Corticospinal Sprouting Differs According to Spinal Injury Location and Cortical Origin in Macaque Monkeys

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The primate corticospinal tract (CST), the major descending pathway mediating voluntary hand movements, comprises nine or more functional subdivisions. The role of subcomponents other than that from primary motor cortex, however, is not well understood. We have previously shown that following a cervical dorsal rhizotomy (Darian-Smith et al., 2013), CST projections originating from primary somatosensory (S1) and motor (M1) cortex responded quite differently to injury. Terminal projections from the S1 (areas 3b/1/2) shrank to <60% of the contralateral side, while M1 CST projections remained robust or expanded (>110%). Here, we asked what happens when a central lesion is added to the equation, to better simulate clinical injury. Monkeys ($n = 6$) received either a unilateral (1) dorsal root lesion (DRL), (2) or a combined DRL/dorsal column lesion (DRL/DCL), or (3) a DRL/DCL where the DCL was made 4 months following the initial DRL. Electrophysiological recordings were made in S1 4 months postlesion in the first two groups, and 6 weeks after the DCL in the third lesion group, to identify the reorganized region of D1–D3 (thumb, index finger, and middle finger) representation. Anterograde tracers were then injected bilaterally to assess spinal terminal labeling. Remarkably, in all DRL/DCL animals, terminal projections from the S1 and M1 extended bilaterally and caudally well beyond terminal territories in normal animals or following a DRL. These data were highly significant. Extensive sprouting from the S1 CST has not been reported previously, and these data raise important questions about S1 CST involvement in recovery following spinal injury.

Key words: axonal sprouting; corticospinal pathway; motor cortex; nonhuman primate; somatosensory cortex; spinal cord injury

Introduction

The corticospinal tract (CST) in the macaque monkey and higher primates is the primary pathway involved in volitional movements of the hands (for review, see Schieber, 2007; Lemon, 2008; Isa et al., 2013), and its importance peaks in humans where finger control is most sophisticated. In macaques, the pathway innervating the cervical cord has ≥ 9 different cortical origins (Catsman-Berrevorts and Kuypers, 1976; Murray and Coulter, 1981; Nudo and Masterton, 1990; Dum and Strick, 1991; Galea and Darian-Smith, 1994; Darian-Smith et al., 1996; Lemon, 2008), including primary motor (M1), primary somatosensory (S1), premotor, and other cortices. This number may be greater in humans. The spinal termination pattern for each cortical region is unique, reflecting a different functional role in hand use (Ralston and Ralston, 1985; Lemon and Griffiths, 2005; Morecraft et al., 2013).

The M1 CST is known to play an important role in the recovery of hand function following hemisection lesions in monkeys

(Galea and Darian-Smith, 1997a,b; Rosenzweig et al., 2010). Rodent studies likewise indicate CST regenerative potential following experimental manipulations (Brus-Ramer et al., 2007; García-Alías et al., 2009; Ghosh et al., 2010; Liu et al., 2010), though the contribution of CST sensorimotor subcomponents is not known, and other tracts (i.e., rubrospinal) may be equally important in recovery.

We have previously shown in macaques, following a unilateral cervical dorsal root lesion (DRL) that removes input from the first three digits (D1–D3), (1) that there is an initial impairment, but subsequent recovery, of digit use over several months (Darian-Smith and Ciferri, 2005); (2) that behavioral recovery is accompanied by local sprouting of spared primary afferents within the dorsal horn (Darian-Smith, 2004); (3) that there is considerable reorganization of the “digit” maps within the cuneate nucleus and S1 (Darian-Smith and Brown, 2000; Darian-Smith, 2009); and (4) that there are major spinal synaptic changes (Darian-Smith et al., 2010). We have also shown that M1 and S1 subcomponents of the CST respond quite differently following a DRL (Darian-Smith et al., 2013), whereby the M1 CST sprouts into the dorsal horn on the side of the lesion, and the S1 CST shrinks to <60% of its original size. This suggests that, following a DRL, the M1 CST plays an important role in postinjury recovery, while the involvement of the S1 CST declines.

Here we were interested in the specific role of S1 and M1 CST projections in postinjury recovery when a central injury is added to the equation, since this model better simulates clinical injuries (e.g., dorsal root avulsions or any spinal injury involving a pe-

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Table 1. Details of monkeys used in the study

Lesion	Monkey ID	Mean weight (kg)	Postoperative times		Tracer and cortical placement (rostrocaudal extent of labeling)	
			Weeks between lesion and cortical surgery	Weeks for tracer uptake	S1	Motor
DRL only	M601	3.00	9.5	6	BDA (C1–C8)	LYD (C1–rostral T5)
	M604	2.02	14	7	LYD (C1–C8)	BDA (C1–rostral T5)
DRL/DCL early	M1106	3.80	DRL/DCL, 11	7	LYD (C1–T4)	BDA (C1–rostral T6)
	M1109	3.16	DRL/DCL, 15	7	BDA (C1–T4)	LYD (C1–T5)
DRL/DCL late	M1107	3.72	DRL, 16; DCL, 6	7	LYD (C1–T3)	BDA (C1–T5)
	M1110	3.90	DRL, 15; DCL, 6	7	LYD (C1–T3)	BDA (C1–T5)

DRL/DCL early animals received both lesions at the same time, whereas DRL/DCL late monkeys received the DCL ~4 months following the initial DRL

Figure 1. Placement of spinal cord DRL and DCL lesions (left side in all monkeys), and cortical maps showing recording sites and neuronal tracer placement. Motor cortex injections were placed according to somatosensory cortex recordings. Sections were processed through all injections to ensure that white matter was not involved and that there was no cross-sulcal contamination. No visible damage or necrosis was observed at the injection sites. Individual injection site volumes were not estimated, given poor definition of the injection borders of the tracers LYD (yellow) and BDA (red). However, injections were found to be equivalent in size and placement across animals. Scale bars, 2 mm.

ripheral and central component). We were also interested in the effect of a small central injury made months following a DRL, since a delay in its timing provides mechanistic insight into the chronic condition and suggests therapeutic strategies that may help optimize postinjury recovery. Our findings show dramatic changes following a combined DRL/dorsal column lesion (DCL), regardless of whether the DCL was made at the same time as the DRL or 4 months later. In both scenarios, the S1 and M1 CST

both sprouted dramatically to (potentially) form new connections. In this way each is likely to be contributing to circuitry remodeling, and each plays a key role in the recovery process.

