Topographically Divergent and Convergent Connectivity between Premotor and Primary Motor Cortex

This study was undertaken to determine the topographic organization of connections between the forelimb representations of the ventral premotor cortex (PMv) and the primary motor cortex (M1). Intracortical microstimulation techniques were used in three experimentally naive squirrel monkeys to delineate the M1 and PMv forelimb representations in the hemisphere contralateral to the dominant hand. Small amounts of biotinylated dextran amine (BDA) were then injected in the PMv distal forelimb representation. Following tangential sectioning, the location of the injection core in PMv and BDA-labeled cell bodies and synaptic boutons in M1 were documented in relation to functional topography. Whereas the injection core was mainly located within the distal forelimb representation in PMv, BDA-labeled cell bodies and terminals were distributed over comparable proportions of proximal and distal forelimb representations in M1. These results suggest that neuronal populations within PMv send topographically divergent outputs to M1 and receive topographically convergent inputs from M1. Finally, we found that PMv projections to M1 were not evenly distributed but rather were directed consistently to three domains within the rostro-lateral portion of M1. To our knowledge, this is the first description of such a consistent clustering of PMv terminals within M1.

Keywords: hand, ICMS, M1, neuroanatomy, PMv, squirrel monkey

Introduction

Based upon numerous neurophysiological and ablation-behavior studies, the ventral premotor cortex (PMv) is thought to be involved in visuomotor integration for motor control of the upper extremity (Hoshi and Tanji, 2004). Microelectrode stimulation studies of PMv have shown that upper limb and orofacial movements can be evoked from this area (Gentilucci et al., 1988; Preuss et al., 1996; Dum and Strick, 2002; Frost et al., 2003). Recording studies in awake behaving monkeys suggest that PMv neurons are involved in both the initiation and control of limb movements in relation to visual and somatosensory cues, and may be modulated by the direction of gaze (Godschalk et al., 1981; Rizzolatti et al., 1983, 1988; Kurata and Tanji, 1986; Gentilucci et al., 1988, 1989; Mushiake et al., 1991; Boussaoud and Wise, 1993; Murata et al., 1997).

PMv shares connections with motor areas of the frontal cortex, somatosensory areas of the parietal cortex, and visual/visuomotor areas of the frontal and parietal cortex (Matelli et al., 1986; Barbos and Pandya, 1987; Huerta et al., 1987; Kurata, 1991; Ghosh and Gattera, 1995). Since the PMv hand area provides one of the most prominent inputs to the M1 hand area (Tokuno and Tanji, 1993) and contains a substantial number of corticospinal neurons (Nudo and Masterton, 1990; He et al., 1993), it is not surprising that stimulation of PMv evokes distal forelimb movements. However, its corticospinal projections are largely to upper cervical segments, where motoneurons controlling proximal muscles are located (Martino and Strick, 1987; He et al., 1993; Galea and Darian-Smith, 1994). This may suggest that PMv’s role in hand motor control is exerted more indirectly, e.g. through its projections to M1. More recent studies conducted in macaque monkeys have demonstrated that PMv can have a powerful facilitatory effect on M1 corticospinal outputs (Cerri et al., 2003; Shimazu et al., 2004), supporting this hypothesis.

Because of the substantial influence of PMv on M1 output, it is important to further define the topographic specificity of PMv connections with M1. To date, no anatomical study has identified the topographic distribution of PMv projections to specific movement fields within the entire M1 forelimb area, or the reciprocal projections from M1 back to the PMv forelimb area. If PMv provides an important pathway for modulation of M1 output, such studies might provide insights regarding the role of this premotor area in motor control.

To document PMv intracortical connections in detail, we injected the bidirectional neuronal tract tracer, biotinylated dextran amine (BDA), into the PMv hand representation of three adult squirrel monkeys. Histological examination of tangential sections allowed us to align the location of BDA-labeled cell bodies and terminals to the M1 motor map. In New World monkeys, the subdivisions of PMv found in macaque monkeys (F4 and F5, respectively), have not been identified (Frost et al., 2003). Thus, we use the collective term “PMv” in the present study.

Materials and Methods

Derivation of Motor Maps

Three adult squirrel monkeys were used in the present study, weighing between ~700 and 1200 g (animals 1893, 1802 and 9409). All animal use was in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Surgeries were performed using aseptic techniques and halothane-nitrous oxide anesthesia (Nudo et al., 1992). Vital signs were monitored and maintained within the normal physiological range. A craniectomy exposed the M1 and PMv hand areas. A plastic cylinder was fitted over the opening and used to contain warm, sterile silicone oil.

