Neurons in the Macaque Dorsal Premotor Cortex Respond to Execution and Observation of Actions

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Abstract
We identified neurons in dorsal premotor cortex (PMd) of the macaque brain that respond during execution and observation of reaching-to-grasp actions, thus fulfilling the mirror neuron (MirN) criterion. During observation, the percentage of grip-selective MirNs in PMd and area F5 were comparable, and the selectivity indices in the two areas were similar. During execution, F5-MirNs were more selective than PMd-MirNs for grip, which was reflected in the higher selectivity indices in F5 than in PMd. PMd displayed grip-related information earlier than F5 during both conditions. In both areas, the number of neurons exhibiting congruent visual and motor selectivity did not differ from that expected by chance. However, both the PMd and F5 neuronal ensembles provided observation–execution matching, suggesting that the congruency may be achieved in a distributed fashion across the selective elements of the population. Furthermore, representational similarity analysis revealed that grip encoding in PMd and F5 is alike during both observation and execution. Our study provides direct evidence of mirror activity in PMd during observation of forelimb movements, and suggests that PMd is a node of the MirN circuit.

Key words: action execution, action observation, dorsal premotor cortex, mirror neurons

Introduction
Dorsal premotor cortex (PMd) is associated with the planning and execution of forelimb movements (Wise 1985; Wise et al. 1997). The discharge of PMd neurons is strongly modulated in relation to parameters of reaching (Caminiti et al. 1991, 1998; Fu et al. 1993; Kalaska et al. 1997; Messier and Kalaska 2000). A representation of distal movements is also housed in PMd (He et al. 1993; Dum and Strick 2002; Raos et al. 2003), and its cells encode aspects of reach-to-grasp actions (Raos, Umilta et al. 2004; Stark et al. 2007; Hendrix et al. 2009; Takahashi et al. 2017). PMd contains neurons that are active during the trajectory formation of both planned and corrected reaching movements (Archambault et al. 2011); it also contains neurons that exhibit a modulation of activity related to the suppression of a programmed arm movement (Mirabella et al. 2011). It was proposed that PMd provides a higher-order control signal to update or suppress a movement plan (Battaglia-Mayer et al. 2014) when the motor output needs to adapt to environmental changes.

PMd neurons respond to the appearance of visual instructonal cues with either the entire action plan, or parts of it, which are combined to an integrated action plan (Riehle and Requin 1989, 1995; Kurata 1993; Hoshi and Tanji 2000). Other researchers reported that the PMd responses to a visual cue represent its motor significance, instead of its visuospatial position (Boussaoud and Wise 1993a, 1993b; Boussaoud et al. 1995). Recent neurophysiological studies reported PMd neurons that discharge when a monkey executes a conditioned task (moving a cursor to capture targets on a computer screen), and when the monkey observes visual stimuli associated with the
performance of the same task; the stimuli may be either replayed or executed by the experimenter (Cisek and Kalaska 2004; Tkach et al. 2007). The response pattern of these neurons resembles that of mirror neurons (MirNs): they fire both when an animal performs a transitive action, and when the same animal observes another agent performing the same or a similar action with a biological effector (di Pellegrino et al. 1992; Gallese et al. 1996; Rizzolatti et al. 1996). MirNs have been found in area F5 of the ventral premotor cortex (di Pellegrino et al. 1992; Gallese et al. 1996; Rizzolatti et al. 1996; Kraskov et al. 2009; Bonini et al. 2014; Papadourakis and Raos 2017), areas PF/FG, AIP of the inferior parietal lobule (Gallese et al. 2002; Fogassi et al. 2005; Fani et al. 2014; Maeda et al. 2015), as well as from the primary motor cortex (Vigneswaran et al. 2013). In the last decade, many investigators proposed that PMd is a node of the MirN circuit (Bonini 2017), providing neuroimaging [monkeys: (Raos et al. 2007); humans: (Molenberghs et al. 2012)] and neuroanatomical data (Bruni et al. 2018) to support this view. However, the presence of neurons in PMd that display MirNs activity, is still controversial.

To resolve this controversy, we investigated the existence of MirNs in PMd by recording the activity of PMd neurons while monkeys were executing and observing object-directed reaching-to-grasp actions. To provide a reference for the PMd neurons, we also recorded the activity of cells in the ventral premotor area F5 of the same monkeys engaged in the same behavior. We demonstrated that PMd houses MirNs, thus providing direct evidence for the inclusion of PMd in the MirNs circuit. PMd- and F5-MirNs were very similar in terms of selectivity, representations and matching between execution and observation responses.

Materials and methods
Subjects and Recordings
Two adult female monkeys (Macaca mulatta) weighing 5 and 7 kg were used in the present study. Animals were purpose-bred by authorized suppliers within the European Union (Deutsches Primatenzentrum, Göttingen, Germany). The experimental protocols were approved by the Veterinary authorities of the Region of Crete (6157/7-5-2014), conducted in the approved animal facility of the Faculty of Medicine of the University of Crete (EL91-Bioexp-06/18-2-2014), and complied with the European (directive 2010/63/EU and its amendments) and National (Presidential Decree 56/2013) laws on the protection of animals used for scientific purposes. To immobilize the monkey’s head, as required for eye tracking and single cell recordings, a head post was fixed to the animal’s skull with mandibular plates and titanium screws (Synthes, Bettlach, Switzerland). When behavioral training was completed, a cylindrical stainless steel recording chamber (diameter: 20 mm) was implanted over the left hemisphere of each monkey. The coordinates for chamber placement were estimated using the McLaren average atlas (McLaren et al. 2009). Surgical procedures were performed under general anesthesia and aseptic conditions. Basic recording procedures have been described previously (Papadourakis and Raos 2017). The recordings were carried out with single microelectrodes. Spike waveforms were sorted, and spike occurrences were stored as binary time series at 1-ms time resolution.