Materials and Methods

Animals used and general procedures. Six young adult male macaque monkeys (*Macaca fascicularis*), averaging 3.3 kg (Table 1), were used in this study. All monkeys were colony bred and between 3 and 4 years of

Figure 2. Photomicrographs showing the core of the DCL made in each of the DRL/DCL monkeys in the present study. Sections were taken from the series stained for Lucifer yellow with DAB. Note that in all cases, the lesion was confined to the cuneate fasciculus, so afferent inputs from the trunk and hindlimb were left intact. M1106 and M1109 were early lesions. M1107 and M1110 were late lesions. Scale bars, 1 mm.

age (where sexual maturity is typically reached at 4–5 years in this species). Though the animals used in the study were relatively young, their sensorimotor pathways mediating hand function were considered mature, and their age is unlikely to have played a significant role in our findings. Monkeys were housed individually at the Stanford Research Animal Facility, in four-unit cages for each monkey (64 × 60 × 77 cm depth × width × height per unit). All procedures were conducted in accordance with National Institutes of Health guidelines and approved by the Stanford University Institutional Animal Care and Use Committee.

Experimental sequence. Monkeys were divided into three groups. Two animals received a DRL alone, and data from these monkeys were reported previously (Darian-Smith et al., 2013). Four monkeys (not used elsewhere) received combined DRL/DCLs but were divided into two groups based on whether the central DCL was made at the same time as the DRL ($n = 2$) or 4 months following an initial DRL ($n = 2$). This delay provided additional insight into the role of the central injury in the CST response. To make lesions (Figs. 1, 2), a laminectomy was used to expose the cervical cord to assess the distribution of sensory input from the first three digits of one hand (the thumb, index finger, and middle fingers or D1–D3). Dorsal rootlets innervating D1–D3 were mapped electrophysiologically and cut to produce the DRL. To make the DCL, the cuneate fasciculus was targeted and cut (Fig. 2). In the final surgery in all monkeys (for timing details, see Table 1), a bilateral craniotomy was made over the S1 and M1 in the region of hand representation. This area was mapped to locate the boundaries of D1–D3, and a series of injections of either the anterograde tracer biotin dextran amine (BDA; Sigma-Aldrich, B-9139), or Lucifer yellow dextran (LYD; Invitrogen, A5750) were made into the S1 or M1 cortex (Fig. 1). Figure 1 and Table 1 specify which tracer was used in each cortical region in each monkey.

Surgery and perfusion. Anesthesia was first induced with ketamine hydrochloride (10 mg/kg) and animals were subsequently maintained throughout surgery with gaseous isoflurane (1–2%)/O₂ (1%) using a standard open circuit anesthetic machine. Atropine sulfate (0.05 mg/kg, i.m.) and cefazolin (20 mg/kg, i.v.) were administered before surgery. A

laminectomy was made during the first surgery in each monkey and the overlying dura cut to expose dorsal rootlets from segments C5–T1. Electrophysiological recordings were made to create a microdermatome map (Darian-Smith and Brown, 2000; Darian-Smith, 2004; Darian-Smith and Ciferri, 2005; Darian-Smith et al., 2013), and this was then used to select rootlets to cut. All rootlets with receptive fields (RFs) on D1–D3 were cut in two places (using iridectomy scissors) to remove 2–3 mm of the rootlet along the length of the lesion. A DCL was made level with the most rostral cut rootlet (Fig. 1), using a scalpel blade (no. 11) that had been scored at 2 mm from the tip. The overlying tissues and skin were then closed in layers. Buprenorphine (0.01–0.02 mg/kg, i.m.) was administered after suturing to provide a postoperative analgesic, and monkeys were returned to their cages and observed closely. Monkeys were awake and alert typically within 1 h, and no postoperative sequelae were observed.

In the cortical surgery, a bilateral craniotomy was made over the central sulcus and electrophysiological recordings (in S1) were used to identify the boundaries of the D1–D3 representation for tracer injection (contralaterally, this was the region of cortical reorganization). A small ~1 cm² bone flap was removed and the dura opened as a series of small windows. This helped maintain a healthy cortex over several hours. Agar (3% in physiologic saline) was used to dampen cortical movement for recording but then removed before tracer injection. Once tracer injections were completed, the bone flap was replaced and the overlying skin sutured. All anesthesia and postoperative analgesic regimens were as described for the laminectomy. Monkeys also recovered rapidly from this procedure.

At the end of each experiment, monkeys were deeply anesthetized and given a lethal dose (intravenously) of sodium pentobarbital (0.44 ml/kg) until spontaneous breathing and corneal reflex had ceased. Animals were then manually breathed and perfused transcardially with heparinized 0.1 M PBS, pH 7.4, followed by 4% paraformaldehyde in phosphate buffer (PB), pH 7.4. The brain and spinal cord were removed, postfixed (same perfusate) for 4 h, and transferred to 30% sucrose for cryoprotection.

