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Callosal connections of dorso-lateral premotor cortex

B. Marconi,[1] A. Genovesio,[2] S. Giannetti,[1,3] M. Molinari[1,4] and R. Caminiti[2]
[1]IRCCS Santa Lucia Foundation, Rome, Italy
[2]Department of Human Physiology and Pharmacology, University of Rome 'La Sapienza', Piazzale Aldo Moro 5-00185, Rome, Italy
Institute of [3]Anatomy and [4]Neurology, Catholic University, Rome, Italy

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Abstract

This study investigated the organization of the callosal connections of the two subdivisions of the monkey dorsal premotor cortex (PMd), dorso-rostral (F7) and dorso-caudal (F2). In one animal, Fast blue and Diamidino yellow were injected in F7 and F2, respectively; in a second animal, the pattern of injections was reversed. F7 and F2 receive a major callosal input from their homotopic counterpart. The heterotopic connections of F7 originate mainly from F2, with smaller contingent from pre-supplementary motor area (pre-SMA, F6), area 8 (frontal eye fields), and prefrontal cortex (area 46), while those of F2 originate from F7, with smaller contributions from ventral premotor areas (F5, F4), SMA-proper (F3), and primary motor cortex (M1). Callosal cells projecting homotopically are mostly located in layers II–III, those projecting heterotopically occupy layers II–III and V–VI. A spectral analysis was used to characterize the spatial fluctuations of the distribution of callosal neurons, in both F7 and F2, as well as in adjacent cortical areas. The results revealed two main periodic components. The first, in the domain of the low spatial frequencies, corresponds to periodicities of cell density with peak-to-peak distances of approximately 10 mm, and suggests an arrangement of callosal cells in the form of 5-mm wide bands. The second corresponds to periodicities of approximately 2 mm, and probably reflects a 1-mm columnar-like arrangement. Coherency and phase analyses showed that, although similar in their spatial arrangements, callosal cells projecting to dorsal premotor areas are segregated in the tangential cortical domain.

Introduction

Goal-directed action often requires a fine interhemispheric tuning through the corpus callosum, the main commissural system of the brain.

Anatomical studies have revealed the patterns of callosal connections in certain areas of the primate frontal cortex (Pandya *et al*., 1969; Pandya & Vignolo, 1971; Jenny, 1979; Jones *et al*., 1979; Jones & Hendry, 1980; Gould *et al*., 1986; Johnson *et al*., 1989; Rouiller *et al*., 1994; Liu *et al*., 2002), such as primary motor cortex (Brodmann's area 4, M1, F1), and supplementary motor area (SMA; Penfield & Welch, 1951; Woolsey *et al*., 1952). Studies on motor cortex have shown that homotopic and heterotopic callosal connections are abundant in the zone of representation of proximal body parts and body-midline, relatively modest in the hand representation (Pandya & Vignolo, 1971; Jenny, 1979; Jones *et al*., 1979; Gould *et al*., 1986; Johnson *et al*., 1989; Roullier *et al*., 1994). SMA has recently been subdivided (Luppino *et al*., 1991, 1993; Matsuzaka *et al*., 1992) into a rostral part, pre-SMA (F6), and a caudal one, 'SMA-proper' (F3), both of which show consistent callosal connections which include the hand representation zones (McGuire *et al*., 1991a,b; Roullier *et al*., 1994; Liu *et al*., 2002).

Data on callosal connectivity of the rostral (PMdr, F7) and caudal (PMdc, F2) dorsal premotor (PMd) cortex (Matelli *et al*., 1985; Barbas & Pandya, 1987) are still lacking (Pandya & Vignolo, 1971; Johnson *et al*., 1989). These two areas have been distinguished, not only on the basis of their cytoarchitectonic (Barbas & Pandya, 1987; Matelli *et al*., 1991) and immunohistochemical (Matelli *et al*., 1985; Gabernet *et al*., 1999; Geyer *et al*., 2000) features, but also for the their differences in functional properties (Wise *et al*., 1983; Wise & Mauritz, 1985; Johnson *et al*., 1996; Fujii *et al*., 2000; for a discussion see Wise *et al*., 1997 and the literature therein; Boussaoud *et al*., 1998; Jouffrais & Boussaoud, 1999) and association connections with frontal (Pandya & Kuypers, 1969; Pandya & Vignolo, 1971; Godschalk *et al*., 1984; Barbas & Pandya, 1987; Luppino *et al*., 2003) and parietal areas (Petrides & Pandya, 1984; Matelli *et al*., 1998; Caminiti *et al*., 1999; Marconi *et al*., 2001).

A study of the callosal connections of areas F7 and F2 has never been performed. Such a study would be of interest to attempt an interpretation of their role in cognitive motor control. We adopted a double-labelling retrograde strategy to describe the laminar and tangential arrangement of callosal cells projecting to F7 and F2. Spectral and coherency analyses were performed for a quantitative evaluation of the fluctuation in the callosal cells density, and for the comparison between the spatial arrangement of the distributions of callosal neurons projecting from different frontal areas to dorso-lateral premotor cortex.

Materials and methods

Anatomical studies

Two rhesus monkeys (*Macaca mulatta*; body weights 3.7, and 3.5 kg) were used in this study. These experiments were approved by the Committee for the control on Animal Experimentation of the Ministry of Public Health of Italy, as well as by that of the Faculty of Medicine of the University of Rome 'La Sapienza'. The animals were pre-anaesthetized with ketamine hydrochloride (15 mg/kg, i.m.) and

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Correspondence: Professor Roberto Caminiti, [2]Department of Human Physiology and Pharmacology, as above.
E-mail: roberto.caminiti@uniroma1.it

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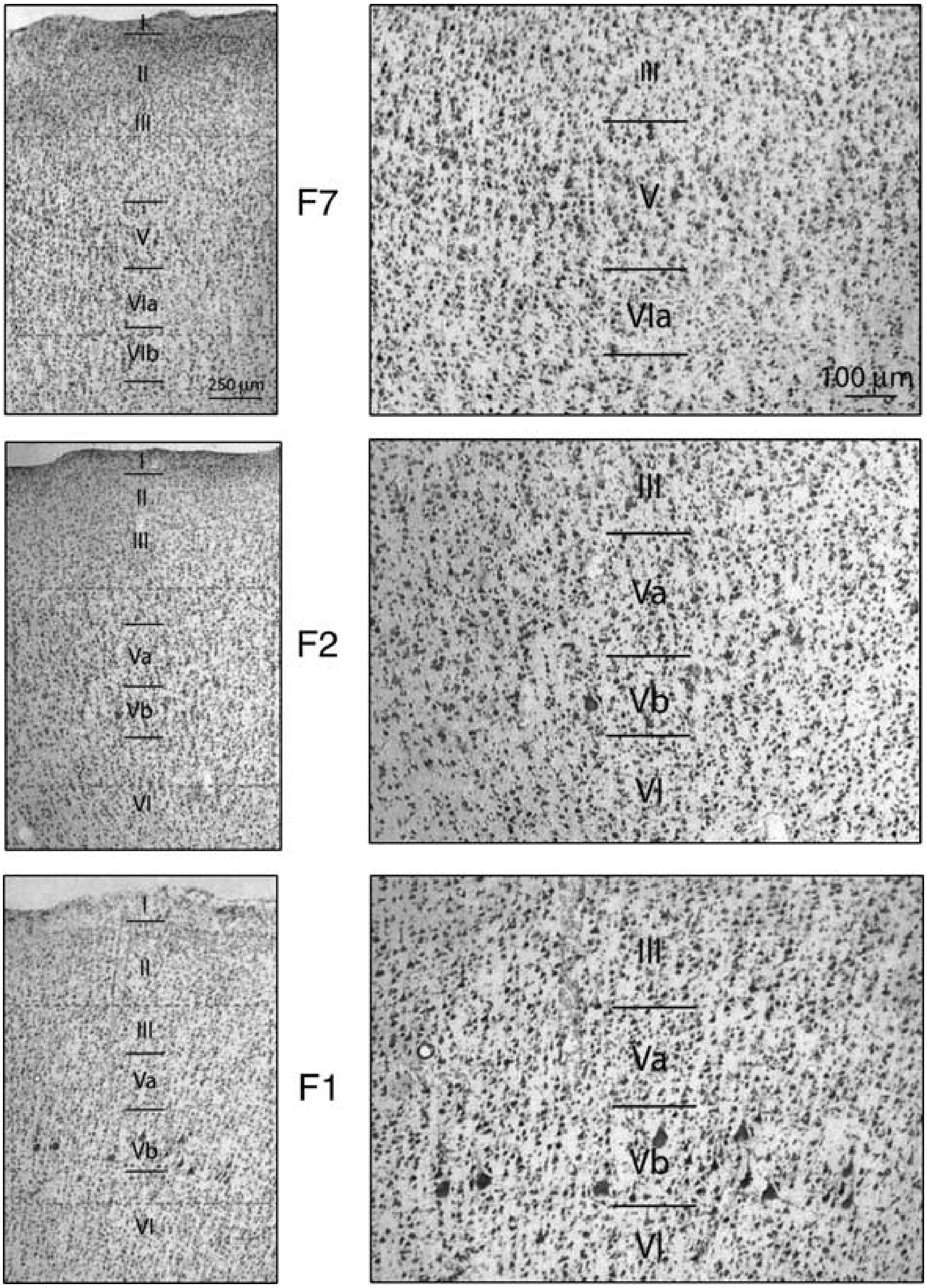


