered saline. The peroxidase
160 was reacted and visualized by incubating the sections in a solution containing 0.01% DAB, 1.0%
161 nickel-ammonium sulfate, and 0.0003% hydrogen peroxide in 0.05 M Tris-HCl buffer, for 15–30
162 min at room temperature. The reaction was terminated by rinsing in Tris-HCl buffer followed by
163 two rinses in PBS. After the DAB-nickel staining, the sections were mounted on gelatin-coated
164 glass slides, some of which were lightly counterstained with 1% Cresyl Violet, dehydrated and

165 coverslipped.

166

167 2.4. Quantification of the number of axons and terminal button-like swellings

168 In order to quantify the number of terminal button-like swellings (hereafter described as
169 “terminal buttons”), three sections separated by 600 μm intervals were selected from the central
170 level of C7. Detailed drawings of CST axons and their terminal buttons, along with a section outline
171 were made using a camera lucida attached to a light microscope (Olympus, Tokyo, Japan). The
172 boundaries between laminae were added to the drawings based on the cytoarchitecture of the gray
173 matter (Rexed, 1952). Descending axons, excluding collaterals, were counted in the dorsolateral,
174 ventromedial and dorsal funiculi of the white matter. The number of terminal buttons (see
175 arrowheads in Fig. 2) was counted in each lamina of the gray matter.

176

177 2.5. Electrophysiological analysis

178 2.5.1 Premedication and surgery

179 Introduction of anesthesia was the same as that of the surgery for attachment of the head
180 chamber, but it was maintained with i.v. injection of α -chloralose (Nacalai Tesque, 60 mg/kg).
181 Pneumothorax and artificial respiration was performed, with the marmosets being immobilized with
182 pancuronium bromide during recording. The expiratory CO_2 was monitored continuously and
183 maintained at 3.3–4.2 %. Rectal temperature was maintained at 36–38°C. A laminectomy of the
184 C2–C7 vertebrae was performed to expose cervical spinal segments. A posterior craniotomy was
185 performed to expose the cerebellum, which was partly sucked out to enable the approach to the
186 caudal brain stem.

187 2.5.2. Stimulation and recording

188 The protocol for electrophysiological analysis of the corticospinal effects on the spinal

189 circuits was based on that of previous studies (Alstermark et al. 2004; Tanaka et al., 2004; Umeda et
190 al., 2010) and is summarized below. Tungsten electrodes (impedance: 100 k Ω , uninsulated tip
191 diameter: 10 μ m) were inserted to stimulate the CST, the reticulospinal tract (RST), and the
192 ascending fibers in the dorsal column (DC) terminating at the cuneate nucleus, all at the medullary
193 level. The electrodes were inclined rostrally by 30° from the vertical axis of the stereotaxic
194 coordinates. Each stimulus was given to the following locations: CST—0.3 mm lateral from the
195 midline, 2.0 mm rostral to the obex, and about 3–3.5 mm deep from the bottom of the fourth
196 ventricle; RST—0.3 mm lateral from the midline, 2.0 mm rostral to the obex and about 1.0–1.5 mm
197 deep from the bottom of the fourth ventricle; and DC—1.3 mm lateral from the midline, and about
198 0.3 mm deep from the bottom of the fourth ventricle. The optimal depth of stimulation was
199 determined by checking the threshold to induce the conduction volley in the individual pathways
200 (see below) (Fig. 5).

201 The bipolar needle electrodes were percutaneously inserted to the peripheral nerve trunks,
202 namely, the deep radial (DR), and median (M) nerves. These nerves are identified by observation of
203 related muscular contractions evoked by stimulations. A combination of one or two of these nerves
204 was selected in each experiment, and the antidromic response of the stimuli was used as guidance to
205 find the lateral motor nuclei.