After the surgical procedures were completed, the halothane was withdrawn and ketamine and Valium (diazepam) administered intravenously. Then, intracortical microstimulation techniques (ICMS) were used to derive neurophysiological maps of movement representations. A microelectrode, made from a glass micropipette tapered to a fine tip and filled with 3.5 M NaCl, was used for electrical stimulation applied at a depth of ~1750 µm (layer V). Stimulation consisted of a 40 ms train of 13 monophasic cathodal pulses of 200 µs delivered at 350 Hz from an electrically isolated, constant current stimulator (Nudo et al., 2005).
1992; Nudo and Milliken, 1996). Pulse trains were repeated at 1 Hz intervals; current was limited to 30 μA, or less. For cases 1934 and 1982, M1 and PMv were mapped at relatively low spatial resolution (~500 μm interpenetration distances); for case 9409, M1 was mapped at high spatial resolution (~250 μm interpenetration distances) and PMv at lower resolution (~500 μm). In both M1 and PMv, the cortical territory was explored with ICMs until sites eliciting digit and wrist/forearm movements were circumscribed by sites eliciting elbow, shoulder, face and tongue movements or by sites where no response was evoked at the maximum current level of 30 μA (Nudo et al., 1992; Nudo and Milliken, 1996; Frost et al., 2003). The cortical surface areas occupied by individual movement representations were then determined using a graphical program (Canvas 3.5; Deneba systems, Inc.) by drawing polygons circumscribing sites where movements of similar body segments were elicited by ICMs. In some analyses, the digit and wrist/forearm representations were grouped into a broader ‘hand representation’ category, consistent with our previous publications.

**Injection of Neuronal Tracer**

A small volume of biotinylated dextran amine (BDA; 5% BDA in saline solution; 10 000 mol. wt, conjugated to lysine; Molecular Probes) was injected into the PMv hand area. The injection in case 1934 was made via pressure injection with a microsyringe pump controller (UPP2-1, WPI instruments) attached to a 1 μl Hamilton syringe (0.2 μl). Injection in case 1892 was made via pressure injection with the same pump controller through a tapered, graduated micropipette (Fisher Scientific Company; 40 μm OD; 0.2 μl). For case 9409, the injection was performed using electrophoresis (6 μA positive current, 7 s on/7 s off for 10 min). All injections were made at different depths in order to label a radial column within PMv through all cortical layers. In case 1892, Fast Blue (Dr. Illing Plastics GmbH) was injected 250 μm caudal to the BDA injection. This injection was made for purposes unrelated to the present study. While some necrosis appeared at the injection site in histological sections, the results were qualitatively similar to the other two cases.

**Histological Procedures**

Twelve days following the injection of BDA, each animal was euthanized with a lethal dose of pentobarbital solution (Euthasol) injected i.P. The animal was then perfused with a heparinized saline solution followed by 3% paraformaldehyde in phosphate buffer (pH 7.4). The brain was then extracted and the cerebral cortex carefully separated from the rest of the forebrain. The cortex was then trimmed to remove and discard the temporal and occipital lobes. The remaining parietal/frontal cortex was extracted and the cerebral cortex carefully separated from the rest of the forebrain. The cortex was then sectioned tangential to the cortical grey matter. Other procedures included the surrounding halo (Fig. 1). The area we defined as the area of transition where cells and terminals could not be easily differentiated. The contour was drawn outside the area of transition where terminal, cell body and fiber labeling started to be identifiable. Thus, the area we defined as the injection core partly included the surrounding halo (Fig. 1).

**BDA Processing**

After sectioning, the tissue was passed through two 10 min rinses in cold 0.05 M potassium phosphate buffer in saline solution (KPBS) followed by two 30 min rinses in 0.4% Triton X-100. Before incubation overnight in the Vectorstain ‘AB’ solution (two drops of ‘A’ and ‘B’ per 5 ml of 0.05 KPBS; Elite; Vector Laboratories, Burlingame, CA), the sections were rinsed (10 min) three times in 0.05M KPBS. The following day, the tissue was rinsed (10 min) four times in 0.1 M KPBS and then incubated in a DAB solution (10 mg of diaminobenzidine [DAB]) in 20 ml of 0.1 M KPBS and 6.7 μl of 30% H2O2 (multiple of the same); formula modified from Dolleman-Van der Weel et al., 1994) for 5–10 min. After three rinses in 0.1 M KPBS, the sections were mounted on subbed slides and dried overnight.

For the DAB intensification process, sections were dehydrated the next day, then transferred to xylene for 4 days. They were then rehydrated, with a final immersion in H2O for 5 min, and incubated in a 1.42% AgNO3 solution for 1 h at 56°C. Sections were subsequently passed through H2O (15 min), 0.2% HAuCl4 (10 min), H2O (15 min), 5% Na2S2O3 (5 min) and H2O (15 min). Sections were finally dehydrated again followed by xylene and coverslipped the next day.

**Verification of Injection Location and Size**

To estimate the location and size of the BDA injections, for each animal, the slide with the largest injection core was visually identified using a dissecting microscope. Then, the injection core was outlined in the section reconstruction (Neurolucida, Microbrightfield Inc.). The dense core was defined as the area of the injection site where cells and terminals could not be easily differentiated. The contour was drawn outside the area of transition where terminal, cell body and fiber labeling started to be identifiable. Thus, the area we defined as the injection core partly included the surrounding halo (Fig. 1).

**Figure 1.** Anatomical analysis. (A) Digital photograph of the slide with the largest dense core area in case 9409. The black arrow indicates the dense core where cells and terminals could not be easily differentiated. The red arrow indicates the zone of dense terminal labeling due to local intrinsic connectivity (halo). Throughout this area, terminal, cell body and fiber labeling were identifiable. Scale bar = 1 mm. (B) Estimation of the injection core. Note that our estimation includes the entire core and part of the surrounding halo, and therefore may slightly overestimate the region of effective uptake. (C) Example of a BDA-labeled cell body found in the rostro-lateral portion M1 following BDA injection in PMv. (D) Example of anterograde labeling of small fibers in M1. (E) Inset showing numerous varicocities considered to be terminals along small fibers (black arrows show two examples). Scale bar = 50 μm.
Analysis of BDA-labeled Cell Bodies and Terminals

For anatomical analyses, a neuroanatomical reconstruction system, consisting of a computer-interfaced microscope (Carl Zeiss, Inc.) and associated software (Neurolucida), was used to record the locations of labeled terminals and cell bodies. This system is based on a stepping motor driven stage and video graphics overlay.

