How are the neurons that directly influence the motoneurons of a muscle distributed in the primary motor cortex (M1)? To answer this classical question we used retrograde transneuronal transport of rabies virus from single muscles of macaques. This enabled us to define cortico-motoneuronal (CM) cells that make monosynaptic connections with the motoneurons of the injected muscle. We examined the distribution of CM cells that project to motoneurons of three thumb and finger muscles. We found that the CM cells for these digit muscles are restricted to the caudal portion of M1, which is buried in the central sulcus. Within this region of M1, CM cells for one muscle display a remarkably widespread distribution and fill the entire mediolateral extent of the arm area. In fact, CM cells for digit muscles are found in regions of M1 that are known to contain the shoulder representation. The cortical territories occupied by CM cells for different muscles overlap extensively. Thus, we found no evidence for a focal representation of single muscles in M1. Instead, the overlap and intermingling among the different populations of CM cells may be the neural substrate to create a wide variety of muscle synergies. We found two additional surprising results. First, 15–16% of the CM cells originate from area 3a, a region of primary somatosensory cortex. Second, the size range of CM cells includes both “fast” and “slow” pyramidal tract neurons. These observations are likely to lead to dramatic changes in views about the function of the CM system.

Muscle representation in the macaque motor cortex: An anatomical perspective

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How are the cortical neurons that control a single muscle arranged in the primary motor cortex (M1), as a highly localized cluster or as a widely dispersed field of cortical neurons? The answer to this question is central to an understanding of how the motor cortex is organized to generate and control voluntary movement. Many physiological experiments have explored this issue, and their results have led to two opposing views (Fig. 6, which is published as supporting information on the PNAS web site). Asanuma and colleagues (1, 2) suggested that the cortical neurons that control a single muscle are focally arranged in M1. They used intracortical stimulation and trains of cathodal pulses from microelectrodes to study the movements and muscle activity evoked with small currents (<10 μA). They found that the lowest threshold sites for evoking contractions of a muscle were confined to a small volume of M1, which they termed a “cortical efferent zone.” The diameters of efferent zones ranged from 0.5 mm to a few millimeters but averaged ≈1 mm. The boundaries of efferent zones were discrete; neighboring efferent zones overlapped only at their edges. Hence, Asanuma (2) viewed the collection of efferent zones for hand muscles as forming a “finely grained mosaic (of single muscle representation) within the depth of M1.”

In contrast, Phillips and colleagues (3–5) proposed that cortical neurons controlling a single muscle are broadly distributed in M1. They initially studied the intracellular potentials evoked in motoneurons after surface stimulation of M1 with anodal pulses (3, 4). Later they monitored the responses of motor units in digit muscles after intracortical stimulation with short trains of cathodal pulses (<80 μA) (5). They termed the population of cortical neurons that make monosynaptic connections with a single motoneuron a “colony.” The colonies innervating the motoneurons of hand muscles occupied broad territories of M1 [e.g., 6.0 × 5.5 mm for extensor digitorum communis (EDC)]. These colonies could display multiple hot spots (3, 5). The colonies for different muscles overlapped extensively. Thus, Phillips and colleagues concluded that “the words ‘discrete’ and ‘mosaic’ . . . have no place in the description of the fine-grained structure of cortico-motoneuronal output. . . .” (ref. 5, p. 31).

To resolve this longstanding controversy, we have taken a unique anatomical approach. We used retrograde transneuronal transport of rabies virus in macaques to identify the location of cortico-motoneuronal (CM) cells that make monosynaptic connections with the motoneurons of three different single muscles that control movements of the hand. Rabies virus is transported exclusively in the retrograde direction by neurons in the central nervous system of primates, and the virus moves transneuronally in a time-dependent fashion (6–8). When rabies virus is injected into a single hand muscle it is transported in the retrograde direction from the muscle to the motoneurons that innervate it (Fig. 1). These motoneurons are located in lower cervical and upper thoracic segments of the spinal cord (9). This process takes ≈3 days. With the procedures we use, only motoneurons that innervate the injected muscle become infected (10–12). Virus is transported by motoneurons of all size ranges, including the smallest “gamma” motoneurons, and virus infects approximately half of the population of cells in a single motoneuron pool (our unpublished observations; see also refs. 8 and 13).