After chamber implantation, the accessible cortical area was functionally explored by means of single neuron recordings and intracortical microstimulation. The available cerebral...
territory included parts of the dorsal and ventral premotor cortex, and extended from the primary motor cortex (area F1) to the posterior part of the frontal eye fields (FEF). The criteria used to characterize areas F1, PMd, F4, F5 and FEF were described in previous studies (Raos et al. 2003, 2006; Raos, Umiltà et al. 2004; Papadourakis and Raos 2017). The location of the recorded areas was confirmed postmortem on the fixated brains of both animals using standard histological procedures (Papadourakis and Raos 2017).

Informal qualitative testing preceded the selection of neurons tested with the behavioral paradigm. Responses to action observation were tested by performing a series of hand grasping actions in front of the monkey. The activity of each recorded neuron was also correlated with the performance of active movements as well as with somatosensory and visual stimulation.

Behavioral Apparatus and Paradigm

A behavioral apparatus, consisting of a rotating uniform hexagonal prism, was positioned in front of the monkey. Each of the six perpendicular rectangle faces of the prism could accommodate a 3D object. Each object was fixed on a metal rod that penetrated the center of each face and allowed a slight horizontal displacement of the object (<0.3 cm) when grasped. A microswitch fixed on the rod was activated during the holding of the object. The distance between the apparatus and the monkey was set at 25 cm or 45 cm, depending on whether the monkey or the experimenter was performing the task, respectively. The relative position of the object, monkey and experimenter is always in the same central position (Fig. 1B). Each object was grasped according to its size and physical characteristics: large sphere (diameter: 40 mm) required whole hand prehension with all the fingers wrapped around the object and the palm in contact with the object; cylinder (length: 40 mm, base diameter: 20 mm) required finger prehension, using all fingers but the thumb; ring (diameter: 15 mm) required a hook grip with the index finger inserted into the ring; cube in vertical groove (side: 10 mm) required an advanced precision grip using the pulpar surface of the distal phalanx of the index finger opposed to the pulpar surface of the distal phalanx of the thumb. Both monkeys used similar grips for grasping the same objects, as judged by the visual inspection. Eye movements were monitored with the scleral search coil technique (Robinson 1963; Judge et al. 1980) at 500 Hz sampling resolution.

Execution task: The illumination of a light emitting diode (LED) located just above the selected object cued the initiation of a trial. The monkey was required to fixate on the LED and press a button, placed at lap level, for a period ranging from 800 to 1200 ms. At the end of this delay period, a brief flash of the LED instructed the monkey to release the button, and to start its movement to grasp the object (while maintaining fixation). The monkey was required to pull and hold the object with its right hand for a period ranging from 600 to 900 ms. A circular tolerance window of 10° diameter centered on the object was used for eye fixation. Turning off the LED signaled the monkey to release the object. The monkey was rewarded with water at the end of each correct trial.

Observation task: In this task, the monkey performed no movements and simply observed the experimenter performing the execution task. The monkeys’ hands were continuously video-monitored to ensure that they remained immobile during the observation task. The trials in which the animals moved their forelimbs were discarded. The experimenter was positioned on the right side of the animal and grasped the same objects with his right hand. The monkey could see the evolution of the experimenter’s hand shape as it approached the object. In this task, the LED above the object was off and the experimenter received instructions from a screen out of the monkey’s view. No restriction was posed on the animals’ oculomotor behavior during the observation task.

The execution and observation tasks were implemented under ambient lighting of normal intensity in blocks of 8–10 trials for each one of the four objects/reach-to-grasp actions. Usually, the blocks of the observation trials for the four reaching-to-grasp actions preceded the blocks of the execution trials. In both tasks, the onset and end of the forelimb movement (the monkey’s in the execution, the experimenter’s in the observation) were signaled by the release of the pushbutton and the turning on of the objects’ displacement switch, respectively. The sequence of the events of both tasks is presented in Figure 1C.

Occasionally, the monkey also observed an intransitive action, in addition to the four transitive ones. This intransitive action consisted of a reaching, non-object-directed movement, with extended wrist and fingers towards a face of the apparatus not occupied by an object.

Analysis of Neural Activity

At least eight trials of each experimental permutation were required for the inclusion of a neuron in the analysis. Two events were used for the alignment of a given neuron’s spike trains: the illumination of the LED, and the beginning of movement. Illumination-aligned spike trains used the period starting 500 ms before and ending 800 ms after the event, whereas the movement-aligned spike trains used windows starting 600 ms before and ending 1450 ms after the event. The two activity periods were concatenated to calculate a discharge frequency for each trial. Discharge frequencies were calculated for each trial in 200 ms long temporal windows, shifted over time with steps of 5 ms. Frequencies occurring at the same sliding windows across trials were averaged to obtain the mean firing rate of each condition-action pair over time.

The temporal profile of the neuronal responses varied across the sectors of the premotor cortex. For example, the peak activity of some neurons followed the movement onset time, whereas other neurons’ peak activity preceded it. To define an epoch around a neuron’s peak response in each condition, we applied the double thresholding method, used previously (Papadourakis and Raos 2017). Initially, the baseline activity (500 ms before trial start) was estimated. Then, we defined the movement modulation epoch: each movement modulation epoch consisted of at least 40 consecutive firing rate data points (200 ms), starting later than 450 ms before the movement and ending earlier than 600 ms after the end of the hold period. Activity also was required to rise above the mean, plus one standard deviation of the baseline epoch. Next, we defined a movement high-activity epoch, consisting of at least 12 consecutive firing rate data points (60 ms), with activity above the mean (plus one standard deviation) of the movement modulation epoch. In addition, an object fixation epoch (from 100 to 400 ms after key press) was used to estimate visual responses to the object.