206 Descending volleys were recorded from the surface of the DC, cord dorsum potential (CDP),
207 in the middle part of the C2–C7 segments, using a silver ball electrode. Recordings were made in
208 each segment for measuring the conduction time of the volley. During intracellular recordings, the
209 CDP was recorded in the same segment as the glass microelectrode. Intracellular recordings were
210 obtained from 57 forelimb motoneurons in C6–C7 using glass micro-electrodes (tip diameter, ~2
211 μ m; impedance, 3–5 M Ω ; filled with 2 M potassium citrate [pH 7.4]). Among these neurons, 13
212 neurons were identified as deep radial (DR) or median (M) motoneurons by antidromic activation

213 from the peripheral nerve. However, even if the neurons were not antidromically identified, they
214 were regarded as motoneurons (unidentified), if they were recorded in the motor nucleus (at a
215 depth of 1.60–2.40 mm with a minimal filled potential of 0.2mV) and had a membrane potential
216 below -30mV and membrane resistance < 2 MΩ. For LFP recordings, similar electrodes were used
217 but with impedance at about 2 MΩ. All signals were digitized (Digidata 1440A; Axon Instruments,
218 Foster City, CA), stored on hard disk, and analyzed off-line using Clampfit (Axon Instruments).

219 To calculate the conduction velocity (CV) of each pathway, volleys were recorded from each
220 spinal segment (CST: C2–C7; RST: C3–C7; DC: C2–C6). The initial positive peak was regarded as
221 the timing of impulse arrival via the fastest conducting fibers, measured as the latency (Illert et al.,
222 1976). CV was estimated from regression analysis and electrode distances (Tanaka et al., 2004).
223 The measurements of the distance were made *in situ*.

224

225 **3. Results**

226 3.1. Anatomical analysis of the CST axons

227 3.1.1. Injection site of BDA

228 Figure 1A is a somatotopic representation of the motor cortex of Ma-A, identified by ICMS.
229 Ma-B, Ma-C, and Ma-D also showed a comparable topographic organization. BDA was injected
230 over a wide area to cover a large portion of the M1 area (Fig. 1A, arrowheads). Figure 1B shows
231 that the visualized tracer was found in the cortical gray matter including layer V of the M1 area in
232 the macroscopic view, and the large pyramidal cells in lamina V were stained densely in
233 photomicrography, as shown in Fig. 1C.

234

235 3.1.2. Axons in the white matter

236 Figure 2A shows the distribution of BDA-reaction products in C7 of four monkeys (Ma-A, B,
237 C, and D). First, the number of CST axons located in each portion of the white matter was counted
238 (Table 1). The majority of descending CST axons was distributed in the DLF on the side
239 contralateral to the injection, and the second largest population of descending axons was found in
240 the ipsilateral DLF. Few axons were observed in the DC and ventrolateral funiculus (VMF). Despite
241 considerable inter-individual differences among the monkeys, 74–91% of the CST axons were
242 distributed in the contralateral DLF, 6–17% in the ipsilateral DLF, 0.5–5.0% in the VMF, and 0.7–
243 3.9% in the DC.

244

245 3.1.3. Axons and buttons in the gray matter

246 As shown in the camera lucida drawings in the right panel of Fig. 2A, collaterals of the CST
247 were distributed both in the contra- and ipsilateral spinal gray matter. We counted the number of
248 terminal buttons of each lamina at the C7 segment (Fig. 2C). The presumed buttons were located
249 mainly in lamina VII (48.7%: the average of four marmosets) on the contralateral side to the
250 injection. There were few projections (3.7%) to the contralateral lamina IX. Note that a
251 considerable number of the collaterals were projected to laminae VII (15.4%) and VIII (5.4%) on
252 the ipsilateral side (Fig 2C). Such bilateral projections were found at all levels of the cervical spinal
253 cord, as shown in Fig. 2B.