Next, virus moves transneuronally to infect “second-order” neurons that make monosynaptic connections with motoneurons. For example, motoneurons are known to receive monosynaptic input from selected group Ia afferents, spinal interneurons, neurons in the magnocellular division of the red nucleus (RNm), and CM cells in layer V of M1 (14–16). Second-order neurons in lower cervical segments of the spinal cord and in dorsal root ganglia are infected 3.5–4 days after virus injections into a hand muscle. Second-order neurons in RNm and layer V of M1 (Fig. 2) are infected a short time (4–5 days) later, presumably because they are a greater distance from motoneurons in lower cervical segments.

At still longer survival times virus will undergo another stage of retrograde transneuronal transport and move from second- to third-order neurons (6, 7, 17). For example, neurons in the interpositus nucleus of the cerebellum become infected after neurons in RNm. Similarly, neurons in layers III and VI become infected after CM cells in layer V. Corticospinal neurons that project to spinal cord interneurons can also become infected at this stage. As a consequence, when labeled neurons are present

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Abbreviations: CM, cortico-motoneuronal; M1, primary motor cortex; ABPL, abductor pollicis longus; ADP, adductor pollicis; EDC, extensor digitorum communis; PTN, pyramidal tract neuron; RNm, magnocellular division of the red nucleus.

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that make disynaptic connections with motoneurons (Fig. 1). To limit our analysis to CM cells, we set the survival period to label only second-order neurons in layer V and excluded animals from analysis if they had any labeled neurons in layers III or VI (i.e., third-order neurons in the cortex).

We injected rabies virus into one of three digit muscles: abductor pollicis longus (ABPL), adductor pollicis (ADP), or EDC. We selected these muscles for analysis because Asanuma and Rosen (1) reported that M1 contained distinct different zones for each of them, whereas Andersen et al. (5) noted that CM cells for EDC were widely distributed in M1. In addition, the three muscles we tested include intrinsic and extrinsic muscles and functional antagonists. Five experimental animals met the criteria for inclusion in our analysis of CM cells (Table 1, which is published as supporting information on the PNAS web site). In each of these animals, labeled neurons were present only in layer V.

Results

Within M1, CM cells that innervate ABPL, ADP, and EDC motoneurons were located predominantly in the central sulcus (Figs. 3 and 4). Indeed, only 1–3% of the CM cells were found in the portion of M1 that lies on the precentral gyrus. Surprisingly, we found a sizeable population of CM cells (15.4 ± 6.3%, mean ± SD) in area 3a at the bottom of the central sulcus.

CM cells of the three muscles were distributed over a broad mediolateral territory that was located between the spur of the arcuate sulcus and the superior precentral sulcus (Fig. 3). This territory represents the classical “arm area” of M1 (18, 19). The M1 regions containing the entire population of CM cells that innervate the motoneurons of an individual muscle were quite large and measured 6.7 × 6.2 mm for ADP (Fig. 3 Top), 11.1 × 4.9 mm for ABPL (Fig. 3 Middle), and 7.0 × 6.8 mm for EDC (Fig. 3 Bottom). Within these regions the density of CM cells varied. For example, ~50% of the entire population of CM cells that innervated ADP motoneurons formed a single dense cluster. However, even this dense cluster measured 2.5 × 1.1 mm.

We noted two interesting disparities between the location of CM cells that innervate digit motoneurons and the types of movements that have been evoked in prior mapping studies of M1 (Fig. 4). The first disparity is between the regions of M1 where stimulation can evoke digit movements and the region of M1 that contains CM cells for digit muscles. We will illustrate this result by comparing the maps of intracortical stimulation from the study of Murphy and colleagues (19) (Fig. 4 A and B) with our maps of CM cells that innervate ADP and EDC (Fig. 4 C). Murphy and colleagues (19) found what others have observed: movements of the thumb and fingers were evoked from a central region of the arm area in M1 that included both the precentral gyrus and the anterior bank of the central sulcus (Fig. 4 A). The lowest threshold sites for evoking these movements were located in the sulcus. A core region of digit representation was surrounded medially, rostrally, and laterally by successive rings of elbow and shoulder representation (Fig. 4 B). The lowest threshold sites for evoking shoulder movements were located medially in or near the central sulcus (Fig. 4 B, circle).