Documentation of Cell Bodies and Terminal Labeling

Cell bodies were roughly round in shape (due to tangential sectioning) with dense black granules of BDA label. We required the labeled cell body to have at least two thin projections, considered to be dendrites or axon. Occasionally, cell bodies were so intensely labeled that they appeared solid dark brown (Fig. 1C). Six to eight sections per case were plotted to document the distribution pattern of cell bodies. Variocities were considered to be terminals or boutons if they appeared as small, darkly labeled spheres attached to a small fiber (Fig. 1D,E). Since the time required to identify each terminal within these sections was prohibitively long, we sampled two slides per animal. To sample both superficial and deep laminae, one section was taken at depths roughly corresponding to layer V (~1600-1800 μm) and the other one at a depth roughly corresponding to layer II/III (~300-600 μm). High-resolution photographs of the remaining sections were scanned visually (but not entered into the computerized neuroanatomical system) to assure representative sampling. Depth was extrapolated from the section numbers. The pattern of cortical connections of PMv in these additional sections was not found to differ in any substantial way from the reconstructed data. Thus, the sampled sections could be used for quantitative comparisons. We sampled the selected slides using a grid pattern overlaid on the section image. If at least two terminals were located within a 100 × 100 μm square of the grid, a marker was placed in the center of the square. As each section had a finite depth (50 μm), the volumetric unit ’voxel’ is used here to report variocities or terminal distribution.

Superposition of Sections Stained for BDA and Neurophysiological Maps

The section stained for BDA reconstructions could be aligned across the entire depth of the gray matter using the distribution pattern of blood vessels. The alignment of the physiological map to section reconstructions was done in a second step by identifying the penetration location of large vessels that were identifiable on the digital photograph of the cortex. Thus, physiologically defined maps of functional areas (i.e. M1 and PMv) could be aligned with the BDA section reconstructions.

Cell bodies and voxels contained in each body representation of the physiological map were compiled and reported in proportion to total connections in the physiologically defined M1 forelimb representation. Cell bodies and voxels containing terminals outside of the physiologically mapped borders were eliminated from further analysis. Proportions were defined by the following formula:

\[ \frac{\text{Total of C or T in representation X}}{\text{Total of C or T in M1 forelimb representation}} \]

where \( C \) = cell bodies, \( T \) = voxels with labeled terminals, \( X \) = the particular body representation, i.e. digit or wrist/forelimb or proximal, and forelimb representation = total forelimb area mapped in M1, excluding non-responsive area.

Visualization and Statistical Analysis of Terminal Distribution in M1

In each case, a list consisting of \( x \) and \( y \) coordinates indicating the location of voxels with \( \geq 2 \) BDA-labeled terminals was produced (Neurolucida). Lists generated from two co-registered sections were combined for analysis in each case. Coordinates outside of the M1 forelimb area were cropped from the dataset. Then, the point distribution was statistically tested for deviation from a random pattern using the Ripley’s \( K(t) \) function, a measure of clustering at many possible distances \( (t) \) and a randomization test (Diggles, 2003). The \( K(t) \) function for the observed terminal locations was compared with \( K(t) \) functions for 500 random data sets. Each random data set had the same number of points distributed randomly, i.e. without clustering, to grid cells on the 100×100 μm sampling grid. Although \( K(t) \) functions are most commonly used to examine clustering in point patterns, they can be used for gridded data when the clustering of interest is considerably larger than the scale of the observation grid, which it is here.

To facilitate visualization of the distribution of terminals in M1, we used a scientific visualization software package (Transform version 3.4; Fortner Software LLC). An arbitrary Zvalue of ‘2’ was assigned to each \( x-y \) coordinate pair indicating BDA-labeled terminals. The visualization software then condensed the original dataset into a 101 × 101 matrix. \( Z \) values within each cell of the condensed matrix were added, providing a relative density distribution. The distribution was then smoothed using a five pass smoothing function resulting in interpolated graphs. Finally, two levels of isodensity contour lines were drawn to circumscribe areas with densities \( \geq \) mean density + 2 SD and mean density + 3 SD, respectively.

To examine common locations of the terminal distribution in the three cases relative to the M1 forelimb map, a warping algorithm was employed (MatLab 6.5). Six anatomically or physiologically defined coordinates were identified in each case to use as ‘control’ or reference points (described in Results). A piecewise linear transformation (Goshin, 1986) was used to map locations in one image (input image; cases 1934 and 1892) to new locations in another image (base image; case 9149). The piecewise linear transformation is a local transformation, since it applies different mathematical expressions (differing in parameters but not in form) to different regions within the image. In each region of the image, an affine transformation was performed, which may include translation, rotation, scaling, and shearing (generally in an affine transformation straight lines remain straight, parallel lines remain parallel, and rectangles may become parallelograms). The transformed point coordinates were given by the following formula:

\[ \begin{bmatrix} x' \\ y' \end{bmatrix} = \begin{bmatrix} t_1 & t_2 \\ t_3 & t_4 \end{bmatrix} \begin{bmatrix} x \\ y \end{bmatrix} \]

The six coefficients \( t_{ij} \) of the affine transformation that was performed in each region of the image were found by solving the above equation for three control point pairs (matching locations, also referred to as landmarks in the input and base images) associated to that region. Over the whole image, six control point pairs were used to infer the coefficients of the regional transformations. At the first step, the algorithm finds a Delaunay triangulation of the control points (Aurenhammer, 1991). Then, using the three vertices of each triangle, it subsequently infers the regional affine mapping from base to input coordinates.