The mean activity in the movement high-activity epoch was used to rank the actions from preferred to non-preferred for each neuron, separately for each task. A two-way ANOVA
followed by Bonferroni post hoc tests (factors epoch (two levels—baseline activity, movement high-activity), and grip (four levels)) was used to verify that a neuron was modulated during around the movement with respect to the baseline (P < 0.05). These comparisons were only considered if the neuron’s mean firing rate was higher than 10 spikes/s during the movement high-activity epoch of at least one grip. The application of the “10 spikes/s” criterion led to the rejection of statistically significant ANOVA results in 5 out of 118 cases in 59 neurons. These neurons were recorded in the same penetrations with the 262 neurons analyzed in this article, but did not qualify as MirNs and were excluded from the database of the present study. This two-way ANOVA was also used to assess the grip selectivity of the neurons. Neurons were considered as grip-selective if both the grip effect, and at least one post hoc comparison between grips in the movement high-activity epoch were statistically significant (P < 0.05). To assess grip selectivity through time, a one-way ANOVA was performed on each sliding window, comparing the means of the four different actions. The onset of grip selectivity was defined as the timestamp of the first of at least 12 consecutive firing rate data points (60 ms) with a P-value < 0.01. The above ANOVA procedures were conducted separately for the execution and observation responses of each neuron.

To obtain the neuron’s net-normalized response for each grip and task, its average baseline activity was first subtracted from the activity of each 200 ms sliding window. The resulting net activity was then divided by the response in the window displaying the neuron’s highest activity across grips and tasks (maximum normalized activity across execution and observation = 1). The average of the net-normalized responses of each rank (preferred to non-preferred) was then calculated separately for each task to obtain the population activity. To assess grip selectivity at the population level, we calculated the average normalized firing rates for each neuron within a 500-ms epoch centered at the time of maximum response. These epoch rates were then compared with the Kruskal–Wallis test (factor: ranked object; P < 0.05).

Quantification Indices

Grip selectivity was quantified for each neuron and task with a preference index (PI), defined as $PI = (n - (\sum r_i/\bar{r}_{pred}))/n - 1$ where $n$ is the number of grips, $r_i$ is the movement high-activity for grip $i$, and $\bar{r}_{pred}$ is the movement high-activity for the preferred grip. PI ranges between 0 and +1; a value of 0 indicates the same amplitude of response for all grips, and a value of +1 indicates preference for only one grip.

The difference between the amplitude of the response of MirNs during grip observation and execution was assessed by the task amplitude index (tAI) that was computed according to the formula $tAI = \frac{\bar{r}_{exec} - \bar{r}_{obs}}{\bar{r}_{exec} + \bar{r}_{obs}}$, where $\bar{r}_{exec}$ is the mean movement high-activity for the execution of the four grasping actions, and $\bar{r}_{obs}$ is the mean movement high-activity for the observation of the four grasping actions. $tAI$ ranges between $-1$ and $+1$, with negative values corresponding to a higher response for observation than execution (and vice versa).

Representational Similarity Analysis

Representational dissimilarity matrices (RDMs) were used to compare the representations between (a) execution and observation activity of MirNs or (b) population activity of PMd and F5 MirNs during execution or observation. In our case, the RDM is a 4 × 4 array containing the pairwise Euclidean distances between the grips either executed or performed. The Euclidean distance between a grip pair is given by:

$$d(a_1, a_2) = \sqrt{\sum_{i=1}^{n} (a_{i1} - a_{i2})^2},$$

where $a_i$ is the mean epoch activity for grip $k (k = 1, ..., 4)$, $n = 1$ in the case of a single neuron RDM, or $n$ is the number of neurons in the case of a population RDM. Before the computation of the RDMs at the population level, the epoch rates of each neuron were normalized by dividing with the neuron’s maximum movement high-activity epoch rate to provide equal contributions from each neuron to the population RDM.

The relationship between two RDMs is measured by correlation coefficients (such as Pearson’s r). To deal with the fact that the pairwise distances are not independent measurements, the significance of the correlation between a pair of RDMs was assessed by the Mantel test (Mantel 1967) as in our previous study (Papadourakis and Raos 2017).

A permutation procedure was used to evaluate the number of neurons with statistically significant correlations between the observation and execution RDMs. The observation responses of each unit were paired with the execution responses of another randomly chosen unit, and then the significance of the relationship between the observation and execution RDMs was measured. This procedure was repeated 1000 times to estimate the null distribution of units with statistically significant correlations. This null distribution was compared with the number of neurons displaying statistically significant correlations between the observation and execution RDMs of the original population to infer its difference from chance.

To explore the effect of the neuronal population size on the population RDMs, neuronal populations of different sizes were created by random selection with replacement. RSA was run 1000 times for each population to approximate $r$ distributions for each subpopulation. The medians of these distributions were used as the dependent variable in a segmented regression model, comprised by a sloping segment followed by a horizontal one, to establish the optimum breakpoint between the size-dependent and size-independent segments (SegReg software [https://www.waterlog.info/segreg.html]). The introduction of the breakpoint, estimated by the segmented regression analysis, resulted in greater explained variance compared with a single linear model.

Procrustes Distance

The standardized Procrustes distance was used as a dissimilarity measure of the neuronal discharge between the two conditions to assess the execution/observation matching of MirNs responses, the so-called congruence. The Procrustes distance is defined as the square root of the minimized sum of squared distances between the corresponding grips in the two conditions: $procrustes = \sqrt{\sum (f_{exec,i} - f_{obs,i})^2}$, where $f$ is the movement high-activity epoch for grip $i$ in execution and observation, and $n$ is the number of grips. The distance is minimized by a linear transformation on the centered data; it ranges from 0 to 1, where smaller values indicate greater matching between execution and observation. A permutation procedure was used to evaluate whether the number of congruent neurons, as defined by a classification threshold of the Procrustes distance, differed from chance. The observation responses of each unit were matched with the
execution responses of another, randomly chosen, unit. The resulting Procrustes distances were then used to define the number of congruent units at a range of thresholds. This procedure was repeated 1000 times, resulting in an estimate of the null distribution for the number of congruent units. The number of neurons in any given threshold of Procrustes distance of the original population was compared with the null distribution to infer its difference from chance.

