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Resting-State Connectivity Identifies Distinct Functional Networks in Macaque Cingulate Cortex

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Subregions of the cingulate cortex represent prominent intersections in the structural networks of the primate brain. The relevance of the cingulate to the structure and dynamics of large-scale networks ultimately requires a link to functional connectivity. Here, we map fine-grained functional connectivity across the complete extent of the macaque (Macaca fascicularis) cingulate cortex and delineate subdivisions pertaining to distinct identifiable networks. In particular, we identified 4 primary networks representing the functional spectrum of the cingulate: somatomotor, attention-orienting, executive, and limbic. The cingulate nodes of these networks originated from separable subfields along the rostral-to-caudal axis and were characterized by positive and negative correlations of spontaneous blood oxygen level-dependent activity. These findings represent a critical component for understanding how the anterior and midcingulate cortices integrate and shape information processing during task performance. The connectivity patterns also suggest future electrophysiological targets that may reveal new functional representations including those involved in conflict monitoring.

Keywords: anterior cingulate cortex, cingulate cortex, functional connectivity, primate, resting-state fMRI

Introduction

The cingulate cortex is a heterogeneous structure along the medial walls of both hemispheres in all mammalian species that have been studied. In human and nonhuman brains, the structure is implicated in diverse higher-order functions ranging from top-down attentional control and conflict monitoring to self-initiated action planning and contextual memory retrieval (Lee et al. 2007; Rushworth et al. 2007; Dosenbach et al. 2008; Beckmann et al. 2009). Studies have suggested a prominent rostral/caudal distinction in anterior cingulate cortex (ACC) organization of primates. The rostral ACC (area 24c) has been shown to have strong connections with dorsolateral prefrontal cortex and is particularly implicated in complex cognitive processes. The caudal ACC (areas 23c and 6c) is connected with primary and supplementary motor areas and is activated in simple sensorimotor tasks (Picard and Strick 1996). In addition, further affective and cognitive divisions of the cingulate have been suggested (Bush et al. 2000). These studies demonstrate that the subcallosal ACC (area 32 and area 25) is both structurally and functionally connected with limbic areas (Ongur and Price 2000) and involved in limbic processes, and the supracallosal is ACC tightly coupled to the middle frontal gyrus and implicated in multiple cognitive processes (Peyron et al. 2000). In contrast, Etkin et al. (2011) recently proposed that the dorsal-caudal ACC is involved in the appraisal and expression of negative emotions, whereas the ventral-rostral ACC plays a role in generating emotional responses.

Despite overall similarities, differences in ACC function have emerged between human and monkey studies (Cole et al. 2009; Mansouri et al. 2009). Human functional magnetic resonance imaging (fMRI) (Botvinick et al. 2001; Kerns et al. 2004) and event-related potential (Yeung et al. 2004) recordings suggest a major role of the ACC in conflict monitoring. In contrast, monkey single unit (Ito et al. 2003; Nakamura et al. 2005), local field potential (Emeric et al. 2008), and lesion studies (Kennerley et al. 2006; Buckley et al. 2009) report signals arising from task switches and enhanced attentional demands (Johnston et al. 2007; Womelsdorf et al. 2010). The differences in functional correlates of neuronal signal illustrate an inherent difficulty with traditional approaches applied in both species. Therefore, approaches applicable to both species that compare the functional connectivity of brain regions independent of task measures are necessary.

Resting-state fMRI (RS-fMRI) examines functional connectivity through correlations of slow blood oxygen level-dependent (BOLD) oscillations originating from active neurovascular events, which are best accounted for by synchronized neuronal activity (Biswal et al. 1995, 1997; Friston et al. 2003; Fox et al. 2006; Fox and Raichle 2007). RS-fMRI can highlight sets of distant brain areas forming integrated networks of information processing (Smith et al. 2009; Honey et al. 2007) and accordingly, has been shown to provide reliable connectivity maps in humans (Beckmann et al. 2005; Damoiseaux et al. 2006) and nonhuman primates (Vincent et al. 2007; Margulies et al. 2009; Hutchison et al. 2011). Recently, novel aspects of human ACC functional connectivity were revealed using a seed-based region of interest (ROI) analysis (Margulies et al. 2007). In addition to the well-described rostral/caudal distinction, Margulies et al. (2007) identified transition zones, which may allow for the integration of affective and sensorimotor processing needed for conflict monitoring. Here, we performed systematic seed-based RS-fMRI followed by cluster analysis of the functional connectivity patterns across the monkey cingulate cortex. We explore the functional connection schemes of cingulate cortical subfields and provide a baseline for further discrimination of their precise involvement in divergent higher cognitive functions.