Electrophysiological recordings in dorsal roots and cortex. Recordings were made from dorsal rootlets (Darian-Smith and Brown, 2000; Darian-Smith, 2004; Darian-Smith and Ciferri, 2005). Briefly, a laminectomy was made and dura resected to expose rootlets from C5–T1. The exposure was photographed so that individual rootlets could be identified for accuracy. A tungsten microelectrode (1.2–1.4 MΩ at 1 kHz) was used to record from each rootlet, and 8–10 single or small multiunit extracellular recordings were made from axons within each fascicle to produce a microdermatome map. Cutaneous RFs were mapped by progressively using hand manipulation, a camel hair brush, and Von Frey hairs. The RF was defined as cutaneous if the stimulus force of ≤2.0 g evoked a response. When the stimulus force of the Von Frey hair was >2.0 g, or where joint movement or hand manipulation were needed to evoke a response, the RF was marked as deep. Where there was ambiguity as to whether the RF was cutaneous or deep, the RF was included as cutaneous for the purposes of defining which rootlets to lesion. All RFs were mapped on to hand/body image score sheets and rootlets with cutaneous RFs on the thumb, index finger, and middle finger were then cut.

Cutaneous RF maps of hand representation were also made bilaterally within the S1 (6–7 weeks before the end of the experiment; Table 1). S1 in this study refers to Brodmann areas 3b and 1, and possibly the rostral tip of area 2 in some monkeys. In M1109, illustrated in Figure 3, the injection sites were contained within cytoarchitectonic regions 3b and 1. Area 3a was not involved in any of our analyses. A craniotomy was made and dura cut to expose a small window of cortex (~1 cm²). Electrode penetrations were then made along the rostral lip of the postcentral gyrus at the approximate border of Brodmann areas 3b and 1 (Fig. 1). Extracellular small multiunit recordings were made in the S1, using stimuli as described above for dorsal rootlets. Similar recordings have been described previously (Darian-Smith and Brown, 2000; Darian-Smith, 2004; Darian-Smith and Ciferri, 2005).

Tracing corticospinal terminal fields within the spinal cord. Anterograde tracers BDA (15% aqueous) or LYD (15% aqueous) were injected bilaterally into the region of D1–D3 representation in S1 and/or M1 (Fig. 1). All injections, were made using a constant-pressure Hamilton syringe

(20 μ l capacity) with a tapered glass micropipette attached (tip diameter, ≤ 30 μ m) with 5 min Araldite. Each injection delivered 0.3 μ l of tracer into the cortex at a 1 mm depth, and the pipette was kept in position for 1 min before being removed (Fig. 3).

BDA was visualized in axon terminals histochemically, with the attachment of peroxidase (ABC kit; Vector, PK-6100), and the chromagen diaminobenzidine (DAB, Sigma-Aldrich). Tissue sections were cut coronally using a freezing microtome (50 μ m) and collected and washed in 0.1 M phosphate buffer and 0.1% Triton (PB-TX), pH 7.4, incubated in ABC for 1 h, rinsed, preincubated in nickel-intensified (0.04% NiNH_4) DAB (0.05% DAB) in 0.1 M PB-TX, and finally incubated with the same solution with 0.01% H_2O_2 until the reaction product was clearly visible (8–10 min).

LYD was visualized with immunohistochemistry. Tissue sections were incubated (4°C) with primary anti-Lucifer yellow (Invitrogen, catalog #A5750; RRID:AB_1501344; 48 h, 1:200), a secondary antibody, biotinylated anti-rabbit (24 h; Vector, BA-1000), and avidin biotin to attach peroxidase (ABC kit; Vector, PK-4000), and reacted with the chromagen DAB (0.05%) plus 0.01% H_2O_2 , 24 h later. Details have also been reported previously (Darian-Smith et al., 1999, 2013).

Pathway analysis. The spinal cord was sectioned coronally and a series of sections taken for histochemical and immunohistochemical processing to visualize BDA and LYD. An additional series was stained for cresyl violet. All sections were cut 50 μ m thick and were separated by either 300 μ m (DRL animals) or 400 μ m (DRL/DCL monkeys), with this interval consistent within individual animals.

The distribution territory was defined as the region occupied by axon terminal boutons labeled following the bilateral injection of either LYD or BDA into S1 or M1 cortex (D1–D3 representation). Terminal boutons were mapped using NeuroLucida (RRID:nif-0000-10294; MicroBright-Field), within a sequential series of coronal sections. Section series were mapped from C1 through T5 (except for DRL animals where sections were mapped from C4 through T1), though statistical analyses were only determined through the lesion zone for sections from C6 through C8 (see Results). A line was drawn to enclose the mapped boutons, and the mean area was calculated (Darian-Smith et al., 2013). Outlying boutons (<5) were not included in the analysis, since they represented $<1\%$ of the population. For calculating terminal distribution territory volumes, the labeled area was multiplied by the interval distance to the next section, and summed.

The gray matter of spinal cord sections used in the statistical analysis (C6–C8) was divided into three regions, as illustrated in the inset in Figure 9, approximating the dorsal horn (Dorsal), the intermediate zone (Medial), and the ventral horn (Ventral; for comparison, see Kuypers, 1981, description of Rexed's laminae; Morecraft et al., 2013). A macro program was written in the image processing software Fiji (National Institutes of Health, previously ImageJ, RRID:nif-0000-30467), and this was used to partially automate the section division (see Fig. 9). The dorsal region was defined by a line drawn between a point at the base of the midsagittal fissure and the most medial border at the base of the dorsal horn. The centroid was then determined for the remaining area beneath this point and a horizontal line drawn dividing this space using medial and ventral regions (by the macro program). Areas were determined for each of these three regions (D_1 , M_1 , V_1), as well as the terminal bouton territory within them (D_2 , M_2 , V_2), and terminal territories calculated as percentages for each region (D_2/D_1 , M_2/M_1 , V_2/V_1).