Once the three cases were warped to the same coordinate space, the visualization software was used as described above to (i) condense the data to a 101 × 101 matrix, (ii) add Zvalues within each cell of the matrix, (iii) interpolate the data using a five-pass smoothing function and (iv) draw isodensity contour lines.

Results

Delineation of PMv and M1 Hand Representations

As previously described for squirrel monkeys, the M1 upper extremity representation was found immediately rostral to the central sulcus (Nudo et al., 1992, 1996; Nudo and Milliken, 1996b). In general, the caudal border of the hand representation was defined by unresponsive sites corresponding to area 3a (Nudo et al., 1992), whereas the medial, lateral and rostral borders were defined by responses of proximal joints. The PMv forelimb representation was located rostral and lateral (ventral) to the M1 hand representation (Fig. 2; Frost et al., 2003). The hand areas of M1 and PMv were typically separated by proximal (movements evoked at the shoulder and elbow joints) and orofacial representations. The PMv hand representation was surrounded by proximal representations at all borders, while orofacial representations were consistently found at its...
caudo-lateral borders. The center of the PMv hand representation was found at an average distance of 6.1 ± 0.7 mm from the center of the M1 hand representation. The rostral border of M1 was 1.4, 2.5 and 2.2 mm from the caudal border of PMv in cases 1934, 1892 and 9409, respectively. Details of the M1 and PMv physiological mapping results are reported in Table 1 for each animal.

**Motor Representations Corresponding to BDA Injection Core in PMv**

We quantified the size and located the core for each BDA injection with respect to the location of the physiological map to facilitate interpretation of variability among animals. In general, we produced one small injection (9409) and two comparatively larger injections (1934 and 1892; Fig. 3A). While the injections in case 1934 and case 1892 were large in comparison to that of case 9409, the actual amount of injected tracer was relatively small with respect to other studies in New World primates (e.g. Stepniewska et al., 1993). In each case, the dense core area was smaller than the PMv hand representation as defined by ICMS procedures, occupying 19.4% of the PMv hand area in the small injection case (9409), and 55.1 and 30.2% respectively in the large injection cases (Fig. 3B, Table 1). Whereas 1934 and 1892 received identical volumes of BDA (see methods), the smaller core in 1892 was most probably due to the different technique used for the delivery of the tracer. In each case, the most rostral portion of the PMv hand representation was not included in the injection core.

Measurements of the PMv topographic areas within the injection core were possible with the graphics program used for reconstruction of the physiological maps (Canvas 3.5, Deneba systems, Inc.). This analysis revealed that the injection core for each animal included different proportions of digit versus wrist/forearm representations (mean = 49.7 ± 28.8 and 29.3 ± 34.2%, respectively; Table 1). Due to the fractionated organization of movement maps in PMv, all injections additionally included a small portion of proximal (elbow/shoulder) representation (mean = 20.6 ± 10.1%; Table 1). The injection did not include the orofacial representations in any of the three cases.

**Motor Representations Corresponding to Locations of BDA-labeled Cell Bodies and Terminals in M1**

In general, the qualitative pattern of PMv projections to M1 was similar in all three cases. Based on our neurophysiological maps of movement representations in M1, the area of M1 sharing connections with the injected PMv hand area was limited to the rostro-lateral portion of the forelimb representation (M1FL; Fig. 4). Although tangential sectioning is not suited for laminar analysis, the density and distribution of BDA-labeled cell bodies and terminals did not appear to differ through the cortical depths that we examined. Thus, laminar specificity is not reported.

Laterally, terminal labeling extended beyond the physiologically defined proximal representation. Based on confidence limits derived from several motor maps derived previously in our laboratory, we determined that this labeling was within the M1 region where proximal representations are typically found (Fig. 4). Labeling also extended beyond the ICMS-defined cortex in rostral M1, in areas where PMd is typically found. Labeling in these physiologically undefined areas was excluded from the quantitative analysis, and thus, these values may somewhat underestimate the proportion of proximal representations in M1 that are connected with PMv distal representations.

Of the connections located within the ICMS-defined M1, 6.7 ± 6.0% of cell bodies were located in M1 digit, 42.5 ± 12.6% in

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### Table 1

<table>
<thead>
<tr>
<th>Case</th>
<th>Weight (g)</th>
<th>M1 hand representation (mm²)</th>
<th>PMv hand representation (mm²)</th>
<th>Injection core area (mm²)</th>
<th>Percentage of PMv hand representation included in the core</th>
<th>Body representations within the injection core</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Wrist/forearm</td>
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<tr>
<td>1934</td>
<td>698</td>
<td>15.0</td>
<td>5.2</td>
<td>3.3</td>
<td>55.1</td>
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<td>1892</td>
<td>1229</td>
<td>12.9</td>
<td>4.0</td>
<td>1.9*</td>
<td>30.2</td>
<td>0</td>
</tr>
<tr>
<td>9409</td>
<td>869</td>
<td>8.5</td>
<td>2.4*</td>
<td>0.9</td>
<td>19.4*</td>
<td>0</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>972 ± 265.9</td>
<td>12.1 ± 3.3</td>
<td>4.8 ± 0.8</td>
<td>2.1 ± 1.7</td>
<td>42.7 ± 17.6</td>
<td>0.4 ± 0.7</td>
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*Approximate because a portion of the dense core in 1892 was missing. This missing portion was considered as being fully contained in the core of the injection site (see methods).