FIG. 1. Low (left) and high (right) power microphotographs of areas F7, F2, F1, from Nissl stained sections. High magnification fields are delimited by the insets (broken lines rectangles) on the brain sections.

anaesthetized with sodium pentobarbital (25 mg/kg; i.v.). Injections of different tracers were made in areas F7 and F2, by using sulcal landmarks, and were later on validated on cytoarchitectonic grounds (Figs 1 and 2). The dura was opened and the fluorescent tracers were injected with a Hamilton microsyringe. In case 1, four Dyamidino yellow (DY; Illing, 2% in saline) equally spaced (2×2 mm) injections with multiple in-depth release sites (0.1 μL each) were placed at the level of the crown of the medial limb of the arcuate sulcus (ASm), in area F7 (Fig. 3, case 1, injection sites inset, a–d), while four equally spaced (2×2 mm) injections of Fast blue (FB; Illing; 2% in saline) with multiple release sites (0.1 μL each) were placed in F2 (Fig. 3, case 1, injection sites inset, e–h), just medial to the spur of the arcuate sulcus (ASs). Data from this case concerning the association connections were published in a previous report (Marconi *et al.*, 2001)

In case 2, four FB injections were placed in the medial bank of the ASm, in area F7 (Fig. 3, case 2, injection sites inset, a–d). In the same animal, four DY injections (0.2 μL each) were made in the cortex lateral to the precentral dimple (pre-CD, Fig. 3, case 2, injection sites inset, e–h), in area F2. The scheme of injections was the same as in case 1. In both cases, all injections were placed between 1.3 and 1.6 mm depths. The dura was then sutured and the animals returned to their cages for the survival period (20 days for case 1; 15 days for case 2), at the end of which they were preanaesthetized as above, then anaesthetized with sodium pentobarbital (50 mg/kg; i.v.), and perfused

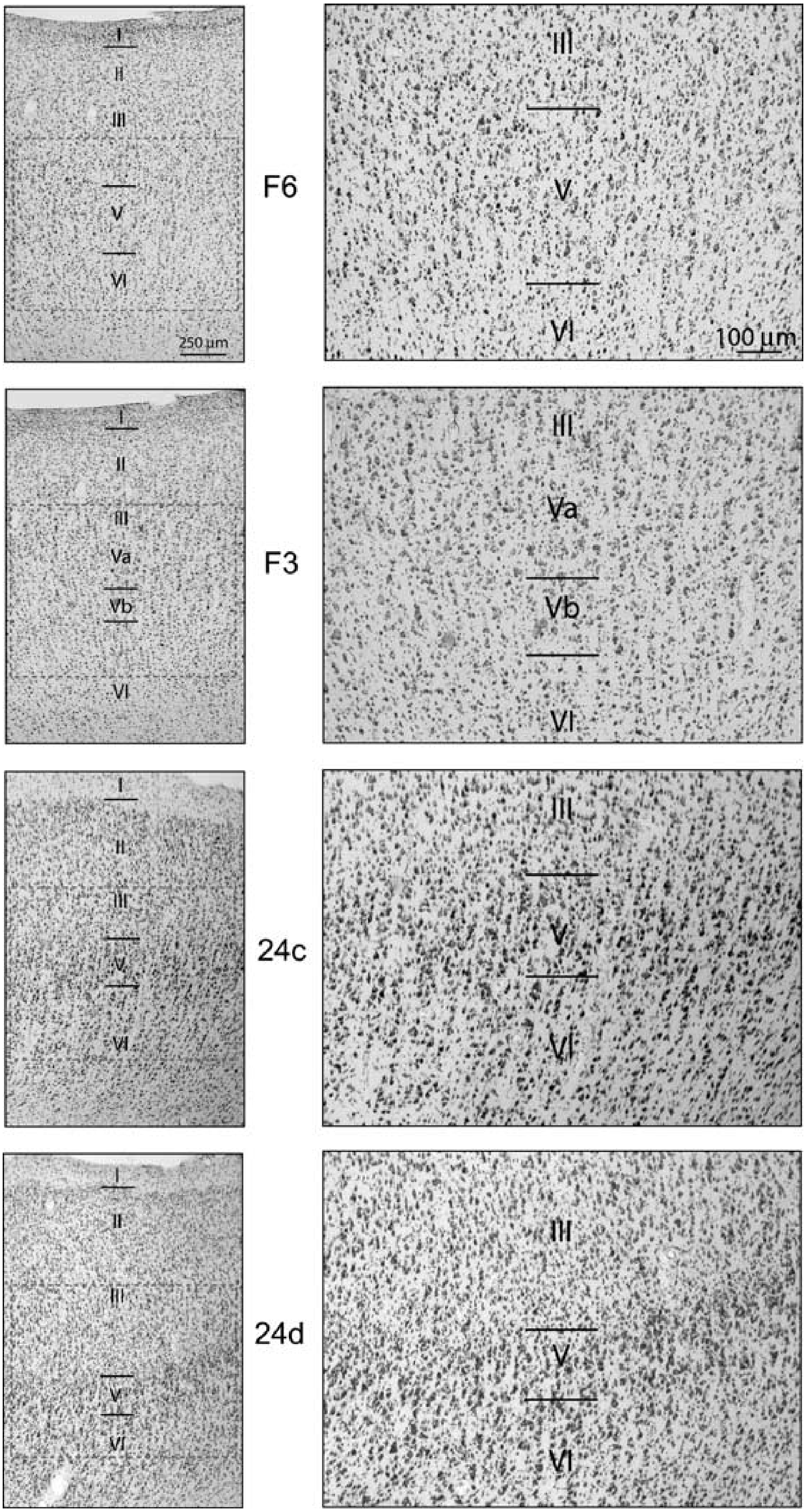
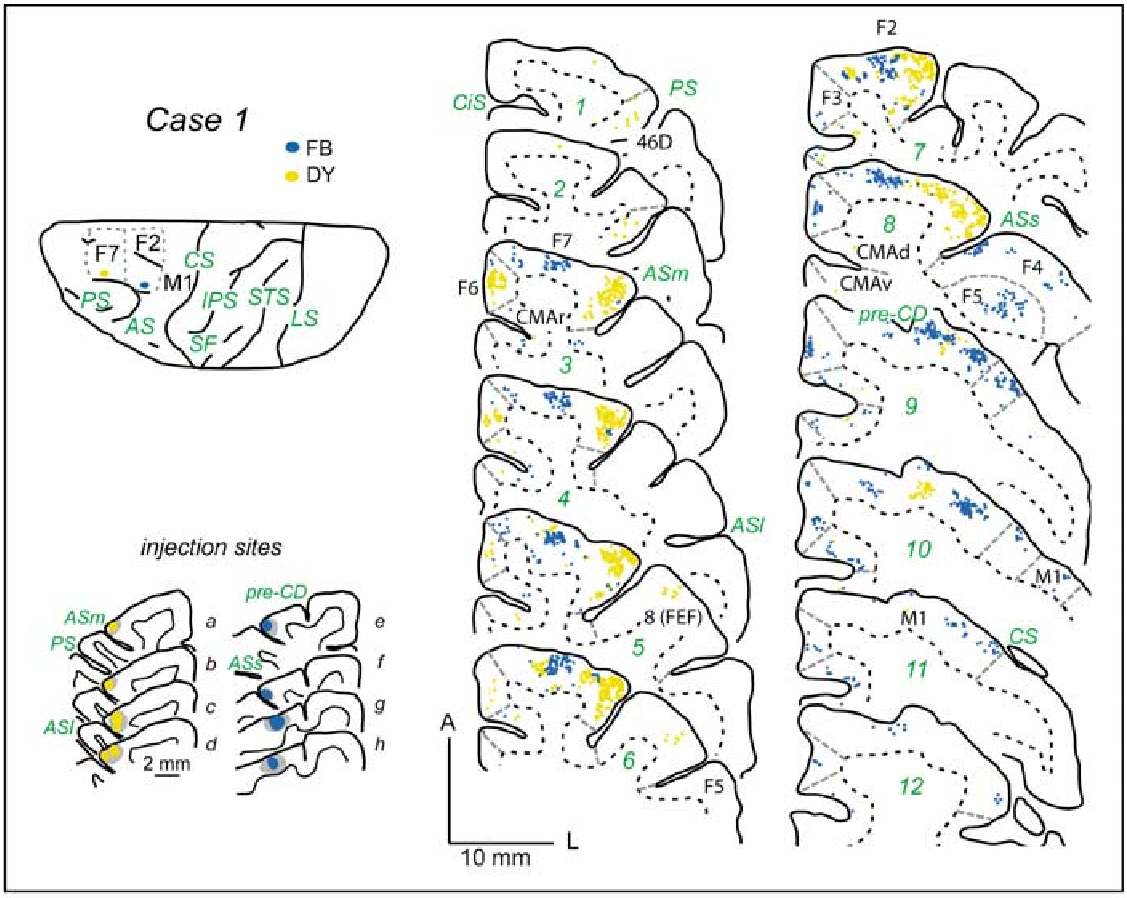


FIG. 2. Low (left) and high (right) power microphotographs of areas F6, F3, 24c and 24d, from Nissl stained sections. Conventions as in Fig. 1.