Statistical analysis. The number of animals used in our analysis was small, which is part of nonhuman primate research. However, this did

Figure 3. Photomontages from adjacent sections showing typical LYD (top) and BDA (bottom) injection sites from monkey M1109. The middle section is Nissl stained with cresyl violet. All injection series were localized to either areas 3b/1 or area 4, and, in some instances, corticocortical labeling was clearly visible. In the BDA-labeled sections, corticocortical labeling demarcates the 3a/3b border (arrow, lower right). Inset shows location of coronal sections. D1–D3, Digits 1 (thumb), 2 (index finger), and 3 (middle finger). Scale bar, 1 mm.

not decrease the power of the statistical analysis used, which took sample size, tracer uptake efficacy, and other variables into account (see Materials and Methods; for discussion of analysis, sample size, and power, see Darian-Smith et al., 2013; Hoenig and Heisey, 2001; Levine and Ensom, 2001).

We analyzed the data as a repeated-measures design within monkeys, where the monkey was the subject and individual slices were repeated measures further subdivided into side, region, and projection. This approach gives far higher power than a simple *t* test approach, providing equivalent power at exponentially lower sample sizes (Grafen and Hails, 2002), not least because it enables us to separate out variance due to individual differences between monkeys, from variance due to biological and technical differences between sections. Such advanced analysis is an essential tool for improving animal welfare in studies of this kind (Russell, 1995).

Our primary variable of interest was the ipsilateral/contralateral staining ratio. When considering the mean ipsilateral/contralateral ratio over multiple repeated measures, this mean is made up of two components. First, the overall mean ratio and, second, the degree to which that ratio is stable when the degree of contralateral staining is particularly high or low. To tease these apart, we used a repeated-measures regression. This enables us to figure the conventional mean ipsilateral/contralateral ratio (by testing the mean ipsilateral staining at the mean level of contralateral staining), controlling for the relative stability of these ratios in the different subareas and treatments (lesion types). Data were analyzed as a Repeated Measures Restricted Maximum Likelihood (REML) mixed model in JMP10 for Windows (SAS). Subject (animal) was nested within treatment (lesion type) as a random blocking factor, and suitable interactions were included to ensure the correct calculation of repeated-measures tests (Newman et al., 1997). Section position was included as a quadratic continuous blocking factor. Subarea (D, M, and V), projection (S1 vs M1), and treatment (lesion type) were included as interactions and non-significant interactions were removed to preserve the integrity of testing marginal terms (Grafen and Hails, 2002). *Post hoc* tests were estimated as REML planned contrasts and, using the Benjamini–Hochberg procedure,

Figure 4. Corticospinal terminal labeling within the spinal segments C1–T5, which originated from the primary motor cortex (D1–D3 representation). Data shown to left (M604) is from a monkey with just a DRL (also reported in Darian-Smith et al., 2013). Data shown on right (M1106 and M1109) is from monkeys that received a combined DRL/DCL lesion at the same time (or “early”; Table 1). The location of each section illustrated is indicated on the schematic of the spinal cord. Blue and green bars indicate the full rostrocaudal extent of terminal labeling for DRL and DRL/DCL early animals, respectively. Green contours demarcate terminal bouton distribution areas. Note that terminal labeling extended ~15 mm farther caudally in DRL/DCL monkeys compared with the DRL monkey. Lesion is on the left side. Scale bar, 1 mm.

ture, corrected for multiple-testing false-discovery rates (Benjamini and Yekutieli, 2001). The assumptions of mixed models (linearity, homogeneity of variance, and normality of error) were confirmed *post hoc* (Grafen and Hails, 2002).

All figures were assembled and created in CorelDraw X4, and photomicrographs adjusted for sharpness, contrast, and brightness within Adobe Photoshop (CS5).

Results

Corticospinal projections were analyzed in six monkeys (Table 1). Injections were always made bilaterally in the region of D1–D3 representation in S1, which was identified electrophysiologically (Fig. 1). M1 injections were made opposite the S1 sites on the opposite side of the central sulcus.

To ensure that labeling did not occur in response to changes in membrane uptake properties following injury, BDA and LYD

were used interchangeably in different animals in the S1 and M1 cortex. Our statistical analysis controlled for confounds due to tracer and confirmed that there were no significant tracer-related effects in the data (Darian-Smith et al., 2013). Tracer-uptake anomalies were therefore not considered a concern in the present study.

Defining cortical injection sites and lesion extent

Injections within the cortex were examined through a series of sections within the region of hand representation in the sensorimotor cortex. Injection sites did not extend beyond the cortical gray matter or across the central sulcus, which could have compromised the data (Fig. 3). Though the boundary of the injection sites was not always sharply delineated (making volume analysis impossible), injections were always matched precisely for location (i.e., within the region of D1–D3 representation, determined

electrophysiologically; Fig. 1), number, and tracer volume injected. Injection sites were typically localized to cytoarchitectonic areas 3b and 1, as defined by adjacent Nissl-stained sections, though some involvement of rostral area 2 cannot be ruled out. Figure 3 shows an example of injection sites from adjacent sections and both hemispheres in M1109. There were no differences in the cortical injection extent between hemispheres in any of the monkeys that could have accounted for the differences in the terminal labeling observed and described below.