To explore the effect of individual unit congruency (as reflected on the Procrustes distance) on the population congruency (as reflected on the relationship between the execution and observation population RDMs), neurons were ranked according their Procrustes distance. Population congruency was then computed, initially for a population consisted of the 50 units with the lower Procrustes distances. Neurons with higher Procrustes distances were gradually added. The same procedure was repeated with the inverse ranking, starting from the population of ≈50 units with the higher Procrustes distances, gradually adding neurons with lower Procrustes distances.

**Gaze-Related Modulation of Activity**

To investigate the dependence of premotor neurons on gaze position, we performed the analysis described by other authors (Boussaoud et al. 1998; Cisek and Kalaska 2002) on 122 PMd and 120 F5 MirNs during execution and observation conditions. Briefly, gaze fixation episodes with a duration of at least 100 ms were identified as time periods in which eye speed did not exceed 50°/s. Long lasting episodes were divided in fragments of 100 ms. For each 100-ms fragment of a fixation episode, the average gaze direction and the average firing rate was calculated within the movement high-activity epoch. The gaze-related modulation was studied separately for each cell and condition using planar regression (Boussaoud et al. 1998), in which the neuronal activity in each fragment of a fixation episode was expressed as a function of the horizontal and vertical components of gaze direction in the 2D linear regression model: response \( = a + b^\text{gaze}_x + b^\text{gaze}_y \) (where \( c \) is the intercept, \( a \) and \( b \) the slopes along the horizontal \( X \) and vertical \( Y \) axes, respectively). The strength of the modulation was assessed by the coefficient of determination \( r^2 \) of the regression \( P < 0.01 \).

**RESULTS**

We recorded the activity of 140 neurons that were active both when the monkeys grasped objects, (execution task, Fig. 1A, top) and during observation of the same behavior (observation task, Fig. 1A, bottom), thus displaying the characteristics of MirNs. The neurons were recorded from the left hemispheres of two monkeys in 57 distinct penetrations, medial of the spur of the arcuate sulcus, corresponding to PMd (Fig. 2). 122 MirNs were also recorded as control in 39 distinct penetrations, lateral of the spur of the arcuate sulcus, corresponding to ventral premotor area F5. The F5 neuronal population was used in an earlier study (Papadourakis and Raos 2017). In both monkeys, the clusters of penetrations in PMd are well distinct from those in F5 (Fig. 2). 87% of the PMd and 75% of the F5-MirNs were located in the upper 3 mm of cortex, measured from the most superficial point at which neuronal activity was recorded. Neurons unresponsive to observation, but active during execution (non-MirNs), were frequently encountered in the majority of penetrations (PMd: 52/57; F5 35/39). Given that our aim was to study the properties of MirNs, we did not proceed with formal behavioral testing for recording sites that did not elicit MirN responses. Occasionally, non-MirNs were recorded simultaneously with MirNs (PMd: 37/140; F5:22/122; Supplementary Figs S1 and S2).

During action observation, the activity of all recorded neurons in both premotor areas was mainly exhibited after the beginning of the experimenter’s movement. MirNs that displayed suppressed activity during observation were sporadically recorded from both areas (PMd: 8%, F5:6%) and excluded from the database. Thus, the action observation-related activity only refers to an increase of the neuronal firing rate as compared with the spontaneous activity. Two-way ANOVA followed by Bonferroni post hoc [factors epoch (two levels—baseline activity, object fixation epoch activity), and grip (four levels)] revealed that none of the neurons displayed any statistically significant activity during the object fixation epoch at the observation task. In contrast, during action execution, 78 (56%) PMd- and 79 (65%) F5-MirNs neurons displayed also a phasic response at the object fixation epoch, in addition to the movement related discharge. Only activity within the movement, high-activity epoch was used for subsequent analyses.

**Response Properties of MirNs**

Figure 3 illustrates the neuronal activity exhibited by two PMd and two F5 example MirNs during observation and execution. The observation response of both PMd–MirNs rises rapidly and peaks during the first half of the experimenter’s approaching movement towards the object; whereas during execution, the neuronal response maximizes before movement commences. Conversely, the observation response of both F5-MirNs takes place during the second half of the movement and at the beginning of the holding phase. The maximum firing rate of both F5-MirNs occurs close to the contact of the experimenter’s hand with the object. The execution responses of both F5-MirNs reach their maximum after the onset of movement. The temporal pattern of the discharge of the neurons presented in Figure 3 is similar for the four grips tested, but the intensity of the response is modulated by the grip type executed by the
and Supplementary Fig. S3 (left columns). This study examines factors: epoch (two levels: baseline, high-activity), and grip (four types: cylinder/finger prehension, sphere/whole hand prehension, ring/hook grip for the cube in groove). Firing rates follow the same color and pattern, indicating that the neuron is selective for the type of prehension associated with the neuron.

The neuron presented in Fig. 3A fires strongly during observation of the advanced precision grip, but significantly less during the observation of the hook grip and the whole hand prehension; even less for the finger prehension (two-way ANOVA; factors: epoch (two levels: baseline, high-activity), and grip (four types)). During execution, the neuron discharges maximally when the monkey executes the advanced precision grip. The activity for the other grips progressively weakens, ending with whole hand prehension ($F_{(2,72)} = 13.3, P < 0.001$). Noteworthy, the type of prehension associated with the neuron’s strongest activity when executed (i.e. the advanced precision grip) is the one maximally activated during observation. The second PMd-MirN (Fig. 3B) is more selective during observation than during execution; the neuron displays a preference for the observation of the advanced precision grip ($F_{(3,72)} = 16.9, P < 0.001$). During execution, the selectivity is associated with pairs of grips ($F_{(3,72)} = 6.6, P < 0.001$): the hook and precision grips evoke the highest activation, whereas the finger and the whole hand prehensions evoke the lowest activation.