As noted above, the CM cells that innervate ADP and EDC were located predominantly within the central sulcus, and very few of these cells were located on the precentral gyrus (Fig. 4 C). This distribution divides the digit representation of area 4 into two regions: a caudal zone located in the sulcus that has CM cells (see also refs. 5, 20, and 21) and a rostral zone located on the gyrus that lacks them (compare Fig. 4 A and C). We did not inject all of the digit muscles, and thus it is possible that CM cells related to other muscles are located on the gyrus. However, prior studies have found that stimulation of area 4 on the gyrus evokes movements related to the muscles we injected (e.g., see figure 2 in ref. 19). Thus, we found a real disparity between the broad region of M1 where stimulation can evoke digit movements and
the more localized region in the central sulcus that contains CM cells for digit muscles. This may be because the motor effects of intracortical microstimulation are not restricted to direct activation of CM cells (16). In fact, it is possible to evoke movements using microstimulation of the motor cortex in animals that lack CM cells (e.g., cats and rats) (16).

The second disparity consisted of the medial extension of CM cells that innervate digit muscles into a region of the sulcus that

Fig. 3. Maps of CM cells that innervate the motoneurons for digit muscles. Each panel displays flattened maps of CM cells (small round symbols) labeled after injections of rabies into ADP (JA25), ABPL (JA30), or EDC (JA3). See Fig. 7 and ref. 25 for details about the construction of these maps. Small arrows are placed at the area 4/6 border and the area 4/3a border. ArS, arcuate sulcus; CS, central sulcus; M, medial; R, rostral; SPcS, superior precentral sulcus.

Fig. 4. Maps of cortical stimulation and maps of CM cells. (A) Finger representation. The small filled circles indicate sites where intracortical stimulation evoked finger movements. The size of a circle is inversely related to the threshold for evoking movement (see the key at the lower right). The solid line at the right is the bottom of the central sulcus. Vertical dashed lines mark various borders. The large dashed circle surrounds a medial region containing the lowest threshold sites for shoulder movements (see B). (B) Shoulder representation. The small filled circles indicate sites where intracortical stimulation evoked shoulder movements. (C) Location of CM cells innervating digit motoneurons. The results from injections of rabies virus into ADP (Fig. 3 Top) and into EDC (Fig. 3 Bottom) have been overlapped to create this figure. Note that the most medial CM cells for digit muscles are located in a region where stimulation evoked shoulder movements at low threshold (dashed circle). The scale for all maps is shown in C. [Reproduced with permission from Kwan et al. (19) (Copyright 1978, American Physiological Society).]
CM cells that was broadly distributed in the caudal portion of M1. Furthermore, the populations of CM cells for different hand muscles were located in the same general region of cortex. These observations indicate that the CM cells controlling a single muscle are arranged as a widely dispersed field of neurons. Moreover, they suggest that the populations of CM cells for different muscles overlap extensively.

It is known that the axons of many CM cells branch and innervate the motoneuron pools of more than one muscle (e.g., refs. 15, 16, and 26–28). Thus, the CM cells we have labeled may have connections with other muscles in addition to the one we injected. On the other hand, Asanuma et al. (29) found that groups of PTNs that were simultaneously recorded from the same electrode sent at least one axon to a common motor nucleus in the spinal cord. They argued that this innervation pattern made it possible to construct focal, cortical efferent zones related to a single muscle even when the neurons in these zones branched widely. Similarly, Cheney and Fetz (28) found that (electrical) stimulus-triggered averages of muscle activity were generally more pronounced but in the same muscles as (spontaneous) spike-triggered averages. They reasoned that this effect could be explained if CM cells in the vicinity of the electrode have the same target muscles. If CM cells that innervate a common target muscle formed discrete clusters in M1, then transneuronal transport of rabies from a single muscle should have revealed a focal accumulation of these cells. However, we found no evidence for distinct clusters of CM cells that would be consistent with single cortical efferent zones averaging 1 mm in diameter (1). Instead, dense populations of CM cells measured at least 2.5 × 1.1 mm in diameter and were generally much larger. We did see variations in CM cell density within a population. These variations could be the substrate for the multiple “hot spots” that have been observed for activation of a muscle with threshold stimulation of M1 (3, 5).