Figure 2 shows that the DCL in each of the DRL/DCL monkeys only involved the cuneate fasciculus in M1107 and M1106 (as far as could be determined from lesion reconstruction), with some additional gray matter involvement in M1109 and M1110. Thus the central injury may have involved cutaneous afferents from the remaining digits (D4 and D5) or forearm that ascend in the dorsal column, but primary afferents for the rest of the body and hindlimb would not have been affected. Importantly, the spinothalamic projection from D4 and D5 was not cut by the lesion, and the central component of the DRL/DCL monkeys largely affected primary afferents already involved by the DRL, as well as secondary neurons originating below the DCL, and any additional fibers traversing this tract. The spinothalamic pathway mainly transmits information from A δ and C receptors (or pain and temperature information), but past studies suggest that this pathway may also encode crude cutaneous and proprioceptive information, either via A δ and C fibers or via spared cutaneous afferents that branch and synapse on spinothalamic afferents within the dorsal horn. Thus D4 and D5 were only partially deafferented in monkeys receiving a combined DRL/DCL lesion, which is unlikely to have translated to a detectable behavioral deficit in these digits. We propose that the addition of the DCL in the DRL/DCL does not significantly affect hand function beyond that already observed following a DRL (Darian-Smith and Ciferri, 2005). Behavioral data obtained from one DRL/DCL monkey, M1106 (not reported here), supports this, since we observed little difference in the deficit or recovery of hand function (tested for D1–D3) in this monkey compared with a DRL alone. Further behavioral data, however, are needed to fully test this.

Analysis of terminal territories

Though the terminal bouton distribution territory was mapped in sections at regular intervals (from C1 to T5 for DRL/DCL animals and from C5 to T1 in DRL monkeys), our statistical analysis was conducted only on sections spanning C6–C8. The reason for this was as follows. DCLs were made level with the rostral DRL border, which was either in caudal C5 (M1109,

Figure 5. Corticospinal terminal labeling within the spinal segments C1–T5, which originated from the primary motor cortex (D1–D3 representation) from monkeys that received a combined DRL/DCL lesion, where the DCL component was made several months following the initial DRL lesion (DRL/DCL late; Table 1). The location of each section and rostrocaudal extent of labeling is indicated with green bars on spinal cord schematic. Conventions as in Figure 4. Lesion is on the left side. Scale bar, 1 mm.

M1110) or rostral C6 (M1106, M1107). C6–C8 was therefore the region of the cord where most of the D1–D3 afferent inputs were mapped (electrophysiologically) and cut when making the DRL during the initial surgery. This is where the greatest changes in circuitry were likely to be occurring and where we observed the greatest differences in the CST labeling patterns on the two sides of the cord. We were able to determine the boundaries of this region very accurately during the initial surgery, and again in the postmortem spinal cord dissection, where the segments, cut rootlets, and dorsal root entry of these to the cord were clearly visible. In sections both rostral and caudal to this region, terminal distribution territories were typically similar between the two sides of the cord, regardless of the animal or lesion type (Figs. 4–7).

Corticospinal projection patterns

The ipsilateral/contralateral staining ratio differed between M1 and S1, between the different subareas, and between the treatments (treatment–subarea–pathway interaction: $F_{(4,10.71)} =$

Figure 6. Corticospinal terminal labeling within the spinal segments C1–T4, which originated from the primary somatosensory cortex (D1–D3 representation). Data shown to left (M601) is from a monkey with just a DRL (also reported in Darian-Smith et al., 2013). Data shown on right (M1106 and M1109) are from monkeys that received a combined DRL/DCL lesion at the same time (or “early”; Table 1). Conventions as in Figure 4. Note that terminal labeling extended ~25 mm farther caudally in DRL/DCL monkeys compared with the monkey receiving a DRL alone. Lesion is on the left side. Scale bar, 1 mm.

5.6784; $p = 0.0105$; see Fig. 10). The following sections explore this result and describe the pattern of terminal labeling within the spinal cord for M1 and S1 CST subcomponents.

Motor cortex

Following a DRL

The data for the DRL monkeys used in this study (M604 and M601) were reported in detail in our recent paper (Darian-Smith et al., 2013). Briefly, and as described in that study, the M1 CST in DRL monkeys responded by sprouting into the dorsal horn in the lesion zone on the side of the lesion. This was observed in three of four monkeys, with the mean terminal territory >110% of the contralateral side. This is summarized for M604 in Figures 3 and 10. Note that following a DRL alone, the pattern of labeling on the side contralateral to the lesion was equivalent to control nonle-

sioned animals as previously described (Galea and Darian-Smith, 1997b; Rosenzweig et al., 2010; Morecraft et al., 2013).

Following a DRL/DCL

The response from the M1 CST following a DRL/DCL was greater than was observed following the DRL alone (Figs. 4, 5; see Fig. 11). In all four monkeys (regardless of whether the DCL component was made early or late) we observed the same extension into the dorsal horn as was observed following a DRL alone. In addition, we also consistently observed more ventral terminal labeling (compared with DRL animals) within the lesion zone, both throughout the ventral horn and bilaterally (Figs. 4, 5; see Fig. 11).

Figure 9 shows the mean histogram profiles of terminal territories (C6–C8) as a percentage of gray matter for the dorsal, medial, and ventral regions of the spinal cord. Each of the lesion

groups and ipsilateral versus contralateral sides of the cord relative to the lesion are shown for both the M1 and S1 CST terminal territories. Not surprisingly, when analyzed as ipsilateral/contralateral ratios (see Fig. 10), data show significantly different overall profiles of terminal labeling for the DRL versus the DRL/DCL animals and for M1 versus S1 CSTs.