The most prominent feature of the F5-MirN presented in the third row (Fig. 3C) is the matching between the observation and execution responses. The grip evoking the strongest activity during observation, the advanced precision grip, evokes the same activity during execution. In addition, the activity for the tested grips in observation and execution displays the same ranking and selectivity in both conditions ($F_{(3,72)} = 6.0, P < 0.001$); ($F_{(3,72)} = 17.1, P < 0.001$). The second F5-MirN (Fig. 3D) is broadly selective in both conditions ($F_{(72)} = 20.7, P < 0.001$); ($F_{(3,72)} = 5.1, P < 0.001$).

The selectivity and the response profile exhibited by the example neurons of the two areas are also reflected at the population level. Figure 4 and Supplementary Fig. S3 (left columns) illustrate that populations of premotor neurons discriminate between the different grips; not only when the grasping actions are executed, but also when they are observed (Kruskall-Wallis test; PMd execution: $H(3) = 49.96, P < 0.001$; PMd observation: $H(3) = 42.51, P < 0.001$; F5 execution: $H(3) = 87.93, P < 0.001$; F5 observation: $H(3) = 42.89, P < 0.001$). Notably, when the ranking of execution responses is used to sort the observation responses (and vice versa), the grip selectivity ceases to exist (Fig. 4 and Supplementary Fig. S3 right columns). This finding indicates that the neurons are selective for different grips in the two conditions (i.e. the neurons are incongruent), and execution and observation responses do not match. Execution/observation congruency at the single neuron and the population level is further analyzed in a following section. During execution, the PMd population reaches maximum activity before the onset of the movement, whereas the activity of the F5 population peaks not far from the end of the movement; presumably, this is while the fingers are closing. During observation, the peaks of the PMd and F5 populations occur in the first and the second half of the observed movement duration, respectively.

To quantify the difference of the magnitude of the discharge between execution and observation we calculated a tAI for each neuron (Fig. 5). In both cortical areas, responses during execution were higher than that during observation ($\tilde{t}AI_{PMd} = 0.10$; $\tilde{t}AI_{F5} = 0.15$). These values of tAI reflect absolute amplitude differences between execution and observation of 20% and 35%, respectively. The distributions of amplitude indices of the two areas were not statistically different (Kolmogorov-Smirnov ($K-S), P > 0.10$).

Statistical analysis of the responses of PMd neurons revealed that 81 (57.9%) and 87 (62.1%) neurons displayed a statistically significant effect for the grip type during observation and execution, respectively ($2 \times 4$ two-way ANOVA, $P < 0.01$). Similarly, in the ventral premotor cortex, 89 (73.0%) neurons were selective when the reaching-to-grasp actions were observed and 97 (79.5%) when executed. A comparison of the proportions of the grip-selective neurons in the two cortical areas and the two conditions revealed that: (i) in both cortical areas, the grip selectivity was stronger during observation than during execution, and (ii) the grip selectivity was stronger in the PMd than in the F5 cortex.
areas, the number of grip-selective neurons during execution is not statistically different from the number during observation; (ii) during observation, both dorsal and ventral premotor cortex have similar quantities of grip-selective neurons; (iii) during execution, the proportion of grip-selective neurons is higher in the ventral than in the PMd [Chi-square(3) = 17.4, P < 0.001, followed by the Marascuilo procedure].

The grip selectivity of each neuron in each task was further quantified by means of a PI, which measures the extent to which the activity for the non-preferred grip type differs from the activity for the preferred one (Fig. 6A). Grip selectivity was similar in the dorsal and ventral premotor cortex during observation (K–S, P > 0.10). In contrast, the neurons in the ventral premotor cortex were more selective than the dorsal premotor neurons during execution (K–S, P < 0.005). This latter finding is explained by the higher proportion of selective neurons in the ventral premotor cortex during execution.

The next step was to assess when selectivity between grip types begins. The onset of grip selectivity was defined as the moment at which the first of at least 12 consecutive sliding windows displayed a statistically significant difference between grips (slide step = 5 ms, one-way ANOVA, P < 0.01). We found that the vast majority of neurons start to be selective after the onset of the observed movement (Fig. 6B). Comparison across areas revealed that the grip selectivity begins earlier in PMd, both in observation and execution (K–S, P < 0.001 for both comparisons).

To compare the execution and observation discharge of MirNs in both areas, we initially applied a congruence criterion used by other studies (di Pellegrino et al. 1992; Gallese et al. 1996; Rizzolatti et al. 1996). According to this criterion, a neuron is congruent if its best response is to the same grip type across conditions (execution and observation). Twenty-six PMd–MirNs (18.6%) and 34 (27.9%) F5–MirNs fulfilled the above criterion, and thus could be considered congruent. However, comparison of the two proportions revealed that they were not statistically different (z-value = 1.6, P = 0.102). Noteworthy, the proportion of congruent F5–MirNs found in the present study is compatible with the proportion of strictly congruent neurons reported in Galles’s study (29/92, 31.5%; z-value = 0.419, P = 0.675).

At this point, it would be legitimate to ask whether the proportions of congruent MirNs found in PMd and F5 are different from chance. If the probability for a grip to evoke the maximum neuronal discharge in either condition was equal across conditions, then the probability of that response for any given grip would be 25%. Based on the detected frequencies of the preferred grips (Table 1), the estimated probability for a neuron to be congruent was 24.8% and 27.1% in PMd and F5, respectively. Therefore, the proportions of congruent neurons found in PMd and F5 populations were not different from chance (PMd: z-value = 1.117, P = 0.264; F5: z-value = 0, P = 1.0).