Case 1
● FB
● DY
F7
F2
CS
M1
PS
IPS
STS
AS
LS
SF
injection sites
ASm
PS
ASl
a
b
c
d
2 mm
pre-CD
ASs
e
f
g
h
CiS
PS
1
46D
2
F7
F6
ASm
CMAr
3
4
ASl
8 (FEF)
5
6
F5
A
L
10 mm
F2
F3
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F5
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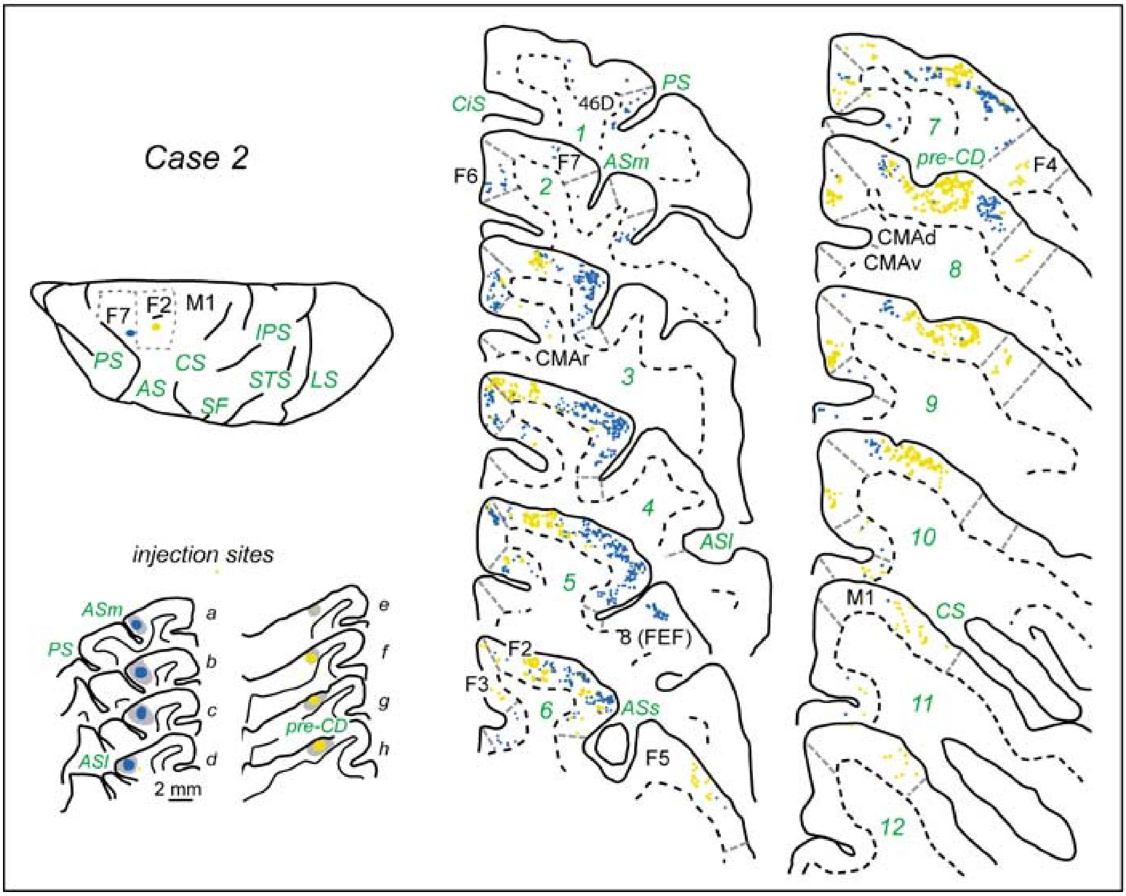
Case 2
F7
F2
M1
IPS
PS
CS
STS
LS
AS
SF
injection sites
ASm
PS
ASl
a
b
c
d
2 mm
e
f
g
h
pre-CD
CiS
PS
46D
1
F6
F7
ASm
2
CMAr
3
4
ASl
5
8 (FEF)
F2
F3
6
ASs
F5
7
pre-CD
F4
CMAd
CMAv
8
9
10
M1
CS
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12

with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The skull was opened and, after removal, the brains were postfixed in the same fixative and then placed in a solution of 30% buffered sucrose until they sank. Frontal cortex was cut in the coronal plane in five series of 50-μm thick sections. Every fifth section was mounted on gelatin-coated slides, air-dried, and coverslipped for retrograde fluorescent labelling analysis. Adjacent sections were stained with thionin (0.025%) for cytoarchitectonic analysis. The architectonic borders of different frontal areas (Figs 1 and 2) were drawn on counterstained sections, using criteria defined elsewhere (Barbas & Pandya, 1987; Matelli *et al*., 1991).

Data analysis

For each animal, a series of frontal coronal sections at 500 μm intervals was plotted. The *x* and *y* coordinates of retrogradely labelled neurons and other landmarks of interest were recorded through a computer-aided microscope (Glaser & Van der Loos, 1965). Cores and halos of the injection sites were defined as described in the literature (Bentivoglio *et al*., 1980; Keizer *et al*., 1983). The present study reports data of callosal labelling. Data on parieto-frontal and fronto-frontal association connectivity has been the object of a devoted paper (Marconi *et al*., 2001).

Laminar analysis

To study the laminar distribution of callosal cells across frontal areas, cytoarchitectonic fields and cortical lamination were defined from counterstained sections. A laminar index (*LI*) between 'supragranular' (*Sg*) and 'infragranular' (*Ig*) callosal cells was calculated as: $LI = Sg/(Sg + Ig)$. The index (*LI*) was computed for those areas containing callosal cells in both upper and deeper layers above a threshold number set at 5% of the total number of callosal cells labelled throughout frontal cortex.

Tangential analysis

To facilitate the analysis of the tangential distribution of the populations of callosal cells, two-dimensional flattened maps of frontal cortex were made, according to the method detailed elsewhere (Johnson *et al*., 1989). Briefly, labelled cells from each section were 'projected' onto a reference line running parallel to the pial surface, at a depth constantly proportional to the distance between this and the white matter. This projection occurred along radial segments mimicking the columnar arrangement of cell bodies in the cortex, as determined from adjacent counterstained sections. The reference line was then flattened and divided into 160-μm wide bins. The sections were finally aligned on a common reference point (the edge of the hemisphere), and the number of cells contained in each bin was expressed through a colour-coded scale. The palette used saturated when cell count in any given bin exceeded the 50% of the number of the cells counted in the bin with highest cellular content. Similarly, an additional cut-off threshold prevented cells representing less than 5% of the highest number of cells present across bins to enter the map.

In order to compare the tangential distribution of the callosal cells labelled from injections in F2 and F7, 2-D 'segregation map' were created by a modification of the two-dimensional flattened algorithm. In these maps, different colour-coded scales were used to represent: (i) bins containing no retrogradely labelled cells, (ii) bins with callosal cells labelled by the first tracer, (iii) bins with callosal cells labelled by the second tracer and (iv) bins containing callosal cells labelled by the first and callosal cell labelled by the second tracer. In each bin, the count of labelled cells was normalized to the maximum number of cells retrogradely labelled by each tracer, as previously described.

Spectral analysis

To study the spatial arrangement of the interhemispheric neurons projecting to F7 and F2, we performed a frequencies domain analysis in the tangential plane of the cortex. A standard fast Fourier transform algorithm (FFT) was used to calculate the discrete Fourier transform (DFT) of the data (Caminiti *et al*., 1985, 1988; for mathematical details see the Appendix in Johnson *et al*., 1989). The binned cell counts along the medio-lateral dimension of each coronal section (41 mm) were used for this analysis. Histograms of cell numbers (bin size 160 μm) vs. tangential location were computed for the different callosal cell populations. The objective of this analysis was to quantify the periodic nature of the fluctuation in cell densities evident in the histological sections and to analyse the mutual spatial relationship of the two distributions by the study of their coherency and phase. The coherency analysis compares two different distributions for desired ranges of frequencies, and assesses the degree to which they are linearly related. It establishes the similarity of two distributions in space by providing their phase relationship, as a measure of the shift between two sinusoids of equal frequency (Johnson *et al*., 1989; Battaglia-Mayer *et al*., 2001; Marconi *et al*., 2001). As we were interested in the portions of the frequency domain that exhibit high values of coherency, we did use the squared coherency, which varies between 0 and 1. The phase range, from $-\pi$ to π, was shown in absolute values varying from 0 to π. Phase relationships were studied only for squared coherency values greater than 0.25.