For the M1 CST, the pattern of ipsilateral/contralateral ratios in the different subareas was significantly different in each treatment (within M1, subarea-treatment interaction: $F_{(8,16,23)} = 9.5265$; $p < 0.0001$). Further *post hoc* tests of this result showed that staining in the terminal territory was significantly greater following a DRL/DCL (mean of both treatments) versus a DRL in the ventral subarea ($F_{(1,18,28)} = 16.6763$; $p = 0.0007$) and the medial subarea ($F_{(1,27,17)} = 12.3093$; $p = 0.0016$), whereas the dorsal region did not differ between treatments ($F_{(1,16,87)} = 0.0873$; $p = 0.7713$).

Terminal labeling was also observed bilaterally from C1 through T5 following the combined lesion (Figs. 4, 5). Terminal labeling in T5 indicated caudal sprouting for ~10–15 mm beyond the (~45 mm–C1–T4; Fig. 4) rostrocaudal territory observed in monkeys with a DRL alone.

As a coarse measure of comparison, absolute terminal territory volumes were also calculated through segments C6–C8 in all monkeys. The overall terminal territory volume for M1 DRL monkeys was found to be a little more than half that calculated for DRL/DCL monkeys (mean, 16.96 mm³; SD, ±4.8) versus 28.89 mm³ (±3.7) ipsilaterally, and 14.66 mm³ (±6.0) versus 24.22 mm³ (±2.6) contralaterally, in DRLs versus DRL/DCLs respectively).

Somatosensory cortex

Following a DRL

As we have previously reported (Darian-Smith et al., 2013), the S1 CST terminal territory was reduced to <60% on the side of the lesion, compared with the contralateral side of the cord. This was in contrast to the M1 CST response in the same DRL animals, and suggests a reduced role for this pathway in circuit reorganization following a DRL alone. Figure 6 shows four sections from M601, and Figure 9 summarizes the reduced S1 CST response in the DRL animals (Darian-Smith et al., 2013).

Following a DRL/DCL

Surprisingly, and counter to our observations following a DRL alone, the S1 CST response was bilateral and dramatic following the combined DRL/DCL. The overall terminal territory volume for the S1 CST in DRL/DCLs (C6–C8) was four times that calculated in DRL animals on the ipsilateral side of the cord (mean, 4.84 mm³; SD, ±2.3; vs mean, 20.95 mm³; SD, ±4.6) and almost three times the DRL volume on the contralateral side (mean, 6.96 mm³; SD, ±2.9; vs mean, 17.94 mm³; SD, ±5.6).

Figure 7. Corticospinal terminal labeling within the spinal segments C1–T4, which originated from the S1 cortex (D1–D3 representation) from monkeys that received a combined DRL/DCL lesion, where the DCL component was made several months following the initial DRL lesion (DRL/DCL late; Table 1). Conventions like those in Figure 4. The location of each section and rostrocaudal extent of labeling is indicated with green bars on spinal cord schematic. Consistent with all DRL/DCL monkeys, terminal labeling was observed caudally to caudal T4. Lesion is on the left side. Scale bar, 1 mm.

Terminal labeling patterns are shown for all four monkeys in Figures 6 and 7, and summarized in Figures 9–11. In all four DRL/DCL monkeys, the S1 CST terminal territory extended ventrally into the medial region or intermediate zone (most obvious within segments C6–C8) and, in three of the four monkeys, this extension was observed into the ventral horn. The absence of this extension into the ventral horn in M1109 (Fig. 6) may simply reflect interanimal variability, since this was not the result of this monkey having fewer cortical injections relative to the other monkeys (Fig. 1).

For the S1 CST, the pattern of ipsilateral/contralateral ratios in the different subareas was significantly different in each treatment (within S1, subarea-treatment interaction: $F_{(8,14,32)} = 10.0787$; $p = 0.0001$). Further *post hoc* tests of this result showed that staining in the terminal territory was significantly greater following a DRL/DCL (mean of both treatments) versus a DRL in the dorsal subarea ($F_{(1,13,75)} = 17.5707$; $p = 0.0009$) and the medial subarea ($F_{(1,16,3)} = 61.9035$; $p < 0.0001$), whereas the ventral region did not differ between treatments ($F_{(1,20,38)} = 2.0692$; $p = 0.1655$).

Figure 8. Photomicrographs (including montages) of labeling in M1106 and M1109 showing terminal distributions following a DRL/DCL, within cervical and thoracic segments. Examples are shown from the S1 (left column) and primary motor (right column) corticospinal tracts. Lesion side is on the left for all images. Scale bar, 1 mm.

In addition, terminal labeling consistently extended rostro-caudally in DRL/DCL monkeys from C1 through T4 (Fig. 8, label in T3; see Fig. 11). The caudal extension (i.e., sprouting) of terminal labeling was bilateral for the S1 CST in all four DRL/DCL monkeys, and extended for ~4 segments beyond what we observed for DRL animals. Terminal labeling was located within the intermediate zone within thoracic segments and gradually diminished caudally, though not always equally on both sides for M1109 and M1107 (Figs. 6, 7, thoracic segments). The extension observed in these animals was twice the total rostrocaudal spread (~50 mm total vs ~25 mm) observed in the DRL monkeys. The assumption was made that terminal labeling extended rostrally to C1 in the DRL animals, as has been described in the literature (Kuypers, 1960; Galea and Darian-Smith, 1997b; Lee and Kim, 2012).