The above criterion only takes the maximum response for each condition into consideration, whereas the rest of the responses are neglected. To investigate the matching between execution and observation discharge (while taking into consideration all tested grips), we used the representational similarity analysis (Kriegeskorte et al. 2008). In this procedure, the activity of each MirN during execution and observation is used to construct RDMs for each condition, containing pairwise Euclidean distances between all grip pairs. Then, the similarity between the execution and observation RDM pair of each neuron was
assessed using a Pearson correlation, and statistically verified with the Mantel test (Mantel 1967). Two correlated RDMs indicate a structural similarity between internal representations of different origin (Shepard and Chipman 1970). Following this procedure, only 6 PMd- and 11 F5-MirNs displayed statistically significant correlations between the observation and execution RDMs, and thus could be classified as congruent. To evaluate whether these results are different from chance, we used a permutation procedure; the sets of the observation and the execution responses were randomly paired to create artificial MirNs. These artificial MirNs were used to construct RDMs and perform RSAs. We found that a chance number of neurons displaying statistically significant RDMs’ correlations (PMd: $P = 0.74$, F5: $P = 0.10$).

In an attempt to obtain a quantitative index of the congruency, we used the Procrustes distance between execution and observation activity. Statistical comparison of Procrustes distance distributions did not reveal any significant difference of the execution/observation matching between the two areas (K-S, $P > 0.10$; Fig. 7). According to the Procrustes distance definition, the lower its value the higher the matching in the two conditions. Instead of using a single Procrustes distance threshold to classify neurons as congruent, we assessed the number of congruent neurons for a variety of thresholds, uniformly distributed between 0.1 and 0.9. For each of these thresholds, we performed the permutation procedure described above, and the resulted populations of MirNs were used to calculate Procrustes distances and define congruent neurons. This process revealed that the number of congruent units was always at chance levels, whatever the Procrustes distance threshold was (Supplementary Table 1). Notably, the 26 PMd- and the 34 F5-MirNs that were classified as congruent (according to the best response criterion) were distributed over the entire span of the Procrustes distance values.

All methods used to evaluate the matching between execution and observation discharge of MirNs revealed that the single neuron congruency, both in PMd and F5, was at chance levels. To assess whether the neuronal ensemble, instead of the individual neurons, provides an observation–execution matching, we employed a representational similarity. This computation is independent of the matching in single neurons, and revealed that the aggregate representations in execution and observation were similar (i.e. there was congruency at the population level). Specifically, we found that in both dorsal and ventral premotor cortex, the high-activity epoch RMDs of MirNs during execution and observation were significantly correlated (PMd: $r = 0.944$, $P = 0.02$, Fig. 8A–C; F5: $r = 0.915$, $P = 0.03$; Fig. 8D–F).

The next step was to explore the number of neurons required to obtain the population congruency (Supplementary Fig. S4). With a segmented regression analysis, we estimated the breakpoint between the size-dependent and size-independent...
segments at 55 and 40 neurons for PMd and F5, respectively. The introduction of the breakpoint provided a better fit to the data than the single linear model [PMd: \( F(2,22) = 19.119, P < 0.01; \) F5: \( F(2,22) = 38.968, P < 0.01 \)].

To explore whether the population congruency is influenced by the degree of execution/observation matching of single neurons, we ranked the neurons in each population according to their Procrustes distance. Then, we calculated the correlation coefficients between the execution and observation RDMs of populations consisting initially of 55 PMd (40 F5) neurons with either the highest or lowest Procrustes distances. These initial populations were gradually augmented by adding five neurons at each successive step, up to the full size of each population. Our prediction was that when starting with a population of neurons with high-Procrustes distances, the correlation coefficient of the RDMs will be low, and will be improved by adding neurons with progressively lower Procrustes distances. On the contrary, when starting with a population of neurons with low Procrustes distances, we predicted that the correlation coefficient of the RDMs will be high and will not be influenced by the inclusion of neurons with higher Procrustes distances. The results of this analysis, presented in Fig. 9, confirmed this prediction.

An advantage of the representational similarity analysis at the population level is that it can be used to compare representations of different origin. As it has been used to examine the execution/observation relationship within each cortical area, it can be also used to evaluate the inter-areal matching of representations under the same experimental conditions. We therefore used RSA to examine the relationship of the neural representations in the dorsal and ventral premotor cortex in each condition. We found that the high-activity epoch RMDs of PMd- and F5-MirNs were significantly correlated both during observation \( r = 0.970, P = 0.026, \) Fig. 8G) and execution \( r = 0.767, P = 0.047, \) Fig. 8H).

To investigate whether the observation of intransitive actions is effective in triggering the discharge of PMd-MirNs, a subset of neurons \( (n = 61) \) were studied during the observation of both transitive and intransitive actions. All tested MirNs responded to the observation of both transitive and intransitive actions. The net response of all recorded MirNs to the observation of the preferred and the non-preferred transitive actions, as well as of the intransitive action, is shown in Supplementary Figure S5. The intensity of the response to the observation of the intransitive action was, for the majority of the cases, lower than or equal to that evoked by the observation of the preferred transitive action (Supplementary Fig. S6). Moreover, the observation of the intransitive action was usually more effective than the observation of the non-preferred transitive one (Supplementary Fig. S6). This is evident both at single neuron (Supplementary Fig. S5) and population levels (Supplementary Fig. S7).