*In this animal the entire map was not exposed in the craniotomy; thus PMv is likely to be larger (estimate = 3.8mm²). The reported 2.4mm² represents the area of the exposed portion of PMv hand area (see Figure 4).

*Based upon an estimate of total PMv size. If only exposed PMv is used to calculate size, the percentage of PMv included in the core becomes 31.1%.

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Figure 2. Location of M1 and PMv forelimb representations in a squirrel monkey brain. Illustration of a squirrel monkey brain illustrating the typical location and size of M1 and PMv forelimb representations as defined by ICMS. Sites whose stimulation evoked movements of the digits, wrist and forearm comprised the hand representation. Stimulation of surrounding sites evoked proximal movements (elbow, shoulder, trunk or face) or no response. Scale bar = 5 mm. Blue = proximal movement; green = wrist/forearm; red = digits; yellow = orofacial; hatched black = non-responsive site at 30 μA.
wrist/forearm and 50.8 ± 6.7% in proximal representations. In comparison, 19.9 ± 6.6% of labeled terminals were located in M1 digit, 39.6 ± 6.9% in wrist/forearm and 40.5 ± 2.8% in proximal representations. Thus, the PMv hand area located within the injection core received only ~49% of its inputs and sent only ~59% of its outputs to the M1 hand representations.

In each case, the proportion of digit area in the PMv injection core was larger than the proportion of terminals and cell bodies...
located in the M1 digit area (average ~ 20% in all cases; Fig. 5A). In contrast, though the proportion of wrist/forearm area varied in the PMv injection core, the proportion of cell bodies and terminals in the M1 wrist/forearm area was ~40% of total M1 labeling (Fig. 5B). Finally, whereas the proportion of proximal area in the PMv injection core was low in all cases, the proportion of cell bodies and terminals in the M1 proximal area was ~40% of total M1 labeling (Fig. 5C). Thus, it appeared that regardless of the motor representations within the PMv injection core, the proportion of digit, wrist/forearm and proximal representations in M1 interconnected with PMv was similar. In other words, a population of neurons within the PMv hand representation sends divergent projections to both distal and proximal representations in M1. Likewise, this population of neurons in the PMv hand representation receives reciprocal, convergent inputs from distal and proximal M1 representations.

Due to the inter-subject variation in motor representations in the PMv injection core in the present small sample, the proportions of specific representations in the injection core were not significantly different from the proportion of labeling in corresponding representations in M1. However, when the digit and wrist/forearm representations were combined, the proportion of hand area (digit + wrist/forearm) in the PMv injection core was significantly larger than the proportion of terminals (t = 10.68, P = 0.009; paired t-test, two-tailed) or cell bodies (t = 11.01, P = 0.008) located in the M1 hand area. The proportion of proximal area in the PMv injection core was not significantly different from the proportion of terminals (t = 2.57, P = 0.12; paired t-test, two-tailed) or cell bodies (t = 3.33, P = 0.08) located in the M1 hand area, though the trend suggests that a larger sample may yield a significant difference.

**Clustering of PMv Terminals in M1**

In each case, the distribution of labeling in M1 did not appear to be evenly distributed, but instead, appeared to form clusters that were particularly discernable in cases with larger injection cores (Fig. 6). In section reconstructions, this clustering was not discernable for the distribution of cell bodies. Therefore, the rest of this section exclusively concerns terminal distribution. Interpolated density plots of the distribution of labeled voxels in M1 revealed that terminal labeling was clustered in multiple locations (Fig. 7). Ripley’s K(t) functions confirmed that the patterns for each individual were strongly non-random (P-value < 0.002 for each individual). Each observed K(t) function was larger than every random K(t) functions at all distances up to 3000 μm.

To objectively identify clusters where terminal (voxel) density was highest, isodensity contour lines were drawn around regions ≥ 2SD and 3SD above the mean density. Based on this analysis, between three and five clusters were found with terminal density ≥ mean +2 SD (Fig. 7C). Isodensity contour lines were then co-registered with the physiologically identified borders (Fig. 7D). In each case, dense clusters appeared to be located in three specific locations: (i) the extreme caudal and lateral aspect of the M1 forelimb representation; (ii) the mid-lateral aspect of the M1 hand representation; and (iii) the rostral aspect of the M1 forelimb representation.