Results

Cytoarchitectonic parcellation of frontal cortical areas

The agranular frontal areas containing callosal neurons projecting to F7 or F2 were identified on Nissl counterstained sections (Figs 1 and 2), according to criteria described in the literature (Barbas & Pandya, 1987; Matelli *et al*., 1991). Briefly, area F7 shows a prominent layer V, and a layer VI subdivided into a pale VIa and a dark and denser VIb (Matelli *et al*., 1991). These features cannot be found in area F2, which is poorly laminated, and unlike M1, contain few giant pyramidal cells in layer Vb (Matelli *et al*., 1991; Johnson *et al*., 1996). Area F6 is characterized by a prominent and well-demarcated layer V, that unlike in M1 and F3, cannot be subdivided into Va and Vb (Matelli *et al*., 1991). Area F3 (Matelli *et al*., 1991) is characterized by a dense layer Va, layer Vb is significantly thinner than that in M1, and contains scattered medium-sized pyramidal cells. M1 is poorly laminated and contains medium-sized and giant pyramids in layer Vb.

Concerning the motor areas of the cingulate sulcus (reviewed in Picard & Strick, 1996), the rostral one (CMAr) lies in area 24c, while the two caudal areas, dorsal (CMAd) and ventral (CMAv), are located

FIG. 3. Distribution of retrogradely labelled callosal cells projecting to F7 (yellow dots in case 1; blue dots in case 2) and F2 (blue dots in case 1; yellow dots in case 2), after injections of DY (yellow oval in F7, case 1; yellow oval in F2, case 2) and FB (blue oval in F2, case 1; blue oval in F7, case 2), in the contralateral hemisphere. Details of the central core (filled area) and halos (grey area) of diffusion of DY (yellow area) and FB (blue area) are shown both on individual sections and in the inset (a–h) of the injection sites at different rostro-caudal levels of frontal cortex. In each section, continuous and interrupted lines indicate, respectively, the cortical surface and the border between layer VI and white matter. Architectonic borders of different frontal areas are indicated by grey dotted lines extending from the cortical surface to layer VI. The name of each cortical area is indicated only in the first section where it appears, while its boundaries are drawn throughout all sections in which it is present. A indicates anterior, L indicates lateral.

in a cortical region defined as area 24d by Matelli *et al.* (1991). These areas differ mostly for a more pronounced columnar arrangement and for the presence of larger pyramidal cells in layers III and V in 24d, as compared to 24c (Fig. 2).

According to Barbas & Pandya (1987), ventral premotor cortex (PMv) can be distinguished from dorsal premotor cortex by the emergence of a distinct granular layer IV. However, their dorsoventral parcellation scheme does not match the currently accepted subdivision of ventral premotor cortex into a caudal field (F4), and a rostral one (F5), which has been substantiated both on histochemical (Matelli *et al.*, 1985), immunohistochemical (Gabernet *et al.*, 1999) criteria, as well as on functional grounds (reviewed in Rizzolatti *et al.*, 1998). We have divided PMv into F4 and F5, on the basis of gross anatomical landmarks.

Distribution of frontal callosal cells projecting to dorsal premotor cortex

Cells projecting to F7

Area F7 received DY injections in its ventral part around the crown of the ASm in case 1 (Fig. 3, brain figurine and injection sites inset, a–d), and FB injections in its caudal part, rostral to the F7/F2 border, in case 2 (Fig. 3, brain figurine and injection sites inset, a–d). All injections sites were confined to the grey matter without diffusion in the underlying white matter (see Marconi *et al.*, 2001). In both cases, the local spread of tracers extended ≈6 mm rostro-caudally, and did not result in retrograde labelling in ipsilateral M1. In the contralateral hemisphere, the highest number of callosal cells was observed in the area F7, where they were mainly located around the crown of the ASm, and to a lesser extent in its medial bank, as well as in the exposed part of the cortex (Fig. 3, case 1, 3–6; case 2, 2–5). In both cases, the foci of retrograde labelling mirrored the injection's locations in the contralateral hemisphere. A second consistent population of callosal cells was detected at heterotopic sites, in area F2, around the crown of the ASm, and in the lateral portions of the cortex (Fig. 3, case 1, 7–10; case 2, 6–10). Smaller contingents of labelled cells were found in F6 (Fig. 3, case 1, 3–6; case 2, 2–5), area 8 (Fig. 3, case 1, 5–6; case 2, 5), CMAr, CMAd, CMAv (Fig. 3, case 1, 3–11; case 2, 3–11), and in prefrontal cortex (PFC, area 46; Walker, 1940; Fig. 3, case 1, 1–2; case 2, 1–2).

The strength of the contribution of each area to the overall callosal input to F7 was expressed as percentage of the total number of callosal cells (Fig. 4A; cases 1–2, light grey). In case 1, homotopic connections represented 47%, in case 2 were 46% of the total callosal input to F7. Heterotopic projections from F2 were 31% in case 1, and 30% in case 2. In both cases 1 and 2, area F6 contributed approximately 18% of the total number of callosal cells. Labelled cells in area 8, 46, and in each cingulate motor area were a smaller fraction of the total projection to F7. It is worth mentioning, however, that a different study (Rouiller *et al.*, 2002) reported more substantial callosal projections from area 46 to F7 than those we found, probably due to differences in injection sizes and location.

Cells projecting to F2

In case 1, area F2 received FB injections in its ventral part (Fig. 3, brain figurine and injection sites inset, e–h), and in case 2, DY injections just laterally to the pre-CD (Fig. 3, brain figurine and injection sites inset, e–h). All injections sites were confined to the grey matter without diffusion in the underlying white matter (see Marconi *et al.*, 2001); the local spread of tracer for both regions extended for approximately 5.5 mm rostro-caudally, and led to retrograde labelling in ipsilateral M1. In both experiments the highest number of callosal cells was observed in the exposed part of area F2 (Fig. 3, case 1, 7–10; case 2, 6–10), rostral to and around the pre-CD, both medial and lateral to it. Their number decreased moving caudally in the cortex.

In F7, callosal cells were observed in the exposed part of the cortex, and on the medial edge of the hemisphere (Fig. 3, case 1, 3–6; case 2, 2–5). Fewer labelled cells were seen in areas F5 and F4 (Matelli *et al.*, 1985). In this region, callosal cells tended to form small clusters across sections (Fig. 3, case 1, 8–10; case 2, 6–9). A small contingent of callosal cells was detected in F3 (Fig. 3, case 1, 7–11; case 2, 6–10). Scattered labelled cells were also found in CMAr, CMAd and CMAv (Fig. 3, case 1, 3–12; case 2, 3–11), as well as in M1 (Fig. 3, case 1, 10–12; case 2, 11–12).

The strength of the contribution of each area to the total callosal input to F2 is represented in Fig. 4A. Homotopic connections were 45% in case 1 and 47% in case 2, while the heterotopic projections from F7 represented 22% in case 1, and 19% in case 2 of the total callosal input to F2. In spite of the consistency across cases, some differences were present in the percentages of projecting cells across those subdivisions (F3, F4, F5) of premotor cortex that contributed smaller contingents (Fig. 4A). These differences could be due to differences in the injection location, and/or rate of transport across cases.

Laminar distribution of callosal neurons

Injections of FB or DY in F7 or F2, resulted in callosal cells distributed throughout the thickness of the cortex, in layers II–III and V–VI. The majority of callosal projecting neurons were medium- and large-sized pyramidal cells (prevalent in layers III and V), and, to a lesser extent, pyramidal cells of layer VI; few small pyramids were present in layer II. Quantitative analysis (Fig. 4B) of the laminar distribution of callosal cells confirmed a general bilaminar distribution, characterized by supragranular dominance, as indicated by *LI* values (>0.6) found in all the areas analyzed (Fig. 4B). Within this pattern, certain differences were noted. The cells of origin of the homotopic callosal input (F7 to F7; F2 to F2) and of the main heterotopic ones (F2 to F7; and F7 to F2) showed a clear-cut unilaminar pattern, characterized by high values of *LI,* and therefore by supragranular dominance. A more bilaminar distribution was observed for the callosal cells projecting heterotopically from other premotor areas. Correlation analyses (Spearman correlation coefficient, r_s) between *LI* and strength of contribution of each area to the callosal input gave $r_s = 0.90$ in case 1, and $r_s = 0.72$ in case 2, thus indicating that the stronger the connection, the higher the probability that it originates mainly from supragranular layers II–III.

Two-dimensional maps of the tangential distribution of callosal cells in the frontal lobe

The tangential distribution of the callosal neurons was also studied through 2-D computer-flattened reconstructions of the frontal lobe (Fig. 5). These maps highlighted the main features of the overall pattern of callosal connectivity. The callosal cells projecting to F7 and F2 were organized in the form of bands or strips running with main rostro-caudal orientation and obliquely along the cortex. These bands were separated by acallosal gaps (Fig. 5A and B).