It should also be noted that while we did not include data from C1–C5 in our statistical analyses, Figures 6 and 7 show bilateral labeling in the intermediate and ventral horns through the most rostral part of C1 in all DRL/DCL animals. In addition, terminal labeling was observed in the dorsal horn on the side of the lesion in rostral cervical segments (in three of four DRL/DCL monkeys), which was not observed contralaterally (Figs. 6, 7; see Fig. 11). Comparable data were not obtained from C1–C3 in DRL monkeys (for S1 CSTs), so it is not known if this labeling was

atypical. Figure 11 summarizes the extensive S1 CST sprouting induced following a DRL/DCL compared with a DRL alone.

Comparing early and late DRL/DCL data

An important observation in the present study was that M1 and S1 CST responses were similar in all four DRL/DCL monkeys even though two monkeys (M1110 and M1107) received the central DCL 4 months following the initial DRL lesion. We performed a series of *post hoc* tests to assess differences between DRL/DCL early and DRL/DCL late animals, including testing for mean overall differences (e.g., is the mean value for all S1 projections different between early and late animals?) and for between-region differences (e.g., whether the difference between medial and ventral M1 scores differed between treatments). None of these tests yielded a significant result. Though the DCL in M1109 and M1110 involved some gray matter, this did not appear to affect the terminal labeling patterns.

Discussion

We have shown that the S1 and M1 CSTs respond very differently following a DRL (peripheral) versus a DRL/DCL (peripheral plus central) in monkeys. With a DRL alone, input to the cervical cord from the S1 cortex diminished on the side of the lesion to ~60%

Figure 9. Histogram showing the data used in the statistical analysis in this study. The CST terminal territory is shown as the percentage of gray matter in the dorsal, medial, and ventral subdivisions. Mean percentages are given for both sides of the cervical cord and for M1 and S1 CSTs. Subdivisions D, M, and V are defined for typical section at right (see text for details). Data were collected for C6–C8. Error bars indicate SEM. Most of the major findings of this study are apparent here. For example, in medial S1 there was significantly more labeling following a DRL/DCL versus a DRL, and in ventral M1, there was significantly more labeling following a DRL/DCL versus a DRL.

of the original distribution territory and the M1 CST remained intact or increased (Darian-Smith et al., 2013). In contrast, with the addition of a central DCL (C5/C6), there was a massive and bilateral expansion of terminal labeling during the following months from both the S1 and M1 CSTs. This response was similar in all monkeys receiving a combined DRL/DCL, regardless of whether the DCL was made at the same time or 4 months after the DRL. The pattern of terminal labeling following the DRL/DCL was also quite different for the S1 and M1 CST subcomponents.

The degree of sprouting observed in our monkeys following the DRL/DCL is unprecedented and, from the reorganized S1 cortex, particularly dramatic. This suggests that the S1 CST response may be as important an indicator of recovery as the M1 CST, at least in primates.

In discussing our findings, certain caveats should be kept in mind. Since the same tracer was injected into both hemispheres, it was not possible to determine the hemisphere of origin of the CST sprouting. This may be important for the M1 CST response, where many fibers cross the midline before and after spinal in-

Figure 10. Summary of the CST terminal labeling patterns from S1 and M1 following a DRL compared with a DRL/DCL, showing the ipsilateral/contralateral distribution territory ratio. Values >100% indicate greater relative staining on the side ipsilateral to the lesion. Values <100% indicate greater relative staining contralaterally. Asterisks indicate values significantly different from 100%.

jury. However, crossover fibers are unlikely to play a significant role in the S1 CST response (at least for hand), since they are not present in normal and DRL animals (Darian-Smith et al., 2013), and are almost nonexistent following the DRL/DCLs (i.e., we observed only 1–2 per section in C1–C3). In addition, terminal bouton density was not determined in this study, since different

Figure 11. Summary of the CST terminal labeling patterns from S1 and M1 following a DRL compared with a DRL/DCL. Columns with green shading show terminal labeling patterns contralateral to the DRL, which are equivalent to nonlesioned terminal distributions. Orange shading shows terminal labeling patterns ipsilateral to the DRL, and blue shows terminal labeling patterns on both sides following the combined DRL/DCL. Major changes are clear. First, the S1 CST plays little part following a DRL, but expands dramatically and bilaterally following a DRL/DCL, with a caudal extension of terminals from C8/T1 to caudal T4. For the motor CST, terminal sprouting extends into the dorsal horn on the side of the lesion following a DRL. Following a DRL/DCL, the terminal sprouting extends bilaterally into the ventral horn, and caudally for an additional segment into T5. Asterisks indicate data not collected in the current study, and predicted from published findings (Kuypers, 1960; Galea and Darian-Smith, 1997a; Lee and Kim, 2012) and more caudal patterns.

tracer injection series were made into the two hemispheres, and a bouton density comparison between sides would be difficult to interpret (Darian-Smith et al., 2013). Having said this, we consistently noted more terminal boutons ipsilateral to the lesion (C5–C8) in the DRL/DCL animals, which is opposite to our observations in DRL monkeys (Darian-Smith et al., 2013).

Interestingly, animals in the early DRL/DCL group and animals in the late DRL/DCL group did not differ statistically in their response (Figs. 9–11), which raises an important clinical question, especially for chronic patients. Could an even smaller central spinal injury awaken and augment the formation of new connections and induce additional recovery many months (or even years) following a disabling rhizotomy or brachial plexus injury? This is a subject for future study.

So what fundamentally changes with the inclusion of a central (dorsal cuneate fasciculus) lesion to induce such a dramatic re-

sponse from both M1 and (especially) S1 CSTs? The answer almost certainly lies with immune response and/or the location of the DCL component of the DRL/DCL.