**Common Domains of PMv Terminals in M1**

To identify common regions of high density terminal labeling relative to the M1 forelimb representation in the three cases, we employed a warping algorithm based on the location of six ‘control’ points identified in the anatomical and physiological data: (i) injection site (center of PMv hand representation); (ii) center of cluster of terminal labeling in SMA (hand representation); (iii) center of the hand-face septum as defined by myelin staining; (iv–vi) the caudo-medial, caudo-lateral and rostro-lateral corners of the M1 hand representation, respectively. The coordinate space of case 9409 was chosen as the reference, and cases 1934 and 1892 were warped to fit 9409. Following the warping transformation, the three datasets were combined and a single, condensed (101 × 101 matrix) dataset was derived. From this dataset, a final interpolated density plot was created. This plot revealed three distinct projection domains in M1 where terminal density was ≥ 2 or 3 SD above the mean density within the M1 forelimb representation (Fig. 8A). This isodensity
contour plot was then co-registered with the physiological map of M1 from the reference case (i.e. 9409, Fig. 8B).

The three domains were identified based on their locations relative to the M1 forelimb map: MC (M1 caudal domain), MM (M1 medial domain) and MR (M1 rostral domain). MC was partially located within the most caudal and lateral portion of the M1 distal forelimb representation, extending somewhat in its lateral aspect into the proximal forelimb representation. MM was located approximately at the rostro-caudal midpoint of the distal forelimb representation extending somewhat in its lateral aspect into the proximal representation. MR was located in the rostral aspect of the M1 forelimb representation, primarily in the proximal representation of M1 and possibly extending into PMd. While the three domains represent the general trend for PMv terminals in M1, it is clear that all cases were not identical. For example, a relatively small MR was found in 1934, while MM and MR appeared to merge in 1892. Finally, in 9409, MM could be divided into two high-density clusters.

Discussion

The present analysis provides evidence to support three main findings: (i) PMv shares the majority of its connections with the rostro-lateral portion of M1 and very few with the caudo-medial aspect. (ii) Neurons within the PMv hand representation send projections to functionally heterotopic portions of M1, including large proportions of proximal representations. Likewise, they receive convergent inputs from heterotopic motor representations in the M1 forelimb area. (iii) The distribution of terminals in M1 was not evenly distributed but rather formed three general domains confined to M1rl. Consequently, large portions of the M1 hand and proximal forelimb representations were not labeled. We thus provide evidence that the organization of PMv connections with M1 is far more complex than originally suspected. These data contribute to our knowledge of structural network interactions between premotor and primary motor cortex and may increase our understanding of both cortical motor control and the internal organization of M1.

Limited Connections to the Caudo-medial Aspect of M1

Contrary to the present results, previous studies in macaque monkeys have reported numerous connections between the caudal portion of the M1 forelimb representation and PMv (Kurata, 1991; Tokuno and Tanji, 1993). It is possible that the incomplete absorption of label by the entire PMv hand representation in the present study could explain the absence of labeling in the caudo-medial M1 hand area. However, because of
numerous methodological differences between various studies (tracer, volume injected, retrograde versus anterograde labeling, M1 versus PMv injections, species differences), based on the available data, it is difficult to disentangle the reasons for this discrepancy.

It should be noted that in the present study, we used a tracer (BDA) that is generally thought to produce a very confined injection core. In addition, the amount of tracer in the present study was approximately an order of magnitude less than that used in many of the previous studies. The resulting small injections were advantageous for the present analysis as they allowed a high degree of specificity in the definition of projection patterns. It is possible that the relatively homogeneous labeling seen in M1 (including caudo-medial M1) in previous studies was due to leakage of tracer into unintended areas.

However, it is equally likely that the small injections used here underestimated the magnitude of connections between PMv and caudo-medial M1, especially if we systematically missed a particular portion of the PMv forelimb representation.

Despite larger injections and the use of different tracers in previous studies, it is interesting to note that in a recent Dum and Strick (2005) study in cebus monkeys, the authors state that ‘the small portion of M1 that is buried in the anterior bank of the central sulcus does not project densely to digit PMd and PMv (data not shown)’. This suggestion appears to be confirmed in the present study in a primate species in which the entire forelimb representation is exposed in an un fissured portion of the cortex, and thus, more precise alignment between physiological borders and labeling patterns can be made in tangential sections. Thus, these data advance the possibility that the PMv

Figure 7. Preferential zones of termination of PMv projections in M1. (A) Distribution of (voxels containing) BDA-labeled terminal boutons in a single section from each of the three cases. Near the central sulcus (caudal M1), terminals were primarily located laterally. Terminals were progressively located more medially in more rostral aspects of M1, displaying a rostro-lateral pattern of projections from PMv to M1. Terminal labeling appeared to be clustered (most clearly seen in the case with the smaller injection: 9409). Note that this method provided a slightly different depiction of the clustering in M1 than that of the raw data shown in the previous figure primarily because high-density regions in Figure 6A comprise BDA-labeled terminals and fibers (and some cell bodies), while the present reconstruction depicts terminal boutons exclusively. (B) To more objectively confirm the clustering of PMv projections, we created interpolated graphs of the terminal locations. This approach revealed zones of high and low density in auto-scaled color contour maps of each case. (C) Contour map illustrating statistically significant zones of high-density terminal labeling. Thin and thick contour lines represent two and three standard deviations greater than the mean density of each case, respectively. (D) Alignment of contour maps with the ICMS- map of M1 forelimb representation. Whereas no obvious topographic relationship was found between the injection core and the zones of intense labeling, three areas showed high-density labeling in all three cases. Despite inter-case variability, the caudo-lateral, mid-lateral and rostral portions of the M1 forelimb representation appeared to be labeled in each case.
the mid-lateral portion (MM) and the rostral edge of the forelimb representation (MR).