In case 1, the callosal cells projecting to F7 (Fig. 5A, green bins) formed two main bands. One was centred on the medial limb of the ASm, in area F7, and extended caudally into the pre-CD region of area F2; the second was located more medially, between the edge of the hemisphere and the dorsal crown of the cingulate sulcus (CiS), in area F6. A smaller cluster was seen rostral to the genu of the AS. In case 2 (Fig. 5A, green bins), the overall pattern of callosal cells resembled that of case 1, with the main difference, however, that the band extending over areas F7 and F2 was fractionated into two main bands, due to one acallosal region observed at the transition zone between

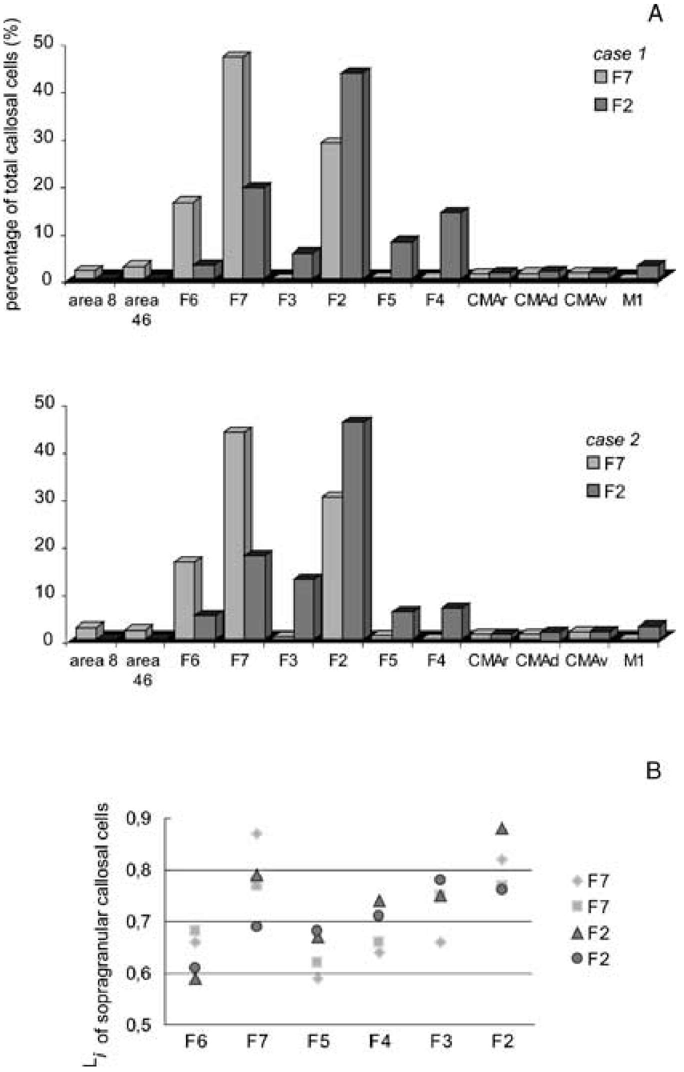

FIG. 4. (A) Histograms of callosal cells counted in each area, expressed as percentage of the total number of cells retrogradely labelled, after injections in F7 (light grey) and F2 (dark grey). (B) Values of laminar index (*LI*) of callosal cells for each cortical area projecting to F2 (black) and F7 (grey).

them. Differences between cases can be seen in the rostro-caudal extent of these maps. This might depend on differences in the rostro-caudal location of the injections sites, to distortions inherent to the topological transformation of the flattening procedure, as well as on individual variations.

In both cases 1 and 2, the pattern of callosal cells projecting to F2 (Fig. 5B, red bins) was characterized by three main bands and by a stripe of cells running obliquely in the antero-posterior extent of the cortex. One caudal band was around and lateral to the pre-CD in F2; the second one was located rostro-medially to the first one, between the edge of the hemisphere and the medial crown of ASm, in area F7; a third band was located in the medial wall of the hemisphere, between its edge and the dorsal crown of CiS, in area F3; finally, an elongated strip extended with a main medio-lateral orientation, in the lateral portion of the cortex, in part caudally to the genu of the AS, across areas F4 and F5.

From these maps an uneven distribution of callosal cells emerges in the frontal lobe, sculpturing bands and stripes of various sizes, shapes and orientation, alternating with zones free of callosal connections.

A direct estimate of the degree of segregation and overlap of callosal cells projecting to F7 and F2 was obtained through a combined 2-D map (Fig. 5C). In both cases 1 and 2, this map showed an almost complete segregation, with very little overlap between populations of projecting cells. In the dorsal aspect of premotor cortex (F7 and F2), the callosal efferent zone projecting homotopically, either to F7 (Fig. 5C, green bins) or to F2 (Fig. 5C, red bins), is always located laterally to that projecting heterotopically. As a consequence, moving rostro-caudally, from F7 to F2, this medio-lateral pattern is reversed, with the F2-callosal zone located laterally and the F7 one medially. This latter pattern is particularly evident in case 2, where moving rostro-caudally, segregated bands of 5-mm width (consistent with that obtained from spectral analysis, see below) appeared rostrally for F7 callosal projections cells, and shift caudally for F2 ones. This reversal of the spatial pattern between the distributions of callosal cells projecting to F7 and F2 is in agreement with the out-of-phase relations found between them through the coherency analysis (see below). A further element that can be seen is that in F7 as well as in F2, callosal cells projecting homotopically were located laterally to those projecting at heterotopic sites.

Overlap of the two populations of cells projecting to F7 and F2 was observed only in restricted zones (see yellow bins in Fig. 5C), as the relative bands were generally sharply separated in the tangential cortical space.

Coherent with the observed segregation was also the finding that double-labelled cells were extremely rare, suggesting that very few, if any, neurons from different premotor areas project to both F7 and F2.

The periodic distribution of callosal cells in the frontal cortex

The density of callosal cells labelled in frontal cortex varied in a periodic fashion along the tangential cortical domain. Their distribution was characterized in a quantitative way through a standard spectral analysis (Caminiti *et al.*, 1985, 1988; Johnson *et al.*, 1989; Battaglia-Mayer *et al.*, 2001). For this, the median power spectra of callosal cells projecting to F7 and F2 throughout frontal cortex were computed. For both the distributions of cells projecting to F7 and F2, most of the power spectrum was contained in the domain of the low (<20th) spatial frequencies, and had two main periodic components (Fig. 6A). The highest peak was always around the fourth Fourier frequency (0.1 cycles/mm), corresponding to a periodicity in cell density with peak-to-peak distance of approximately 10 mm. This indicates an arrangement of fronto-frontal callosal cells in 5-mm width bands. A second smaller elevation was observed in the domain of high spatial frequencies (around the 20th Fourier frequency, 0.5 cycles/mm), corresponding to a periodicity in cell density with peak-to-peak distance of approximately 2 mm. This is suggestive of a columnar-like (1 mm width) arrangement (Jones *et al.*, 1975) of callosal cells in frontal cortex.

The coherency analysis provided a measure of the similarity, in the frequency domain, of the distributions of callosal cells projecting to F7 and to F2. Squared coherency values for all individual frontal sections are shown in Fig. 6B. In the range of the low (<10) spatial frequencies (corresponding to the band-like arrangement described above) and for most of the rostro-caudal extent of the frontal lobe (0–14 mm), the squared coherency between the distributions callosal cell projecting to F7 and F2 was high (0.3–0.8; Fig. 6B, case 1–2). These data suggest a remarkable similarity between bands of callosal cells projecting to F7 and F2. At the rostral and caudal edges of the cortex, only one distribution was present and therefore the coherency could not be calculated. In the same spatial frequency domain (<10), the distribution of the callosal cells projecting to F7 or to F2 were out of phase (Fig. 6C) to varying degrees, phase values ranging between 1/4π and ½π. This fits that observed in the 'segregation maps' (Fig. 5C), and confirms that callosal bands projecting to F7 and F2 are segregated in the tangential cortical space.