254

255 3.2. Electrophysiological properties of descending and ascending pathways

256 3.2.1. Conduction volleys

257 Figure 3 shows the arrangement of the electrophysiological experiments. Figure 4 shows the
258 profile of evoked responses recorded at the C4 cord dorsum by stimulation at different depths of the
259 caudal medulla (2 mm rostral to the obex, 0.3 mm lateral from the midline). The short latency peaks

260 (shown as an open circle), representing the response of the RST, are prominent at a depth of about
261 1.5 mm. On the other hand, the long latency triphasic component (black circle), which was greatest
262 with stimulation at a depth of about 3.0 mm, represents the CST.

263 Figure 5B shows evoked responses by stimulation of the CST, RST, and DC (stimulation sites
264 are indicated by black circles in Fig. 5A), from which recordings were made at cervical segments
265 C2–C7, C3–C7 and C2–C6, respectively. As shown in Figs. 4B and 5B, the volleys were composed
266 of triphasic waves. The first positive peak (indicated by circles in these figures) corresponds to the
267 arrival of action potentials of the fastest fibers, and was used to calculate the CV of each tract (see
268 the next section).

269

270 3.2.2. Conduction velocities

271 Figure 5C shows plots of the latencies of the first positive peak of the triphasic volley of
272 individual tracts against the distances between the stimulation and recording, fitted with linear
273 regression lines. The CVs (mean $\pm$ SD), estimated from the slope of the regression lines, were 22.5
274 $\pm$ 0.8 m/s for CST (n=6), 66.2 $\pm$ 5.9 m/s for RST (n=5), and 33.2 $\pm$ 6.9 m/s for DC (n=4).

275

276 3.2.3. Corticospinal field potential

277 We performed LFP recordings along the dorso-ventral axis from the dorsal surface of the
278 spinal cord to a depth of 2.20–2.40 mm, with 200 μ m intervals, for several recording tracks with
279 different laterality from the midline (as shown in Fig. 6A), to cover the half of the spinal gray
280 matter contralateral to the pyramidal stimulation. Figure 6B shows the average LFPs at each
281 recording site. The responses locked to the first stimulus are indicated in black, and those to the
282 third stimulus are indicated in red. A typical LFP recording, showing two distinct negative peaks, is
283 shown in Fig. 6C; a large negative peak was observed just after the stimulation (onset latency of the

negativity was 0.8 ms from the stimulus). This component was consistent through the repeated stimulus, and the onset latency was the same as the initial positive peak of the volley in the CDP (see the blue continuous vertical line in Fig. 6C). Considering the time course of the transmission from the pyramid to the spinal cord, the first peak was taken to indicate the action potential of the descending volley. On the other hand, the third pulse of the repetitive stimulation induced a negative component with a latency from the stimulus of 1.6 ms (see the red trace in Fig. 6B; lateral 750 μ m, depth 1.2 mm). Accordingly, the segmental latency of this negative component (as measured between the continuous blue vertical line and first blue dotted line) was 0.96 ms, which was supposed to be in the monosynaptic range. Therefore, the first slow, and then sharp negativity of this late negative LFP component was thought to be the result of the summed effect of spiking responses of the neurons around the tip of the electrode overriding the excitatory postsynaptic potentials (EPSPs) induced by the monosynaptic inputs from the CST. To evaluate the magnitude of these effects and graphically represent their distribution, we analyzed the peak-to-peak amplitude of each peak in the time windows described below. We set a time window of 0.8 ms for action potentials (AP) whose onset corresponded with the first negative peak of CDP, representing the arrival of CST descending action potentials (as described above). Then, the subsequent 1.0 ms window was assigned for monosynaptic current inflow (Mono). We chose these windows so that the second window excludes the action potentials but is long enough to include the second peak. Figure 6D shows the contour map of AP and Mono. The peak of AP was located at the DLF, which corresponds to the distribution of the CST axons in the white matter (Fig. 2A), while the peak of Mono was in the lateral part of lamina VII, where the largest number of CST terminals was located (Fig. 2C).