Although we defined the populations of CM cells for only three muscles, the muscles we examined should have made the “best case” for cortical efferent zones (1). We sampled two muscles that control movements of the thumb, a finger with an especially high degree of movement independence (30). Indeed, cortical efferent zones were thought to be part of the neural substrate for independent movements of the fingers. However, even biasing our sample in this way failed to yield any evidence for a discrete mapping of muscle representation in M1. Although fine control of hand movement depends on M1 and its CM cells (for references and review see ref. 16), it does not require focal representations of single muscles (see ref. 31).

The classical hand area of M1 includes both the precentral gyrus and the anterior bank of the central sulcus (18, 19). Cortical neurons that project to the spinal cord originate from both surface and sulcal portions of the hand area (for references and review see ref. 22). Prior studies have found important differences in the pattern of inputs to the rostral part of M1 on the gyrus versus the caudal part of M1 in the sulcus. Both parts receive input from “cutaneous” and “deep” (muscle and/or joint) peripheral afferents. However, neurons that respond to cutaneous input are concentrated in the caudal part of M1, whereas neurons that respond to deep input are concentrated in the rostral part of M1 (32–36). The basal ganglia and cerebellum also project to both parts of M1 (37), but the caudal part receives its densest input from thalamic subdivisions that are the target of output from the globus pallidus. In contrast, the rostral part of M1 receives its densest input from thalamic subdivisions that are the target of cerebellar output. Data from the present study provide additional evidence that rostral and caudal parts of M1 differ in their output organization. Although both regions project to the spinal cord, the caudal part of M1 has CM cells for digit muscles, whereas the rostral part lacks them. Thus, based on both its input and output organization, M1 is not a homo-

Fig. 5. Size of CM cells. The graphs show the size of CM cells in M1 labeled after virus transport from ADP (Top), ABPL (Middle), and EDC (Bottom). Cell size in these graphs represents the average of a cell’s maximum and minimum diameter (see Methods for details).
The finding that 15–16% of the CM cells were located in area 3a was unexpected. Although there is a prior report that efferents from area 3a terminate in the ventral horn (42), stimulation of area 3a in macaques failed to evoke overt motor effects or to facilitate motoneuron activity (43). Thus, area 3a has not been viewed as a source of descending commands for motor output. What then is the function of the CM cells in area 3a? The absence of motor effects from area 3a stimulation leads us to speculate that CM cells in this cortical region make monosynaptic connections with gamma motoneurons that control the sensitivity of muscle spindle afferents. The corticospinal system makes monosynaptic connections with gamma as well as alpha motoneurons (44). In addition, area 3a receives a prominent input from group Ia spindle afferents (45, 46). Thus, the CM cells in area 3a could be involved in efferent control over the propioceptive information ascending to higher centers, including area 3a itself. This function is similar to that proposed for corticospinal efferents from other areas of somatic sensory cortex (see, e.g., ref. 4).

Finally, our results have important implications about how the two-dimensional distribution of CM cells in M1 might be involved in the generation and control of hand movement. Individual CM cells with branching axons can coactivate a set of muscles and generate functional synergies. However, the synergies created by this mechanism are limited. CM cells generally influence the motoneurons of a few muscles that have similar actions about a joint, and they rarely innervate the motoneurons of functional antagonists. The widespread distribution of CM cells of a single muscle and the extensive intermingling of CM cells that influence different muscles could provide the neural substrate to create a broader range of functional synergies than that generated by the branching patterns of individual cells. This is illustrated by the presence of CM cells for hand muscles at sites where stimulation produces shoulder movements at lowest threshold. These sites could contain the building blocks for movements requiring coactivation of hand and shoulder muscles. To quote Hughlings Jackson, the CM cells innervating different hand motoneurons “are represented . . . in thousands of different combinations” across the two dimensions of M1: “that is, as very many movements; it is just as many chords, musical expressions and tunes can be made out of a few notes” (ref. 47, p. 400).

Methods

These experiments were performed on rhesus monkeys (Macaca mulatta) (Table 1). All experimental procedures were conducted in accordance with National Institutes of Health guidelines and were approved by the relevant institutional animal care and use and biosafety committees. The procedures for handling rabies virus and animals infected with rabies have been described previously (6, 7) and are in accordance with the recommendations from the Department of Health and Human Services (Biosafety in Microbiological and Biomedical Procedures).