relatively small portions of the PMv proximal representation, in a large portion of the PMv hand representation, and included across cases. Even though the injection cores extended across containing labeled terminals and cell bodies was rather constant across cases. Even though the injection core was confined to the PMv digit representation in case 9409 (reference case). Thin and thick contour lines depict significantly higher density of PMv terminals in relation to the M1 forelimb representation. (A) Composite color-coded contour map illustrating the location of three domains of high-density terminal labeling in the M1 forelimb representation. A warping function was used to identify consistencies in the pattern of terminal distribution across the three cases illustrated in Figure 7. Control points were used to warp the cases into a common coordinate frame (see text). After combining terminal locations for all three cases, a composite interpolated density plot was derived. From this analysis, three zones of significantly dense labeling were evident in the M1 forelimb representation. (B) Domains of significantly higher density of PMv terminals in relation to the M1 forelimb representation in case 9408 (reference case). Thin and thick contour lines depict densities &gt;2 and 3 SD, respectively. Clusters were found in the caudal portion (MC), the mid-lateral portion (MM) and the rostral edge of the forelimb representation (MR).

forelimb area projects primarily to the rostro-lateral portion of the M1 forelimb area, at least in New World monkeys.

Divergence and Convergence of PMv/M1 Connections

The present data suggest that small areas of the PMv hand representation share connections with comparatively widespread, and functionally heterotopic portions of the M1 forelimb representation. If connections between different motor areas were topographically segregated, as has been found in somatosensory cortex (Burton and Fabri, 1995; Manger et al., 1997; Florence et al., 1998; Fang et al., 2002), we should have found a linear relationship between the proportion of a particular body representation (e.g. digits) within the injection core and the amount of labeling found in the same representation in M1. Instead, the proportion of body representations in M1 containing labeled terminals and cell bodies was rather constant across cases. Even though the injection cores extended across a large portion of the PMv hand representation, and included relatively small portions of the PMv proximal representation, in M1 the connections were observed in comparable proportions of distal and proximal representations.

It thus appears that the rule of homotopic connectivity does not generalize to cortical motor areas, at least not to the specificity of intracortical connections between PMv and M1. This result is consistent with the notion that topographic relationships in the motor system are maintained on a more global scale and are less specific with regard to finer grain detail. For this reason, the M1 hand area has been referred to as a 'shared neural substrate' for motor control of the hand (Sanes et al., 1995). Divergence can also be found in the corticofugal projections of M1. Stimulation of a limited region within the M1 hand area results in the contraction of multiple muscles located in the arm and forearm, demonstrating divergent patterns of corticospinal termination (Humphrey, 1986). Even individual corticomotor-neuronal cells can facilitate (or suppress) several different motoneuron pools that innervate muscles of both distal and proximal joints (Cheney and Fetz, 1985; McKiernan et al., 1998; Park et al., 2001, 2004). Thus, with regard to topographic specificity, our results suggest that comparable divergence occurs in the connectivity of intracortical projections from premotor to primary motor cortex.

Functional Significance of Divergent/Convergent Connections between PMv and M1

It is surprising that in all cases, the M1 digit representation is sparsely interconnected with the PMv. This is true even when most of the injection core was confined to the PMv digit representation. In squirrel monkeys, digit representations are typically more numerous within the caudal portion of M1 (Friel et al., 2005) but PMv interconnects most densely with rostro-lateral areas. The sparse interconnectivity between PMv and digit representations in M1 raises important questions regarding the function of premotor areas, particularly considering that digit movements can be evoked from a large portion of the PMv hand representation (half to two-thirds of the total area; see Table 1).

Because corticospinal neurons in PMv are thought to project primarily to higher cervical levels (Martino and Strick, 1987; He et al., 1993; Galea and Darian-Smith, 1994), it has been proposed that digit movements elicited by stimulation in area F5 of macaque PMv are the result of facilitation of PMv projections to M1 (Cerri et al., 2003; Shimazu et al., 2004). Here, we provide evidence that in squirrel monkeys, neurons in digit representations of PMv send relatively few projections to M1 digit representations, but larger numbers of projections to M1 wrist/forearm and proximal representations. Thus, strictly based on our anatomical data, it is tempting to suggest that not all digit movements evoked in PMv are mediated solely through activation of M1 corticospinal neurons.

It has been demonstrated that stimulation of premotor regions with long train bursts (up to 1 s) can elicit complex hand-to-mouth movements (Graziano et al., 2002). The anatomical divergence observed in the present study may provide an anatomical substrate for complex movements evoked by stimulation of PMv.