In contrast to peripheral injuries, CNS injuries result in an enhanced immune response that involves a cascade of acute to chronic events, a massive astrocytic and microglial proliferation, and the eventual formation of a cellular scar (Fitch et al., 1999; Darian-Smith, 2009; Rolls et al., 2009; Karimi-Abdolrezaee and Billakanti, 2012). The cellular and molecular environment changes dramatically at the site of injury (Yang et al., 2006) and well beyond, and, while many factors associated with this altered environment are essential for protection and repair of surviving neurons (David et al., 2012), others can inhibit CST sprouting (e.g., chondroitin sulfate proteoglycans; Fitch and Silver, 2008; Rhodes and Fawcett, 2004; ephrins; Miranda et al., 1999; Fabes et al., 2006). While our understanding of the immune response over time following different spinal injuries remains poor (particularly in primates), determining the CST response following a DCL injury alone will help test its importance. If similar M1 and S1 CST sprouting is observed, then clearly the central injury and changing CNS environment are key to our understanding of the mechanisms involved. If, however, terminal sprouting from the S1 and M1 CSTs is not observed following a DCL alone, then there may be an interaction between the peripheral and central injuries that will require further investigation.

Another explanation for the different CST sprouting response following the two lesions is that different circuitry was affected by each. A DRL alone removes all primary afferent input from the affected digits but leaves the central circuitry intact. Though the DRL/DCL largely cuts the same primary afferents already cut by

the DRL, it also involves more caudal, primary afferents of the hand. The spinothalamic input remains intact below the dorsal rhizotomy, as do all descending projections (i.e., bulbospinal, reticulospinal, rubrospinal, etc.), so D4–D5 are only partially deafferented. Most sensory reafference, which requires input from the periphery, is lost for D1–D3 in both lesions. However, the central cuneate fasciculus lesion in the DRL/DCL blocks additional intraspinal connections not cut by the DRL alone, and this may contribute to the different CST responses following the two different lesion models.

From where do these atypical projections originate? In normal macaque monkeys, as much as 98% of the motor “hand” CST projection arises from the contralateral cortex and descends in the dorsolateral CST (dlCST), with 2% descending in the ipsilateral dlCST (Morecraft et al., 2013). M1 CST axons are also known to send collaterals to the contralateral side of the cord. Previous

studies in macaque monkeys that looked at the M1 CST response following spinal hemisection suggest that these collaterals play a role in postinjury sprouting and the recovery process (Galea and Darian-Smith, 1997b; Rosenzweig et al., 2009). In keeping with these earlier reports, we also clearly observed M1 CST axons crossing the midline in cervical and thoracic segments following DRLs and DRL/DCLs (Fig. 8). However, their hemispheric origin and the importance of their contribution to bilateral sprouting and postinjury recovery remains unclear.

In nonlesioned monkeys, the S1 CST terminates in a confined region of the dorsal horn and there is no evidence of crossover collateral branching. Following a DRL/DCL in this study, a few labeled S1 CST axons were observed in the midline in C1–C3 (Fig. 8), but none were observed below these segments in either the DRL/DCL or DRL monkeys, so S1 CST crossover axons are unlikely to play a significant role in reorganization after injury. Given this, what could be driving the bilateral response following the combined DRL/DCL? One possibility is that callosal connections to the ipsilateral hemisphere drive the CST terminal sprouting in the spinal cord contralateral to the lesion. While callosal projections connect the hand region of M1 (Brodmann area 4) interhemispherically in macaques, they are surprisingly sparse in the hand region in S1 areas 3b and 1 (Killackey et al., 1983; Iwamura, 2000). They are, however, reasonably dense for areas 2 and 5, where bilateral RFs are commonly found (Iwamura, 2000). It is therefore feasible that the partially deafferented S1 hand region receives callosal input either directly from area 2, or indirectly (i.e., via corticocortical connections), and that callosal input helps drive M1 and S1 CST sprouting in the contralateral cord.

Surprisingly, following a DRL/DCL, the M1 and S1 CST terminal distribution extended caudally well into the thoracic cord. For the S1 CST, terminal labeling extended as far as T4 (i.e., four segments) or double the distance (i.e., a total of 50 vs 25 mm) observed in normal monkeys (Galea and Darian-Smith, 1997b) or those with a DRL alone (Darian-Smith et al., 2013). For the M1 CST, terminal bouton labeling was observed as far as rostral T6 (M1106), or an additional segment beyond what we observed in DRL monkeys. The role of these caudal connections is not known, but they may enhance intraspinal and propriospinal connectivity supporting hand–trunk coordination following injury.

At the rostral pole of the cervical cord, dense terminal labeling was also observed (for both S1 and M1 CSTs in DRL/DCL monkeys) in the internal basilar nucleus close to the midline (Figs. 4–7, 10). Though little is known about the role of this nucleus, it is continuous with the cuneate nucleus (Cliffer and Giesler, 1989). It receives input from ulnar and radial nerves (LaMotte et al., 1991) and from the cervical enlargement (Cliffer and Giesler, 1989). It also receives CST input from the sensorimotor cortex (Kuypers, 1960; Antal, 1984; Bortoff and Strick, 1993; Valtschanoff et al., 1993). It seems likely that this region may also contribute to postinjury reorganization and recovery.

Our results raise important questions. What spinal neurons do newly formed terminals synapse with and are they functional? What is the time course for their formation? How does the cellular and molecular microenvironment change over time? And how do our findings correlate with behavioral recovery?

Our findings indicate that the role of the S1 CST in reorganization following a DRL/DCL may be as important as the M1 CST in the recovery process following a central spinal injury. They also suggest that the S1 CST may present an important additional biomarker of recovery following spinal injury. Certainly, the role of the CST subcomponents in recovery following spinal injury

warrants further study, and should be considered in the interpretation and design of future experiments.

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