Materials and Methods

Animal Preparation

All experimental procedures were carried out in accordance with the Canadian Council of Animal Care policy on the use of laboratory animals and approved by the Animal Use Subcommittee of the
University of Western Ontario Council on Animal Care. Data was obtained from 6 macaque monkeys (Macaca fascicularis, 4 females). The weight ranged from 3.6 to 5.3 kg (mean ± standard deviation = 4.58 ± 1.4 kg). The data has been previously published (Hutchison et al. 2011). Prior to the imaging experiments, an MRI-compatible custom-built acrylic head post was anchored to the skull with 6-mm ceramic bone screws (Thomas Recording, Giessen, Germany) and dental acrylic that served to restrain the head during image acquisition.

In preparation for image acquisition, each monkey was injected with intramuscular injections of atropine (0.4 mg/kg), iotropium (0.025 mg/kg), and ketamine hydrochloride (7.5 mg/kg), followed by intravenous administration of 3 ml propofol (10 mg/ml) via the saphenous vein. Anesthesia was then maintained using 1.5% isoflurane mixed with oxygen following oral intubation with an endotracheal tube. Animals were spontaneously ventilating throughout the duration of scanning. The monkey was then placed in a custom-built monkey chair with its head immobilized using the head post and inserted into the magnet bore, at which time the isoflurane level was lowered to 1%. Physiological parameters were continuously monitored throughout the duration of scanning (rectal temperature via a fiber-optic temperature probe [FISO, Quebec City, QC] = 36.5 °C; respiratory via bellows [Siemens Corp., Union, NJ] = 25-30 breaths/min; end-tidal CO$_2$ via capnometer [Covidien-Nellcor, Boulder, CO] = 24-28 mm Hg). Animal body temperature was maintained using a heating disk (Snugglesafe, Littlehampton, West Sussex, UK) and thermal insulation. Anesthesia eliminates motion, physiological stress, and training requirements of the animals. Isoflurane has been shown to have vasodilator properties (Farber et al. 1997) that can potentially affect cerebrovascular activity. Therefore, coherent resting-state fluctuations and network connectivity have been robustly reported in previous studies using an isoflurane regime in monkeys (Vincent et al. 2007; Margulies et al. 2009; Hutchison et al. 2011).

**Data Acquisition**

Data was acquired on an actively shielded 7-T 68-cm horizontal bore scanner with a DirectDrive console (Varian, Yarnton, UK; Walnut Creek, CA) and a Siemens AGHI gradient subsystem (Erlangen, Germany) operating at a slew rate of 350 mT/m/s. An in-house designed and manufactured conformal 5-channel transceive primate head RF coil was used for all experiments. Magnetic field optimization ($B_0$ shimming) was performed using an automated 3D mapping procedure over the specific imaging volume of interest. For each monkey, 2 runs of 300 continuous echo-planar imaging (EPI) functional volumes (time repetition [TR] = 2000 ms; time echo [TE] = 16 ms; flip angle = 70°, slices = 30, matrix = 72 x 72; field of view [FOV] = 96 x 96 mm; acquisition voxel size = 1.5 x 1.5 x 1.5 mm) were acquired. Acquisition time of each scan was 10 min. EPI images were acquired with GRAPPA at an acceleration factor of 2. Every image was corrected for physiological fluctuations using navigator echo correction. A high-resolution $T_2$-weighted anatomical reference volume was acquired along the same orientation as the functional images using a fast spin echo acquisition scheme (TR = 5000 ms; TE = 38.6 ms; echo train length = 5, effective echo = 3, slices = 30, matrix = 256 x 256; FOV = 96 x 96 mm; acquisition voxel size = 0.375 mm x 0.375 mm x 1.5 mm). A representative $T_2^*$ functional image and $T_2$-weighted anatomical image is displayed in Supplementary Figure 1.