Reliability of ICMS-defined Motor Maps and Implications for Interpreting Divergence/Convergence of PMv-M1 Connections

Variability in the movements evoked by ICMS can be introduced via a variety of sources (e.g. individual variation, depth of...
stimulation, anesthetic level, previous stimulation, initial posture), as we and others have discussed in previous publications (Stoney et al., 1968; Neafsey et al., 1986; Nudo et al., 1990). We attempted to control each of these sources of variability. For example, ICMS mapping was conducted only during stable periods of anesthesia and was temporarily halted during occasional periods of excessive muscle tone or deep narcosis marked by movement threshold elevation. Previous studies demonstrated that multiple maps derived in the same animal with no intervening manipulation differ from site to site, but the locations of representations, and proportions of digit, wrist/forearm and proximal territories remain remarkably constant (Nudo et al., 1996a). Also, we have found no substantial differences in ICMS motor maps derived under different anesthetic conditions (Plautz et al., 2000). While no direct comparisons of maps derived via ICMS in anesthetized and awake animals have been reported, the control of anesthetic state, the similarity of motor maps using different anesthetic agents, the stability of ICMS maps over time and the low current levels required to evoke responses (as low as 1 μA, but more typically 10–15 μA) suggests that ICMS evoked responses derived under ketamine anesthesia are probably comparable to those derived in the awake monkey. Finally, there appears to be a reasonably close correspondence between the muscles facilitated by repetitive ICMS and individual corticomotoneuronal cells (as defined by spike-triggered averaging in awake monkeys; Cheney, 2002). However, the ICMS technique as employed here does not display a complete picture of M1 outputs. By defining the movement evoked at near-threshold current levels, we may underestimate the overlap of various forelimb representations in both M1 and PMv. Thus, at least part of the divergence seen in the present study may be due to proximal representations in the injection core that were missed.

Comparison of Convergent/Divergent Patterns of Connections between PMv and M1 in other Non-human Primate Species

Finally, it is possible that the unique distribution of PMv connections with M1 is specific to squirrel monkeys and is not present in other non-human primate species, such as macaques or humans. PMv in macaques has been subdivided into two components. The more caudal component, called F4, is thought to be functionally related to arm, neck and face movements in peripersonal space (Rizzolatti et al., 1988). The rostral component, called F5, is related to sensory and motor transformations involved in grasping and manipulation. Thus, to a certain degree, distal and proximal representations are segregated in the two areas. Such a parcellation of PMv has not been demonstrated in squirrel monkeys (Frost et al., 2003). It is possible that F4 and F5 are a single area in squirrel monkeys, resulting in a high degree of divergence from PMv to M1. It will be informative to determine if such a divergent pattern exists from F5 in macaques, or instead, if the projection pattern in squirrel monkeys is comprised of the sum of the projection patterns from F4 and F5 in macaque monkeys.

PMv Projection Domains in M1

PMv terminations within M1 were not homogeneously distributed and appeared to cluster at three locations (Figs 6–9). These locations consistently received significantly more terminals from the PMv hand representation (Fig. 9). Spatial warping of the cases into a common coordinate space confirmed the consistency of the clustering in MC, MM and MR domains across individuals. These domains of densely innervated cortex alternated with regions of sparsely innervated tissue.

The only other description of similar clustering of the connections between PMv and M1 was reported in a paper by Matelli et al. (1986). In that study, the bidirectional tracer wheatgerm agglutinin conjugated to horseradish peroxidase was injected in the presumed PMv hand field of a single macaque monkey. After injections, reciprocal connectivity was generally found. However, while three islands of retrogradely labeled cells were observed in M1 (fig. 3 of that paper); anterograde labeling appeared throughout the entire labeled area (fig. 2 of that paper). This observation is in contrast to the present results, in which clustering was seen in the terminal labeling pattern but not in the retrograde labeling pattern. This discrepancy is likely due to at least two differences between the studies: (i) BDA is not an efficient retrograde tracer and may not have labeled enough cells to demonstrate a clustering pattern; and (ii) Matelli et al. (1986) did not quantify the density of anterograde labeling. In the present study, the quantitative distribution of voxels with labeled terminals allowed us to determine relative densities of termination across the M1 hand area. As in the Matelli paper, we observed labeled terminals throughout the rostro-lateral portion of M1.

In the present study, while the pattern of projection from the PMv hand area appeared to be segregated into three clusters in the M1 forelimb area, considerable individual variability was evident with respect to specific movement representations located within the clusters. As we found in previous studies (Nudo et al., 1992; Nudo et al., 1996a), the mosaical maps of distal forelimb movement representations in primary motor cortex were highly idiosyncratic. In general, map size and spatial complexity (fractionation of movement representations)
are variable, and appear to be related to an individual's motor experience.

Whereas a complete quantitative report of labeled terminals in M1 would surely be more accurate and possibly display the clustered domains more intensely, this method would be prohibitively time consuming. We chose a method that would maintain the topographic information regarding the location of labeled terminals, while providing a rough estimate of terminal density. One limitation of this approach is the size of the analysis grid (100 μm by 100 μm; see Materials and Methods). While a given voxel was considered labeled if only two labeled terminals were found, the total number of labeled terminals within voxels exceeded 90 in some areas [ex. MM: 78 ± 12 (n = 4)]. Thus, there may be additional heterogeneity in the terminal distribution that could not be resolved using this approach. Nonetheless, this resolution was sufficient to show the location of separate domains, particularly when the data were treated with spatial visualization software using a smoothing function. While the data provided by the current set of experiments provide little insight regarding the functional role of this specific clustered organization of interconnections between PMv and M1, it is possible that PMv influences different aspects of motor control based on differential sensorimotor processing that occurs within each of the three domains. However, the present study of three animals did not have enough statistical power to demonstrate any consistent relationship between motor map topography and the location of the domains. Thus, at this point, it is not possible to know if the specific clustering pattern found in individual animals underlies specific aspects of their motor function or if that the variability is due to subtle differences in the precise location of the BDA injection in PMv. Clearly, this issue should be explored in future studies.

Notes

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Funding to pay the Open Access publication charges for this article was provided by ...