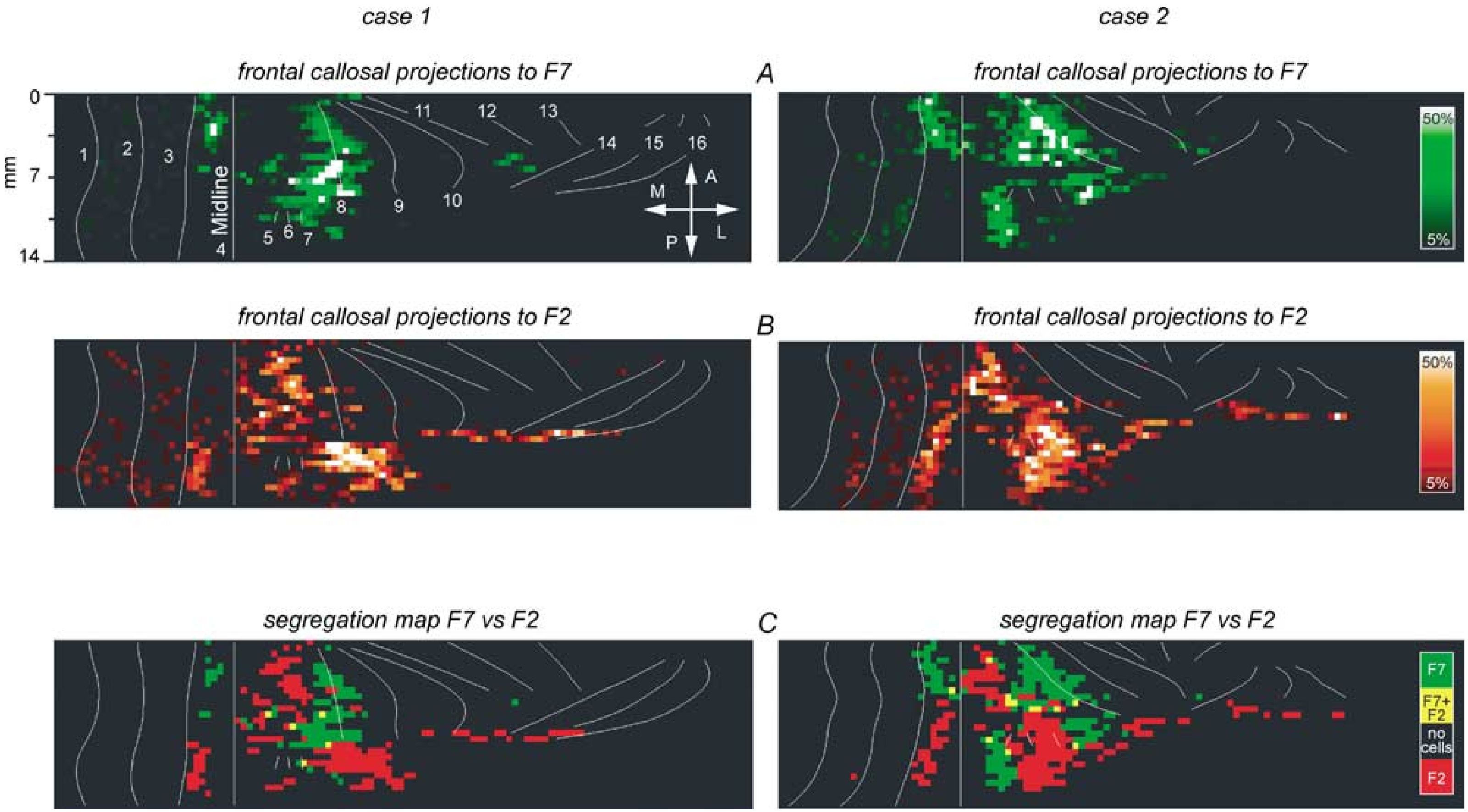

Fig. 5. Two-dimensional flattened views of the frontal lobe showing the tangential distribution of frontal callosal cells. For the generation of these maps, all retrogradely labelled neurons of each section were projected, along radial line mimicking cortical columns, onto a reference line running parallel to the pial surface at a depth constantly proportional to the distance between the cortical surface and the bottom of layer VI. This line was then flattened and divided into 160-µm wide bins. The labelled cells contained in each bin were then expressed as coloured pixels, by using a green-scale from black (zero cells) to white for callosal cells projecting to F7 and a red-scale from black to white for callosal cells projecting to F2. Sections were aligned on a common reference point, the edge of the hemisphere (straight line, 4). In each map, numbers indicate the following anatomical landmarks: crown of the ventral bank of the CiS (1), fundus of the CiS (2), crown of the dorsal bank of the CiS (3), edge of the hemisphere (4), medial crown of pre-CD (5), fundus of pre-CD (6), lateral crown of pre-CD (7), medial crown of the ASm (8), fundus of ASm (9), lateral crown of ASm (10), medial crown of lateral limb of the arcuate sulcus (ASl) (11), fundus of ASl (12), lateral crown of the ASl (13), medial crown of the principal sulcus (PS) 14, fundus of PS (15), lateral crown of PS (16). A is anterior, P is posterior, M indicates medial, and L the lateral part of the cortex. Scale bar, 5 mm.

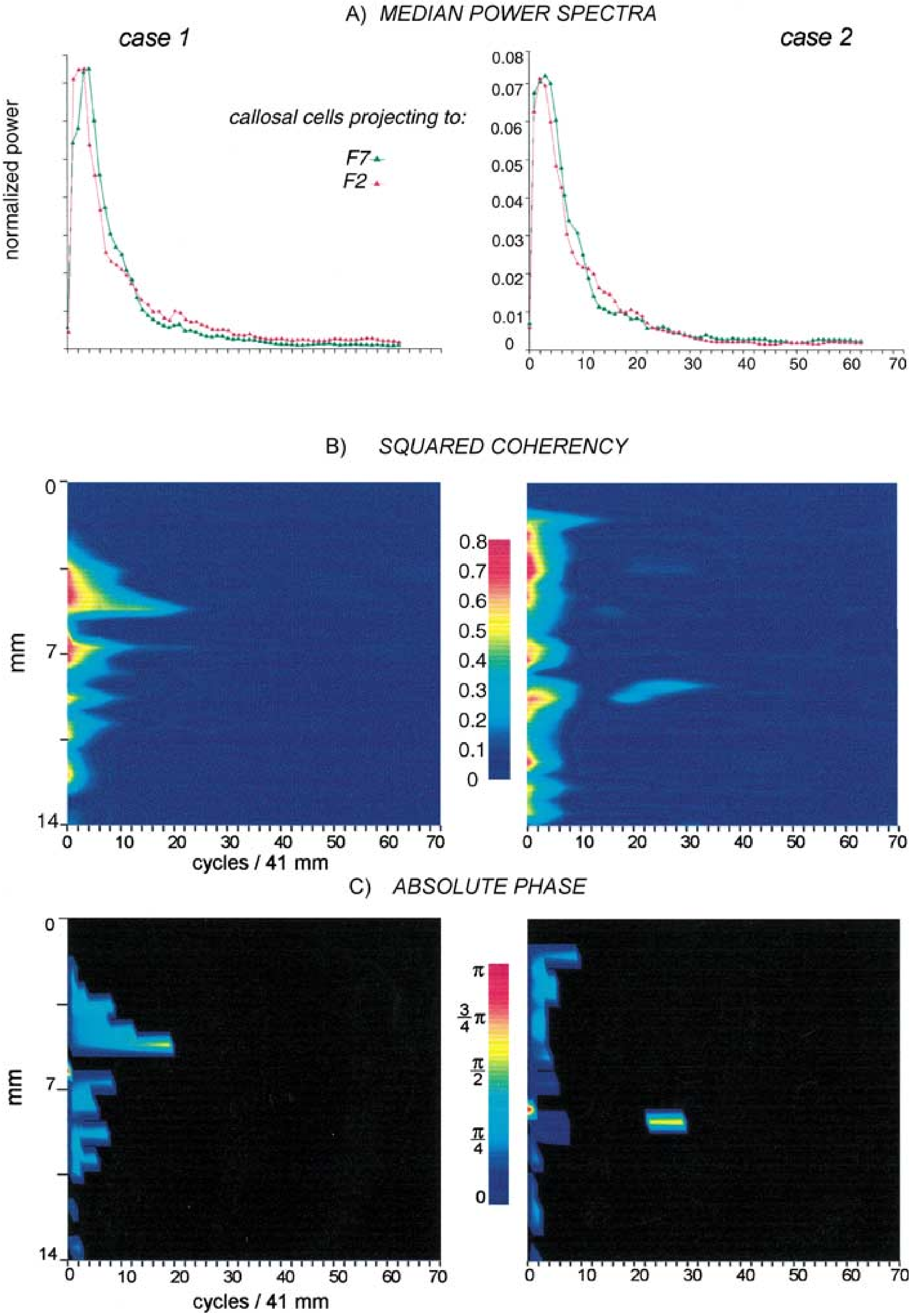

FIG. 6. Spectral analysis of the distribution of callosal cells in frontal cortex. (A) Power spectra of callosal populations projecting to F7 and F2. (B) Squared coherency and (C) absolute phase relationships of the distributions shown in (A). Coherency and phase values in the antero-posterior (A–P) dimension (mm) of the cortex are expressed as a function of the Fourier frequency (cycles/mm). Squared coherency values and phase relationships are colour-coded. The black background in the phase-insets indicates the regions where the coherency values were lower than 0.25 and therefore the phase relationships were meaningless.