**Image Preprocessing**

Functional image preprocessing was implemented in the FMRIB Software Library toolbox (FSL; http://www.fmrib.ox.ac.uk). This consisted of motion correction (6-parameter affine transformation), brain extraction, spatial smoothing (Gaussian kernel of full-width at half-maximum of 8 mm), normalization (12 DOF linear affine transformation) to the F99 atlas template (van Essen 2004; see http://sumsdb.wustl.edu/sums/macaquemore.do). The F99 atlas, while designed and manufactured conformal 5-channel transceive primate scanner with a DirectDrive console (Varian, Yarnton, UK; Walnut Creek, CA) and a Siemens AGHI gradient subsystem (Erlangen, Germany) operating at a slew rate of 350 mT/m/s. An in-house designed and manufactured conformal 5-channel transceive primate head RF coil was used for all experiments. Magnetic field optimization ($B_0$ shimming) was performed using an automated 3D mapping procedure over the specific imaging volume of interest. For each monkey, 2 runs of 300 continuous echo-planar imaging (EPI) functional volumes (time repetition [TR] = 2000 ms; time echo [TE] = 16 ms; flip angle = 70°, slices = 30, matrix = 72 x 72; field of view [FOV] = 96 x 96 mm; acquisition voxel size = 1.5 x 1.5 x 1.5 mm) were acquired. Acquisition time of each scan was 10 min. EPI images were acquired with GRAPPA at an acceleration factor of 2. Every image was corrected for physiological fluctuations using navigator echo correction. A high-resolution $T_2$-weighted anatomical reference volume was acquired along the same orientation as the functional images using a fast spin echo acquisition scheme (TR = 5000 ms; TE = 38.6 ms; echo train length = 5, effective echo = 3, slices = 30, matrix = 256 x 256; FOV = 96 x 96 mm; acquisition voxel size = 0.375 mm x 0.375 mm x 1.5 mm). A representative $T_2^*$ functional image and $T_2$-weighted anatomical image is displayed in Supplementary Figure 1.

**Seed Selection**

A total of 40 spherical seed ROIs (radius = 1.5 mm, volume = 14.14 mm$^3$) were created in the F99 atlas space (van Essen 2004; Fig. 1 and Table 1). Using the cingulate sulcus as a reference, 2 parallel curves were created within the cingulate cortex along the midline ($x = 0$ mm), one 3.52 mm dorsal to the sulcus and the other 3.02 mm ventral to the sulcus. Eight equidistant (−5 mm distance) seeds were then placed along the dorsal (s1-s8) and ventral (i1-i8) lines to encompass the precentral and superior frontal gyri on the medial wall (s1-s8) and the cingulate gyri (i1-i8). Two additional seeds were placed in the subgenual cortical areas (i9 and i10). Eight seeds were also placed laterally ($x = 3$ to +4 mm) in each hemisphere, tracing the cingulate sulcus (m1-m8 and r1-r8, respectively). In addition, seeds (radius = 1.5 mm) were placed bilaterally in the in the prefrontal cortex, lateral intraparietal cortex, primary visual cortex, caudate, putamen, hippocampus, and superior colliculus to examine correlation values outside the cingulate cortex as well as the anterior bank of the arcuate sulcus and in the central sulcus (see Supplementary Fig. 3) for comparison with a previous resting-state study in monkeys (Vincent et al. 2007).