Surgical Procedures. Surgical procedures were performed under aseptic conditions on monkeys anesthetized with inhalation anesthesia (1.5–2.5% isoflurane in 1–3 liters/min O2). We incised the skin to expose the target muscle. For ABPL, which is a deep muscle lying mostly under EDC, we also opened the fascia compartment of EDC and gently retracted it. Each muscle was identified by its anatomic position and tendinous insertion. In addition, we evoked its characteristic movement by electrical stimulation applied directly to the muscle (0.2-ms pulses at 25 Hz for 1 s, at a maximum intensity of 15 V). We determined the lowest threshold site for evoking movement for each muscle, which presumably is near the motor endplate region of the muscle.

Once the muscle was identified, we injected it with a fixed strain of rabies virus [N2C, 1 × 107 plaque-forming units/ml, provided by B. Dietzschold and M. Schnell (Thomas Jefferson University, Philadelphia)] using a 1 ml-syringe with a 26-gauge needle. Only a single muscle was injected in each animal. Typically, small amounts of virus were injected at multiple locations along one needle track for ADP and EDC and along three tracks for ABPL. We deposited a total amount of 0.25 ml of virus into the ADP and 0.5 ml of virus into the ABPL and the EDC. The syringe needle remained in place for 2–3 min after each injection. In some instances, the needle hole was sealed with surgical glue. The incision over the muscle was closed with subcuticular sutures, and animals were given an analgesic (buprenorphine, 0.01 mg/kg, i.m.) Then the animals were transferred to an isolation room (biosafety level 2) for the entire survival period (98–115 h). Animals were monitored at regular intervals for early signs of infection (e.g., a change in temperature).

Histological Procedures. At the end of the survival period, animals were deeply anesthetized (25 mg/kg ketamine i.m. and 37 mg/kg Nembutal i.p.) and perfused through the heart with fixatives according to a three-step procedure adapted from ref. 48. The perfusates were 0.1 M phosphate buffer (pH 7.4), 10% buffered formalin, and a mixture of 10% buffered formalin and 10% glycerin at 4°C. After the perfusion, the brain was stored overnight in 10% buffered formalin plus 10% glycerin at 4°C. Then it was placed in 10% buffered formalin plus 20% glycerin at 4°C for 6–8 days. A frozen block of tissue containing the cortical motor areas in the frontal lobe was serially sectioned (50 μm) in the coronal plane or a near sagittal plane that was altered to be perpendicular to the central sulcus. Every 10th section was processed for cytoarchitecture by using a Nissl stain. To identify neurons infected with rabies, free-floating tissue sections were processed according to the avidin–biotin peroxidase method (Vectastain, Vector Laboratories). Rabies antigen was detected by using a monoclonal antibody directed against the nucleoprotein of rabies virus [SDF12, diluted 1:100, supplied by A. Wandel] (Animal Diseases Research Institute, Nepean, Ontario, Canada)]. Reacted tissue was mounted on gelatin-coated glass slides, air-dried, and coverslipped with Artmount.

Analytical Procedures. We examined ~200 reacted sections from each experimental animal using bright-field, dark-field, and polarized illumination. Section outlines and labeled neurons were plotted with a computer-based charting system (MD2, Accustage). Salient features, such as sulcal landmarks and cytoarchitectonic borders, were added to these charts. The charts were used to reconstruct a flattened map of the distribution of labeled neurons in layer V of the motor cortex (for details see Fig. 7, which is published as supporting information on the PNAS web site, and ref. 25). Area 3a was distinguished from area 4 by...
(i) the presence of a thin, interrupted granular layer IV; (ii) a decrease in thickness of the gray matter; and (iii) a sharpening of the boundary between the gray and white matter (32). Area 6 was distinguished from area 4 by a decrease in the density of large, pyramidal-shaped neurons in layer V (22).

Digital microphotographs were “captured” from the microscope by using a video camera coupled to a high-resolution video-processing board in a personal computer. Custom software enabled us to generate high-resolution composites from multiple images. The soma diameter of labeled neurons with a complete nucleus was measured at $\times 400$ by using a calibrated scale. Cell size was expressed as the average of the cell’s maximum and minimum diameters (24, 25). We made no corrections for tissue shrinkage.

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