In addition, we performed LFP recordings on the side ipsilateral to the stimulation. For the majority of recording sites, we did not observe distinct LFP components that could be related to

specific physiological events. However, when the tip of the recording electrode was located at a depth of 1.80 mm (Fig. 7A), in the track of laterality 750 μ m on the ipsilateral side, a single stimulus induced no clear response, which corresponds to a lack of massive fiber termination in this area (Fig. 7B, upper); however, repetitive stimulation induced a spiking response with a segmental latency of about 1.80 ms (Fig. 7B, lower). This may indicate oligosynaptic corticospinal linkage to this neuron. Such spiking responses were found in two distinct recording sites on the ipsilateral side to the stimulus.

3.2.4. Motoneuronal recordings

We obtained intracellular recordings from 57 motoneurons (11 neurons showing an antidromic response to the DR nerve stimulation, two to the M nerve stimulation, and 44 unidentified but interpreted as motoneurons owing to their location in the motor nuclei, see Methods). In all these cells, a single pyramidal stimulus at 100–200 μ A induced no response. However, when repetitive stimuli were applied, EPSPs were found in 46 neurons (Fig. 8), while inhibitory postsynaptic potentials (IPSPs) were recorded in 13 neurons (Fig. 9), which were reversed by passing negative current. Among the latter group of neurons, EPSPs preceded the IPSPs in two neurons. The segmental latency of the EPSPs ranged from 1.6 to 2.8 ms (mean $\pm$ SD, 2.2 ± 0.30 ms) and that of the IPSPs ranged from 1.8 to 2.9 ms (mean $\pm$ SD, 2.3 ± 0.35 ms).

These results suggested that the shortest synaptic linkage from the CST to the forelimb motoneurons is disynaptic; a monosynaptic connection is lacking. The disynaptic pathway is mainly excitatory, but existence of IPSPs with a segmental latency of less than 2 ms suggests the existence of a disynaptic inhibitory pathway from the CST to forelimb motoneurons.

332 4. Discussion

333 The significance of corticospinal effects to forelimb control is widely examined in cats (for a
334 review, see Alstermark and Isa, 2012) and monkeys (see Lemon 2008; Isa et al., 2013). In this study,
335 we characterized the organization of the corticospinal tract in common marmosets, by histological
336 and electrophysiological approaches, to contribute to the establishment of a novel animal model for
337 neuroscience. Our results indicate 1) weak or no direct monosynaptic connections to hand/arm
338 motoneurons, and 2) possible corticospinal contribution to bilateral forelimb control in marmosets.

339

340 4.1. Lack of monosynaptic CM excitation

341 We performed LFP and intracellular recordings from the spinal gray matter at the lower
342 cervical level (C7) and forelimb motoneurons, and investigated the responses to electrical
343 stimulation of the medullary pyramid. We found no sign of monosynaptic EPSPs in motoneurons.
344 Histological assays in this study also showed very sparse corticospinal termination of the CST in
345 lamina IX. Collectively, these results indicated no evidence for the existence of direct CM
346 connections in the common marmoset.

347 This clarification is important because it has been proposed that CM connections are
348 essential for the capacity of skilled digit movement. It is well known that the Old World macaque
349 monkey has direct CM connections and very well-developed dexterity (Heffner and Masterton,
350 1983). On the other hand, the ability to perform skilled digit movement varies among New World
351 monkeys. Marmosets use only power grip for retrieving food pellets (Takemi et al, 2014). Among
352 the other New World monkeys, squirrel monkeys possess weak direct CM connections (Nakajima et
353 al. 2000), and can perform tasks requiring the skilled use of digits; however, their ability for
354 independent digit movements such as forming a precision grip is limited (Nudo et al., 1992). In

355 contrast, the Cebus monkey can form a precision grip between the index finger and thumb (Spinozzi
356 et al., 2004). Cebus monkeys have abundant CM connections (Bortoff and Strick 1993).
357 Considering this knowledge, the present finding adds further evidence to the correlation between
358 the existence of direct CM connections and the ability of independent finger control. However, it
359 should also be kept in mind that the direct CM pathway is not the sole system that controls
360 independent finger movements. Recent studies in macaque monkeys showed clear evidence that
361 some groups of spinal cord interneurons are involved in the control of precision grip (Takei and
362 Seki 2010; Kinoshita et al. 2012).