**Statistical Analysis**

The mean time course for each seed region was extracted for each animal and each scanning session. Statistical analysis was then carried out in 3 levels. First, mean time courses for each seed region were used as predictors in a regression model for multiple regression at the individual subject level for each scanning session. The model also included nuisance covariates for white matter, cerebrospinal fluid, and 6 motion parameters. The results of the individual subject regression analysis are individual maps of both positively and negatively predicted voxels for each regressor. Functional connectivity maps across the 2 scans for each monkey were calculated using a second-level fixed-effects analysis. A third-level group-level analysis was then carried out using a fixed-effects model producing z-score maps of connectivity associated with each cingulate seed ROI. We chose fixed-effects analyses to share the same genus as the monkeys used in the present study (Macaca) is not of the same species (mulatta). Stereotaxic atlases are often used interchangeably between these 2 macaque species, and we believe the difference did not significantly affect normalization (see Supplementary Fig. 1). Global mean signal regression, a preprocessing method to correct for the global variability of the BOLD signal, was not implemented. It has been shown that this type of preprocessing can cause a bias toward finding anticorrelations between time series (Murphy et al. 2009) and may be coupled to the underlying neural activity (Schölvinck et al. 2010).
analyses instead of random-effects analyses as employed in a similar human study (Margulies et al. 2007) because of the relatively low number of subjects (6 monkeys vs. 24 subjects in the human study). A random-effects analysis has as many degrees of freedom as number of subjects included in the study. A larger sample size increases the study’s power because the standard error of the mean decreases by the square root of the number of subjects. The result of simulated task-based human data has shown that with percent signal changes of approximately 0.5% and spatial smoothing at a FWHM of 5 mm, a minimum of 12 subjects are needed to ensure 80% power at $z = 0.05$ at the single voxel level. Furthermore, a more conservative alpha required twice as many subjects to maintain this level of power (Desmond and Glover 2007). A fixed-effects analysis, however, treats observations (time points) as the unit of analysis and as such, has greater degrees of freedom, though it limits inferences to the sample instead of the population. Corrections for multiple comparisons were implemented at this step in both human and animal fMRI experiments including previous resting-state investigations of the macaque (Vincent et al. 2007; Margulies et al. 2009) that, like the current study, used a smoothing kernel of 5 mm FWHM. By definition, smoothing will cause blurring, often into white matter tracts. This was observed in our data set in addition to minor intersubject anatomical variability that was not compensated for by spatial normalization. To illustrate the potential effects of spatial smoothing, the entire analysis procedure was repeated using the same preprocessing and analysis methodology with the exception of smoothing.

### Cluster Analysis

Unweighted average-linkage hierarchical cluster analysis was applied to identify the functional subdivisions of the cingulate cortex. The algorithm utilizes a pairwise distance matrix to form a hierarchy of clusters, progressively merging clusters from the individual data elements based on the mean distance between elements. To first construct the distance metric for the cingulate cortex, we calculated the correlations among the 54 cingulate seed ROIs. The extracted time course for each seed region was correlated with the time course of all other seeds to obtain a correlation matrix. Each element of the matrix was averaged across both scans and then across the 6 monkeys. The Euclidean distance between each pair of group-averaged correlations was then computed to assess the similarity among the cingulate seed ROIs. A hierarchical cluster tree, referred to as a dendrogram, was then derived by applying the linkage criterion to the Euclidean distance matrix (Michener and Sokal 1957). This method does not require the number of clusters to be specified, however, following the analysis, a distance threshold must be selected and each linked component connected below that threshold forms a cluster. In the present study, a threshold value of 1.5 was used as this best captured the hierarchical grouping shown in the dendrogram. It is important to note that lower thresholds could have been chosen, resulting in greater partitioning of the networks. In addition, the average-link analysis was repeated using correlation matrices of both scans individually (averaged across all monkeys) to determine the interscan reproducibility of the cluster partitioning.

To verify the reliability of the group data partitioning, $k$-means clustering was also employed. The algorithm groups the observations into an prior number of clusters ($k$ were selected based on the average-link cluster results) in which data elements belong to the cluster with the nearest mean. The initial means are randomly selected and the elements assigned to one of the clusters based upon the minimum distance. An iterative process occurs in which the centroid of the new cluster becomes the new mean and the cluster membership recalculated. The centroid is recalculated every time an element is added to the cluster and this continues until all the components are grouped into the cluster with the closest center, minimizing the objective squared error function (Hartigan and Wong 1979). The final cluster membership can be sensitive to the initial, randomly selected cluster mean locations, as local optimums can result in the case by which moving any single point to a different cluster increases the total sum of distances. To account for this, the algorithm was repeated 3000 times with random initial mean locations to ensure the optimal cluster membership.