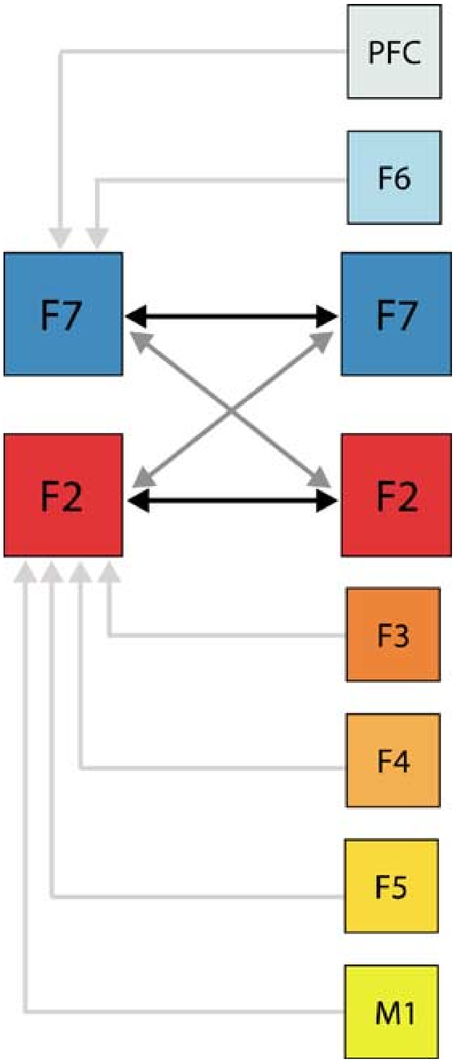

FIG. 7. Schematic representation of the main frontal callosal connections of areas F7 and F2. The strength of connectivity was determined by counting all callosal cells retrogradely labelled. The percentage of cells projecting from any given area is indicated by light grey (<20%), dark grey (21–40%), and black (>40%) lines.

In a range of periodicities that well-matched a columnar-like organization, the coherency values were around 0.2 (Fig. 6B; case 1–2) for a very limited number of sections, where phase values ranged between ½π and 3/4π (Fig. 6C). This indicates a nonoverlapping, but rather alternating arrangement of callosal cell columns projecting to F7 and F2. In most of the sections, coherency values were very low (< 0.1) along the rostro-caudal tangential domain, making it impossible to determine phase relationships in this frequency domain.

Discussion

The callosal system of the dorsal premotor cortex: a comparison with association connections and its relevance to the definition of dorsal premotor areas

In the frontal lobe, callosal connections have been extensively investigated in primary motor and supplementary motor cortices (Jones & Powell, 1969; Pandya *et al.*, 1969; Pandya & Vignolo, 1971; Jenny, 1979; Jones *et al.*, 1979; Jones & Hendry, 1980; Caminiti *et al.*, 1985; Gould *et al.*, 1986; Johnson *et al.*, 1989; McGuire *et al.*, 1991a,b; Roullier *et al.*, 1994; Liu *et al.*, 2002). In some of these studies, lesions or injections of tracers have also been made in a cortical region that has been recognized as dorsal premotor cortex (reviewed in Wise *et al.*, 1997), but never by taking into account its proposed subdivision into a rostral (F7) and a caudal (F2) field, or in isolation from motor cortex. The present study describes the organization of callosal connections of each of these two subdivisions of agranular frontal cortex, and offers a quantitative evaluation of their mutual relationships.

The callosal connections of F7 and F2 seem to conform to the general rule governing the topography of interhemispheric connectivity (reviewed in Innocenti, 1986), as each field has a main homotopic callosal connection, as well as heterotopic projections to other cortical areas (Fig. 7). The main heterotopic projection of each of the two dorsal premotor areas is addressed to the other subdivison of premotor cortex. Thus, F7 is linked homotopically to F7, and heterotopically to F2, while F2 projects homotopically to F2 and heterotopically to F7. When smaller contingents of heterotopic projections are taken into account, a marked difference of callosal connectivity emerges. In fact, F7 connects with pre-SMA, and prefrontal cortex, while F2 connects with SMA, both rostral (F5) and caudal (F4) subdivisions of ventral premotor cortex, and motor cortex. A preliminary study (Rouiller *et al.*, 2002) has reported heterotopic callosal projections from prefrontal cortex to F7 more substantial than those found in the present manuscript. As we have not observed clear-cut differences in the distribution of callosal cells projecting to the rostral and caudal parts of F7, this discrepancy is probably due to variations in injection locations along the dorso-ventral extent of this area. Significant differences in the strength of ipsilateral connections have been reported (Luppino *et al.*, 2003) between prefrontal cortex and ventro-caudal and dorso-rostral parts of F7, the latter containing the supplementary eye fields (SEF). It is therefore possible that the discrepancy between our results and those of Rouiller *et al.* (2002) depends on the fact that in this last study tracer injections involved the part of F7 containing the SEF, which receives almost half of its cortico-cortical projection from prefrontal cortex, while F7-non SEF receives only approximately 10% of ipsilateral cortical afferents from it (Luppino *et al.*, 2003). F7-SEF was not injected in our study.

Beyond that, our study and that of Rouiller *et al.* (2002) offer a very similar pattern of callosal connections of F2 and F7. Interestingly, this pattern matches that of frontal association connectivity of these areas (Muakkassa & Strick, 1979; Barbas & Pandya, 1987; Luppino *et al.*, 1993, 2003; Lu *et al.*, 1994; Johnson & Ferraina, 1996; Marconi *et al.*, 2001). In fact, association connections link F2 and F7. In addition, they link F7 mostly to cortical regions that do not project to M1 (pre-SMA/F6, prefrontal cortex) and F2 to cortical regions projecting to M1 (SMA/F3, ventral premotor areas F4 and F5, cingulate motor areas) In turn, this pattern of cortico-cortical connectivity match the observation (Dum & Strick, 1991; He *et al.*, 1993, 1995) that F2, SMA, F4, F5, and the cingulate motor areas project to the intermediate zone and ventral horn of the spinal cord, as does M1, while F7 and F6 project to the brain stem (Keizer & Kuypers, 1989).

It has recently been suggested (Picard & Strick, 2001; Dum & Strick, 2002) that area F7 is not a premotor area proper, but a cortical region with cognitive rather than motor functions. We believe that the significant cortico-cortical link between F7 and F2, and the arrangement of functional properties in the tangential domain of these areas (Johnson *et al.*, 1996; Fujii *et al.*, 2000) indicate that F7 is part of a network with gradient-architecture involved in the planning of combined eye-hand movements. As of its position within the gradient and by virtue of its cortico-cortical connections with prefrontal cortex, F7 might be a crucial node in the process of linking the plans for eye-hand actions to the cognitive aspects necessary to interpret different behavioural contexts and successfully operate within them. Further physiological studies are needed to assess the potential role of F7 as link between the parieto-frontal and prefrontal network, a role so far inferred mostly on the basis of its cortico-cortical connectivity.

The tangential and laminar arrangement of callosal neurons projecting to dorsal premotor cortex

The callosal connections of dorsal premotor cortex conform to the general organization of interhemispheric connections in another aspect, that of their discrete architecture (reviewed in Innocenti, 1986). Frontal callosal cells projecting to premotor cortex are organized in a 'column-like' fashion (Jones *et al*., 1975). The tangential reconstructions made in this study show that these discrete cell aggregates are arranged in the form of 'bands' or 'stripes' that extend irregularly in the tangential cortical domain. It is worth stressing that the particular shape and orientation of these bands has to be interpreted by taking into account both the plane of histological brain section and the spatial distortion inherent to topological transformations. These aspects of what seems to be a common pattern of organization across cortical areas (Caminiti & Sbriccoli, 1985; Johnson *et al*., 1989) were reflected in the results of the spectral analysis, that showed how the distribution of callosal cells is characterized by a main elevation in the domain of the low spatial frequencies, corresponding to an organization in the form of bands, and by a smaller one in the domain of the higher spatial frequencies, reflecting an arrangement of callosal cells in the form of clusters. Furthermore, the coherency analysis showed that in frontal cortex the 'bands' of callosal cells projecting to F2 and those projecting to F7, although similar in their periodic components, are largely segregated in space, confirming the different pattern of callosal connectivity of these agranular frontal areas, and suggesting for them, participation to different cortico-cortical circuits.

The laminar index, introduced as quantitative measure of the relative contribution of supra- and infragranular layers to the callosal projections from any given area, was higher for homotopic connections, and decreased progressively for heterotopic ones, obeying the positive correlation found between its value and the strength of callosal connectivity. These results resemble those reported by a study (Barone *et al*., 2000) where the percentage of association cells projecting from supragranular layers has been used as a way to define the relative position of cortical areas in the visual hierarchy (Felleman & Van Essen, 1991; Young, 1992). It has been found that the higher this percentage, the closer the projection distance between two areas. If interpreted within this conceptual frame, our results would suggest that F6 and F5 are at the higher hierarchical level than F4 and F3 (discussed in Rizzolatti *et al*., 1998). Of the two dorsal premotor areas injected in this study, F7 would be at a higher level than F2, as also suggested by its overall pattern of cortico-cortical connectivity (also discussed in Picard & Strick, 2001). These results support the contention that in nonhuman primates callosal connections between areas at a similar hierarchical level, therefore homotopic ones, tend to originate mostly from supragranular layers, while those of heterotopic connections with higher order areas, or with lesser architectonic differentiation (Barbas, 1986; Barbas & Rempel-Clower, 1997) tend to have a more bilaminar distribution or a prevalent origin from infragranular laminae (see Innocenti, 1986; Caminiti & Sbriccoli, 1985; Manzoni *et al*., 1986; Innocenti *et al*., 2002). When interpreting these results in terms of information processing within a distributed system, it must be remembered that while in the visual system there is a tight coupling between information flow and hierarchy, in the parieto-frontal system the composition of motor commands is probably based on a progressive match of information, with parietal and frontal premotor areas operating at a relatively earlier stage than motor cortex. It remains to be determined which is the direction of the information flow across the hemispheres in frontal cortex, although it is reasonable to assume that this is a task-dependent process.