**Gaze-Related Modulation of Activity in Dorsal and Ventral Premotor Cortex**

As is the case in the majority of electrophysiological studies of MirNs (Gallese et al. 1996; Umiltà et al. 2001; Caggiano et al. 2009, 2012; Kraskov et al. 2009), the oculomotor behavior of the monkeys in the observation condition was unconstrained. Animals’ eye movements were recorded during the observation task only to ensure that the monkeys observed the presented action without any intention to pose restrictions on animals’ oculomotor behavior. It is therefore plausible that gaze during observation may have influenced the differential discharge of MirNs. To investigate the effect of orbital eye position on the neuronal activity, we performed planar regression analysis (Boussaoud et al. 1998; Cisek and Kalaska 2002) in which the neuronal activity during fixation periods was expressed as a function of the horizontal and vertical components of gaze direction. The strength of the modulation was assessed by the coefficient of determination \( r^2 \) of the regression. The results of this analysis are summarized in Supplementary Table 2. It is evident that more than half of the neurons in each area and condition do not display any statistically significant modulation of the activity by the gaze. Furthermore, for 75% of the remaining cells, the statistically significant gaze effect accounted for <15% of the observed response variance. Thus, the gaze-related modulation in the present dataset is weak, and is unlikely to account for the selective activity reported during both execution and observation. Analogous results were obtained also by Cisek and Kalaska (2004) who investigated the strength of gaze-related modulation of PMd neurons similar to ours that discharged both when a monkey executed a conditioned task, and when the monkey observed visual stimuli associated with the performance of the same task either replayed or executed by the experimenter.

Recent studies reported that about 50% of the F5-MirNs were gaze-dependent (their discharge was stronger when the monkey observed the action than when it did not observe it). In addition, the response of these gaze-dependent MirNs was more intense and started earlier when the fixation onset occurred before than after hand-target contact (Maranesi et al. 2013). Thus, to verify the animals’ engagement in the task, an eye position window of 9.5° in diameter, centered at the object, was used to count the trials at which monkey’s gaze was within this window before the contact of the hand with the object. We found that this was the case in the vast majority of the trials (98.4% for PMd and 96.2% for F5). This high-
percentage indicates that our monkeys reliably gazed at the reference window before the end of the observed movement and guarantees that action observation evoked the optimum response of the gaze-dependent MirNs.

Discussion

In this study, we demonstrate that area PMd contains MirNs that respond during the observation of biological actions. PMd–MirNs are similar to F5-MirNs in terms of selectivity, amplitude and matching of the responses between execution and observation. Moreover, the encoding of grip types in PMd and F5 was alike, during both observation and execution. Therefore, our study provides direct evidence suggesting the inclusion of PMd in the MirNs circuit.

Methodological Considerations

The viewpoint from which an action is seen and the location at which an action takes place, relative to the observer, influences the discharge of F5-MirNs (Caggiano et al. 2009). More recently, it has been demonstrated that these two factors are combined in the observation-elicited discharge of F5-MirNs (Maranesi et al. 2017). To exclude the contribution of these factors in the differential discharge of MirNs, we kept them constant throughout the experiment; the experimenter performed the action at the monkey’s extrapersonal space, and the monkey observed it from a 45° perspective. The extrapersonal space was chosen because it allowed the unimpeded unfolding of the action by the experimenter and discouraged the monkeys to plan/execute any movements towards any objects, since they were located well beyond their reaching distance. The fact that in the majority of the electrode tracks, in both cortical areas, MirNs and non-MirNs were encountered along the same penetrations and sometimes recorded simultaneously, allowed us to verify the animals’ behavior throughout the course of the experiment. In accordance with previous studies employing similar behavioral paradigms, the animals performed no movements during the observation task (Gallese et al. 1996; Cisek and Kalaska 2004; Raos, Evangeliou et al. 2004; Kraskov et al. 2009; Bonini et al. 2014; Maranesi et al. 2015).

When the monkey was executing movements, the concurrent view of the object and the instructional cue evoked activity...
Figure 9. Effect of single neuron matching on population congruency. Correlation coefficients of the population congruency obtained by ensembles consisted of neurons displaying different degrees of observation/execution matching as expressed by the Procrustes distance. (A, B) PMd, (C, D) F5. The initial populations consisted of neurons with higher (A, C) or lower (B, D) Procrustes distances. Circles denote the correlation coefficients of the population congruency, solid circles mark the statistically significant correlations (Mantel, \( P < 0.05 \)). Squares symbolize the median Procrustes distance of the neurons comprising the population in each case.

during the object fixation epoch in a considerable proportion of MirNs in both dorsal and ventral premotor cortex. This activity resembles the object elicited responses displayed by non-MirNs both in PMd (Fogassi et al. 1999; Raos, Umilta et al. 2004) and F5 (Murata et al. 1997; Raos et al. 2006). In contrast, when the monkey was observing, the view of the object per se was not sufficient to trigger a response. This finding is in agreement with the original studies reporting that MirNs do not respond to the presentation of objects (Gallese et al. 1996; Rizzolatti et al. 1996) and in disagreement with a recent article describing the existence of F5-MirNs that are activated by both action observation and object fixation (Bonini et al. 2014). We speculate that the way the object is presented (with or without the cue) and/or the space sector at which the presentation occurs (peripersonal or extrapersonal) may constitute factors that modulate the neuronal discharge during the object-fixation epoch.

MirNs in PMd

The existence of PMd–MirNs documented in our study is in agreement with the neurophysiological studies reporting that PMd neurons discharge both during performance of a familiar task and subsequent observation of the task related events (Cisek and Kalaska 2004; Tkach et al. 2007). Although these neurons displayed activity that resembled that of MirNs, they were not considered MirNs because the monkeys were not observing movements executed with natural effectors (such as the hand of a conspecific or human); instead, they were observing the cursor, operating as a surrogate for hand position. Their activity has been suggested to reflect mental rehearsal of a learned motor action acquired through learned stimulus–response associations rather than processes related to action recognition (Cisek and Kalaska 2004). The latter function is supposedly subserved by MirNs (Gallese et al. 1996; Rizzolatti et al. 1996). Our study demonstrates that PMd contains neurons that fulfill the “mirror” neuron criterion as defined in the original studies: they fire both when the animal performs goal-directed actions, and observes another agent performing similar actions with natural effectors (di Pellegrino et al. 1992; Gallesse et al. 1996; Rizzolatti et al. 1996). The fact that no cues were visible by the monkey during action observation in our study renders any influence of visual instructional signals on the timing and the intensity of the action observation-related activity implausible. Our findings directly support neuroimaging studies both in monkeys (Raos et al. 2007) and humans (Molenberghs et al. 2012), as well as neuroanatomical (Bruni et al. 2018) and opinion (Bonini 2017) articles that have proposed the inclusion of PMd in the MirN circuit.