363 Recent studies suggested that expression of secreted phosphoprotein 1 (SPP1) mRNA in the
364 motor cortex is correlated with the existence of direct CM connection (Higo et al. 2010; Yamamoto
365 et al. 2013). SPP1 mRNA is expressed in layer V neurons in the sensorimotor cortex of rhesus
366 macaques, Cebus monkeys, and humans but only weakly or not expressed in squirrel monkeys,
367 marmosets, and rats. Thus, again, the present study supplied additional evidence for the hypothesis
368 that SPP1 expression is involved in the functional or structural specialization of highly developed
369 corticospinal systems (Yamamoto et al., 2013).

370 Monosynaptic excitation from the motor cortex to spinal motoneurons could not be found in
371 our study, but instead, we observed di- or oligosynaptic excitation or inhibition from the CST in a
372 majority of motoneurons. Identification of intercalated neurons mediating such indirect effects,
373 either segmental interneurons, propriospinal neurons, or reticulospinal neurons, is not clear at this
374 moment, but as shown in Fig. 2, a large number of CST terminations could be observed in lamina
375 VII of all levels of cervical segments, where the majority of premotor neurons are known to exist
376 (Alstermark and Kümmel 1990; Kinoshita et al. 2012). These results suggest it is likely that spinal
377 interneurons such as segmental interneurons and propriospinal neurons mediate the cortical
378 command to motoneurons, as is the case in cats and macaque monkeys (Alstermark and Isa, 2012;

379 Isa et al. 2013).

380

381 4.2. Bilateral spinal terminations of the corticospinal tract

382 Our results suggested corticospinal contribution to bilateral forelimb control in the marmoset.

383 The present study showed that on average, 85% of CST axons descend in the dorsolateral tract of

384 the spinal cord, contralateral to the hemisphere of origin, while 10.7% of axons descend in the

385 dorsolateral fasciculus that is ipsilateral to the hemisphere of origin. In the gray matter of the lower

386 cervical spinal cord (at least from C2–C8), CST axons send bilateral axonal projections. Abundant

387 button-like swellings were found along its extent. This remarkable bilaterality of the CST projection

388 is quantitatively distinct from observations in rats (Rouiller et al., 1991; Brus-Ramer et al., 2007).

389 In contrast, the anatomy revealed in the present study is similar to the CST organization of the Old

390 World monkey (Rosenzweig et al., 2009; Yoshino-Saito et al., 2010; Morecraft et al., 2013) and of

391 humans (Nathan et al. 1990). Furthermore, in this study, we observed the spiking response of spinal

392 neurons induced by repetitive CST stimulation on laminae VII–VIII of the spinal gray matter

393 ipsilateral to the pyramidal stimulation with relatively short segmental latency (1.8 ms), which

394 suggested oligosynaptic linkage from the CST. These results suggested that the CST could exert

395 synaptic effects bilaterally on the spinal cord in the marmoset.

396 The bilaterally projecting CST axons may provide a possible substrate for recovery after

397 spinal cord injury. A recent study in the macaque monkey showed that spinally decussating

398 corticospinal fibers might have sprouted within the cord following spinal hemisection (Rosenzweig

399 et al., 2010). It has been reported that humans sustaining spinal cord hemisection (Brown-Sequard

400 syndrome) recovered the ability of locomotion and to partially use the affected hand (Little and

401 Halar, 1985; Roth et al., 1991). Therefore, the presence of abundant bilateral corticospinal

402 projections in the marmoset suggests the usefulness of this animal species as the model animal to

403 study the neuronal mechanism of and therapeutic strategy for functional recovery after human
404 spinal cord injury.