The callosal efferent messages of dorsal premotor cortex: treatment of directional information?

How this pattern of callosal connections relates to the functional organization of premotor cortex can only be inferred from this study. So far, only preliminary analysis of single cell properties is available from F7 (Fujii *et al*., 2000), and a complex representation of the motor periphery has been found in F2 (Godschalk *et al*., 1995; Fogassi *et al*., 1999; Raos *et al*., 2003). Useful information on the tangential organization of these cortical regions also comes from studies of the distribution of hand and eye directional signals in F2 (Johnson *et al*., 1996), and F7 (Fujii *et al*., 2000). Studies of association projections to F7 and F2 from parietal areas that had previously been characterized physiologically offer additional indirect information (Johnson *et al*., 1996; Battaglia-Mayer *et al*., 2001; Marconi *et al*., 2001). Overall, these studies indicate that different eye and arm movement related signals, such as those concerning the process of target localization, eye and arm movement direction and position, are represented in a gradient-like fashion along the rostro-caudal extent of F7 and F2 (Johnson *et al*., 1996; Fujii *et al*., 2000). These signals, often in combination, influence the activity of neurons that in F2 have been found in a cortical region located laterally to the precentral dimple. Our results show that this region is rich in callosal connections. Similarly, the rostro-caudal regions of F7 related to saccadic eye and arm movement (Fujii *et al*., 2000) seem to have abundant callosal connections.

Treatment of directional information in agranular frontal cortex seems to be a widespread phenomenon. In F2 (Cisek & Kalaska, 2002; Cisek *et al*., 2003), as well as in M1, a good proportion of cells are modulated by movements of both arms and their directional tuning has been compared across different epochs of instructed-delay reaching tasks, performed separately with either the contra- or ipsilateral limb. In most premotor and motor cortical neurons, the preferred direction does not differ between arms during the presentation of the visual instruction-signal, while it tends to change in F2 during movement-time, an epoch in which it significantly shifts between arms in M1. In motor cortex (Steinberg *et al*., 2002), the directional tuning of bilateral neurons remains unchanged when movement is performed with one or both arms, and population vectors predict movement direction of both unilateral and bilateral arm movements, also when the arms move simultaneously in different directions.

It is therefore reasonable to conclude that, in these areas, visuospatial directional signals, related to reaching movements, have access to the interhemispheric transfer.

The potential role of callosal connections of premotor cortex in visuomotor behaviour and intermanual transfer

Motor behaviour in primates often consists of movements that require interhemispheric transfer of visual and motor information, as prerequisite both for intermanual transfer of motor learning and bimanual actions. How the motor system achieves this goal is still an open issue. Activation studies performed in humans, when confronted to single cell analysis in monkeys, provide information on the potential role of callosal connections from different areas, including premotor cortex.

An interesting question concerns the interhemispheric transfer required during simple forms of visuomotor behaviour, an issue recently addressed through fMRI (Tettamanti *et al.*, 2002) using the Poffenberger paradigm (Poffenberger, 1912; Marzi, 1999). This paradigm compares hand movement reaction-times to visual stimuli in an uncrossed condition, where the visual information is lateralized and processed by the hemisphere that commands the moving hand, vs. a crossed condition, that requires a longer processing time due to the interhemispheric transfer of such information. During this task, the areas involved in the callosal transfer form a distributed network that also includes premotor cortex (Tettamanti *et al.*, 2002). These results do not imply a preferential role, but rather participation, of premotor cortex in such a process. In fact, interhemispheric transmission times (Berlucchi *et al.*, 1995) are unchanged in patients with section of the anterior part of the corpus callosum, where premotor callosal fibres supposedly cross the midline (De Lacoste *et al.*, 1985), as well as in patients with posterior callosotomy. On the contrary, interhemispheric times are increased in cases of callosal agenesis (Jeeves, 1965, 1991; Berlucchi *et al.*, 1995), and complete surgical section of the commisure (Berlucchi *et al.*, 1995). This suggests a distributed processing of crossed manual responses throughout the entire corpus callosum (Berlucchi *et al.*, 1995).

Referring to bimanual coordination, PET studies in humans point to a preferential role of SMA and right premotor cortex (Sadato *et al.*, 1997) that, however, display significantly more activation during parallel than mirror movements. Furthermore, Andres *et al.* (1999), by using EEG recordings, have shown that the interhemispheric coherence between the dorsal premotor areas, primary sensory-motor cortices, supplementary motor areas, cingulate motor areas, posterior parietal cortices is enhanced during the acquisition of new sequences of bilateral finger movements, while it decreases with practice. It has been suggested that the higher interhemispheric coupling during bimanual skills acquisition reflects enhanced interhemispheric integration, necessary to face to computational load inherent to learning a new task.

On their part, neurophysiological studies in monkeys show that neural activity related to bilateral arm and/or hand movement is not specific to any given cortical area, but can be revealed across a multiplicity of areas, including SMA (Tanji & Kurata, 1985; Tanji *et al.*, 1987, 1988; Donchin *et al.*, 1998, 2002; Kermadi *et al.*, 1998), M1 (Donchin *et al.*, 1998; Tanji *et al.*, 1988; Kermadi *et al.*, 1998; Cardoso De Oliveira *et al.*, 2001; Donchin *et al.*, 2002; Steinberg *et al.*, 2002), dorsal premotor cortex (Kermadi *et al.*, 2000; Cisek & Kalaska, 2002; Cisek *et al.*, 2003), posterior parietal cortex (Kermadi *et al.*, 2000), and cingulate motor areas (Kermadi *et al.*, 2000).

Tanji *et al.* (1987, 1988) have shown that when monkeys are required to press a key with the right, left, or both hands, movement- and instruction-related activities of neurons in SMA and premotor cortex differ significantly from those of primary motor cortex. The activity of a large fraction of neurons is related to either bilateral movements only, or both bilateral and unilateral movements. Donchin *et al.* (1998, 2002) have found cells related only to bimanual movements, and have reported that their number is almost equal in MI and SMA.

These areas seem to be part of a network that probably contributes to intermanual transfer and bilateral motor control (reviewed in Swinnen, 2002). This network is by far more widely distributed than previously suggested by studies focused on the pivotal role of SMA (Brinkman, 1984; Tanji *et al.*, 1987, 1988). The observation that in monkeys transient inactivation or lesion of some nodes of this network (Kermadi *et al.*, 1997), such as SMA and dorsal premotor cortex, does not impair animals' performance during execution of the tasks used to reveal the activity-types associated to bimanual movements (Kermadi *et al.*, 1998; Kermadi *et al.*, 2000) also points to a similar conclusion. All together, these observations suggest that, in addition to unimanual movement, neural activity in MI, PMd, and SMA also reflects cortical processing associated with bimanual motor control.

To conclude, callosal connections seem important for visuomotor coordination and essential for the intermanual transfer and coordination. So far, beyond somatosensory (Manzoni *et al.*, 1984) and posterior parietal (Caminiti & Sbriccoli, 1985) cortex, anatomical studies in monkeys had documented the existence of interhemispheric connections between the hand representations of SMA, and pre-SMA (Roullier *et al.*, 1994; Liu *et al.*, 2002). In area F2, many neurons along a strip of cortex located caudally to the spur of the arcuate sulcus are active during hand movement, as also indicated by microstimulation effects (Fogassi *et al.*, 1999; Raos *et al.*, 2003). In our study, this region is rich in callosal connections, suggesting that they can contribute to the intermanual transfer of motor learning, probably those based on arbitrary or conditional visuomotor mapping (Wise *et al.*, 1997) as well as to intermanual coordination.

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Abbreviations

ASm, medial limb of arcuate sulcus; CiS, cingulate sulcus; CMAd, dorsal cingulate motor area (area 24d); CMAr, rostral cingulate motor area (area 24c); CMAv, ventral cingulate motor area (area 24d); DY, dyamidino yellow; F2, caudal part of dorsal premotor area [PMdc, area 6DC (part)]; F3, supplementary motor area [SMA-proper, area MII (part)]; F4, caudal part of ventral premotor area [PMvc, area 4c (part)]; F5, rostral part of ventral premotor area [PMvr, area 6Va (part)]; F6, presupplementary motor area; pre-SMA, [area MII (part)]; F7, rostral part of dorsal premotor area; PMdr, [area 6DR (part)]; FB, fast blue; FEF, frontal eye fields (area 8) M1, primary motor cortex (area 4) F1; pre-CD, precentral dimple; SEF, supplementary eye fields.

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