An early study that investigated the visual responses in PMd reported the existence of visual neurons that were activated by stimuli moving or rotating along the tangential plane in the monkey’s extrapersonal space (Fogassi et al. 1999). Although it was not reported whether action observation had been tested as a visual stimulus, we hypothesize that at least some of the reported extrapersonal visual neurons could actually represent MirNs neurons. Back then, it was believed that MirNs respond only to object-oriented actions (Gallese et al. 1996). Thus, the extrapersonal neurons would be excluded from any further investigation for mirror properties due to their activation by the observation of intransitive movements. However, our recent finding that MirNs respond also to the observation of intransitive actions (Papadourakis and Raos 2017) and current study support the above hypothesis. At this point, we would like to emphasize that the activation of MirNs by the
observation of intransitive actions, considered along with their differential discharge evoked by the observation of object-directed actions, rules out any possibility that MirNs firing is elicited by moving stimuli in an unspecific manner.

Possible Sources of Action-Related Information During Observation

The presence of dorsal premotor neurons that are influenced by the mere observation of a motor act poses the question of action-related input areas to PMd. The cortical areas containing MirNs, i.e. the ventral premotor area F5 (di Pellegrino et al. 1992; Gallese et al. 1996; Bonini et al. 2014) and areas PF/PFG and AIP in the rostral half of the inferior parietal lobule (Fogassi et al. 2005; Rozzi et al. 2008), are the first to be considered. Area F5 is heavily and reciprocally connected with the lateral part of F2 (Marconi et al. 2001; Bruni et al. 2018); thus, the exchange of action-related visual information between these two premotor areas is possible. On the other hand, the possibility that the parietal cortical areas transmit this kind of information to PMd cannot be excluded, although the parieto-frontal projection is weak (Rozzi et al. 2006). Moreover, the medial rostral cortex, which contains the partner-type neurons that selectively encode the action of others (Yoshida et al. 2011), may convey higher-order agent-related information to the dorsal and ventral premotor cortex (Matelli et al. 1986; Luppino et al. 2003; Gerbella et al. 2011).

The PMd–MirNs neurons of the present study reside in a part of the PMd cortex that presumably corresponds to the ventral part of area F7 and the ventrostral part of area F2 (F2v). This part of PMd receives input from MST (Luppino et al. 2001), an area that is critical for the processing of visual motion (Komatsu and Wurtz 1988); MST is also the target of projections from the adjacent motion-sensitive visual area MT/V5 (Maunsell and Van Essen 1983; Ungerleider and Desimone 1986). Consequently, areas of the superior temporal lobe could provide motion-related information directly to the PMd. Supporting evidence has shown that observation of others’ actions activates components of the motion complex in the STS, including MST, FST and MT (Klintari et al. 2014).

Another possible hypothesis is that visual information is provided to the PMd by superior parietal areas endowed with visual properties. Parietal visuolateral input to F2v is provided by areas V6A and MIP of the superior parietal lobe, whereas ventral and caudal parts of F7 are targets of area PGm/31 in the medial wall of the hemisphere and the ventral part of V6A (Petrides and Pandya 1984; Cavada and Goldman-Rakic 1989; Johnson et al. 1996; Matelli et al. 1998; Shipp et al. 1998; Marconi et al. 2001; Camerini et al. 2009; Passarelli et al. 2018). Motion-related visual information is conveyed to these posterior parietal areas from extrastriate areas of the occipital lobe, including V6, and from the visual areas of the superior temporal sulcus (Galletti et al. 2001; Camerini et al. 2009; Passarelli et al. 2011). It should be noted that posterior parietal areas V6A, MIP and PGm, proposed to be nodes of the circuit transmitting visual information to the PMd, were activated by the observation of others’ transitive and/or intransitive actions (Evangelio et al. 2009; Raos et al. 2014).

Relationship Between Observation and Execution Responses of MirNs

One of the main properties of MirNs that is usually neglected is the congruence between the observed action that triggers their discharge and the motor response. Although the term “mirror” implies that there is a close match between the execution and the observation domains, this is not the case. We found that the frequency of strictly congruent MirNs (those responding to identical observed and executed actions) is not different from chance. This weak one-to-one correspondence between observed and executed actions displayed by MirNs has been considered incompatible with the direct matching hypothesis: i.e. the mechanism proposed for the transformation of the sight of an action into a corresponding motor representation (Csibra 2008; Jacob 2009). Our finding, that representations of action in MirN populations were significantly correlated during execution and observation, suggests that matching may be achieved in a distributed fashion across selective elements of the neuronal ensemble. Thus, although the mirror property is not a feature of each MirN individually, it is a characteristic of the MirN population as a whole. It should be noted that the higher the congruency displayed by the neurons of an ensemble, the greater the matching at the population level (provided that the group consisted of at least 55 PMd/40 F5 neurons). Our findings are in agreement with those of a recent study, in which it has been demonstrated that although individual MirNs in the premotor cortex displayed different degrees of execution/observation similarity in the timing and amplitude of their activity, the population tended to display similar hidden neural states sequences and trajectories during execution and observation (Mazurek et al. 2018).

In conclusion, our study provides strong direct evidence of MirNs activity in the PMd during the observation of biological actions. The MirNs activity in PMd was quantitatively compared with that in F5 at single cell and at population level, and found to possess similar properties. Future studies will reveal the distinct role of each of the two premotor areas in the MirNs circuit, as well as their dynamic interaction.

Supplementary Material

Supplementary material is available at Cerebral Cortex online.

Notes

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