405

406 **Acknowledgement**

407 This work was supported by grants of Japan Orthopaedics and Traumatology Research
408 Foundation, Inc. No. 245 to Y.K., Grant-in- Aid for JSPS Fellows (23-40018) to Y. K., Research
409 Center Network for Realization of Regenerative Medicine from by the Japan Science and
410 Technology Agency (JST) to H.O., and Keio Gijuku Academic Development Funds to J. U.

411

Figure 1. Injection sites of BDA into the primary motor cortex. (A) The results of ICMS

mapping and injection sites (arrowheads). The movements induced in each location with the lowest threshold either in the wrist (W), elbow (E), shoulder (S), trunk (T), jaw (J) or hindlimb (H) are indicated. Open circles indicate the locations where no movement was induced with the current intensity lower than 70 μ A. (B) BDA-reaction product at the injection site. (C) High-magnification view of BDA-labeled pyramidal neurons in layer V at the injection site in Ma-A. Note that the number of penetration points for ICMS was reduced in Ma-C and Ma-D for minimizing the physical load.

Figure 2. Camera lucida drawing of descending fibers and terminal button-like swellings in

the lower cervical segments following BDA injection into the primary motor cortex. (A) Photographs of whole BDA-labeled sections of the C7 segments (left); camera lucida drawing of the BDA-labeled terminal button-like swellings to show their laminar distributions (right). (B) Photographs of the whole BDA-labeled sections of the C3, C5, C7 spinal segments in Ma-B and Ma-C. (C) Histogram indicating the absolute number of terminal button-like swellings in individual laminae of Rexed in the C7 segments of individual marmosets. The numbers over three sections were averaged. The error bars indicate SD.

Figure 3. Schematic drawing of the experimental arrangement. (A) A schematic drawing

illustrating the position of the electrodes to stimulate the medullary pyramid on the left side and the deep radial (DR) nerve, silver ball electrodes for cord dorsum potential (CDP) recordings, and a glass microelectrode for intraspinal recordings. (B) A typical antidromic response of a DR motoneuron.

436 **Figure 4.** Depth profile of the evoked volley along a track for stimulation at the caudal
437 medulla. (A) Transverse section of the medulla (2 mm rostral to the obex, 0.3 mm lateral to the
438 midline) showing the electrode track used for medullary stimulation to activate the CST and RST.
439 (B) CDP recordings at the C4 segment in response to stimulation of the medulla at 200 μ A. The
440 depths of the stimulating electrode were varied from 0.0 mm (surface) to 3.5 mm at 0.5 mm
441 intervals. The black circle and white circle indicate the onset of the first negative component of the
442 corticospinal and reticulospinal volleys, respectively.

443
444 **Figure 5.** Properties of conduction volleys evoked by stimulation of the corticospinal tract
445 (CST), reticulospinal tract (RST) and ascending fibers in the dorsal column (DC). (A) A transverse
446 section of the medulla. Stimulation sites of CST, RST, and ascending fibers in the DC are indicated
447 with black circles. (B) Examples of volleys recorded at different cervical segments for each tract.
448 The onset of the first negative components, regarded as the timing of arrival of the action potential
449 of the fastest conducting fiber of the tract, are indicated by white circles. (C) Plots of the onset
450 latencies against the conduction distance from stimulation to recording for each tract in Ma-E, F, G,
451 H, I, and J.

452
453 **Figure 6.** Local field potentials evoked by stimulation of the medullary pyramid. (A,B)
454 Transverse section from the C5 segment and four electrode tracks for recording the LFP placed at
455 different horizontal locations: 250 μ m, 500 μ m, 750 μ m and 1,000 μ m lateral to the midline. Each
456 track contains 11 recording points at intervals of 0.20 mm (from 0.20 mm to 2.20 mm). (B) The
457 average of 15 LFP recordings at different recording sites of the spinal cord, immediately after (for 2
458 ms) the first (black) and third (red) pulse of the repetitive stimulation (intervals of 3.3 ms) of the
459 medullary pyramid at 200 μ A stimulation. The baselines were adjusted to each other. Note that the

460 third pulse evoked peaks with longer latency at some recording sites. (C) Fifteen sweeps of LFP
461 recordings and the average at 750 μm (lateral) and a depth of 1.60 mm from the surface (upper, the
462 same as the corresponding recording site in Fig. 7B), and CDP recording (lower). The continuous
463 blue vertical line indicates the onset of the volley and the first dotted vertical blue line represents
464 0.8 ms from the onset. We considered this time window as the range of the action potentials of the
465 descending CST fibers (AP). The second time window, between the two dotted vertical blue lines is
466 presumed to correspond to the range of monosynaptic responses (Mono), the duration of which is
467 1.2 ms. (D) Contour map of the peak-to-peak value measured in the time window determined as
468 above. Linear interpolation was applied between each recording site. AP (action potentials)
469 represents the distribution of action potentials of the descending CST axons and Mono represents
470 monosynaptic current inflow.

471

472 **Figure 7.** Example of responses to the ipsilateral pyramidal stimulation. Extracellular
473 responses to the pyramidal stimulation recorded at 750 μm ipsilateral and 1.80 mm from the surface
474 (A; black circle). (B) The spiking response recorded at this location. Quadruple pulse stimulation
475 applied to the ipsilateral medullary pyramid evoked spiking responses of a spinal neuron.

476

477 **Figure 8.** Intracellular recordings from forelimb motoneurons. (A) An example of disynaptic
478 excitatory postsynaptic potentials (EPSPs). Fifteen sweeps of an intracellular recording (middle)
479 and the average (top) from a deep radial (DR) motoneuron showing the effect of single, double,
480 triple and quadruple pyramidal stimulation at 200 μA . CDP records are shown below. (B)
481 Distribution of segmental latencies of pyramidal EPSPs.

482

483 **Figure 9.** Intracellular recordings from forelimb motoneurons; inhibition. (A) The effect of

current injections at -10 nA (left), 0 nA (middle) and $+10$ nA (right). The same arrangement is used as in Fig. 8A. (B) Distribution of segmental latencies of pyramidal inhibitory postsynaptic potentials (IPSPs).

Table 1. Number of descending axons in each component of the white matter. BDA-labeled axons of three sections in each component of the white matter at the C7 levels in individual marmosets with BDA injection to the primary motor cortex. Data are presented as means $\pm$ SD (percentage). DLF, dorsolateral funiculus; VMF, ventrolateral funiculus; DC, dorsal column.

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 612

Table. 1

	DLF		VMF	DC
	Contra	Ipsi		
Ma-A	505 ± 64 (89.0%)	36 ± 8 (6.3%)	5 ± 1 (0.8%)	22 ± 3 (3.9%)
Ma-B	936 ± 95 (90.7%)	84 ± 5 (8.1%)	5 ± 1 (0.5%)	7 ± 1 (0.7%)
Ma-C	1051 ± 56 (86.0%)	140 ± 31 (11.5%)	16 ± 5 (1.3%)	15 ± 6 (1.2%)
Ma-D	994 ± 41 (74.4%)	224 ± 15 (16.8%)	66 ± 6 (4.9%)	52 ± 2 (3.9%)

Figure 1

Figure 2

A

Ma-A

Ma-B

Ma-C

Ma-D

B

Ma-B

C3

Ma-C

C3

C5

C5

C7

C7

C

Ipsi

Contra

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

A

B

Figure 8

A

B

Figure 9

A

B