### Cluster Networks

A four-level cluster network-level analysis was computed across group-level seed-based networks encompassed by each cluster identified with the average-link clustering algorithm (fixed effect, $z > 2.3$; cluster significance: $P < 0.05$, corrected). The analysis allowed the calculation of shared functional connectivity across each of the cluster’s subnetworks. In other words, functionally connected brain areas that are common across the individual seed networks for all of the identified seeds in a given cluster will be revealed. The same analysis was also applied to individual monkey’s scan averaged data to identify the consistency across the individual animals using the cluster membership identified with the group-averaged hierarchical clustering.

### Unsmoothed Data

Spatially smoothing greatly improves the signal-to-noise ratio of data while minimizing the effects of functional anatomical variability at the cost of reducing spatial precision. Smoothing is a standard preprocessing step in both human and animal fMRI experiments including previous resting-state investigations of the macaque (Vincent et al. 2007; 2010; Margulies et al. 2009) that, like the current study, used a smoothing kernel of 5 mm FWHM. By definition, smoothing will cause blurring, often into white matter tracts. This was observed in our data set in addition to minor intersubject anatomical variability that was not compensated for by spatial normalization. To illustrate the potential effects of spatial smoothing, the entire analysis procedure was repeated using the same preprocessing and analysis methodology with the exception of smoothing.
Network Reproducibility
The correlation coefficient of connectivity maps was calculated to quantify intersubject and interscan reproducibility of the functional connectivity maps. For interscan comparison, a group-level analysis was carried out for each scan separately for all cingulate cortex seeds (i1-i10) (fixed effect, $z > 2.3$; cluster significance: $P < 0.05$, corrected). The thresholded whole-brain voxel $z$-scores of both scan functional maps were then correlated to produce a conservative similarity measure. Intermonkey comparison was implemented in a similar manner, although the pairwise correlations between all individuals were calculated for medial-lateral cingulate cortex seeds (m1-m8) as well as the individual monkey cluster networks.

Voxelwise Correlation Analysis
Calculation of $z$-scores using multiple regression analysis does not allow the evaluation of the correlation strength between significant brain areas. Therefore, correlation values of cluster networks were calculated using a similar 4-level analysis procedure to that described above. The mean time course of each seed region was correlated with every brain voxel at the individual subject level for each scanning session. The results were then averaged across sessions after a Fisher $z$-transform and then averaged across monkeys and finally across the seed ROIs within each cluster identified with the group-averaged hierarchical clustering before being converted back to correlational values.

Results

Resting-State Functional Connectivity
We performed seed-based resting-state fMRI analysis on data obtained from 6 anesthetized macaque monkeys in a 7-T scanner (see Materials and Methods). For initial comparison of our data set with a previous resting-state fMRI study in monkeys (Vincent et al. 2007), we placed spherical seed ROIs (radius = 1.5 mm) in the anterior bank of the arcuate sulcus, corresponding to the location of the frontal eye fields (FEFs) in macaques (Bruce et al. 1985) to show functionally correlated cortical networks (Fig. 2). In addition to positively correlated...
activity near the seed ROI, positively correlated activity was also found in the principal sulcus, middle frontal and superior frontal gyrus, intraparietal sulcus, parieto-occipital sulcus, lunate sulcus, supramarginal gyrus, and posterior bank of the superior temporal gyrus and middle temporal gyrus. On the medial wall (Fig 2A,D), positively correlated activity was found in the cingulate sulcus, posterior cingulate gyrus, pre-central and superior frontal gyrus, and in the frontal pole (Fig. 2B,D). Functional connectivity analysis also revealed strong anticorrelated activity. This included regions in the lateral sulcus, occipital gyrus, fusiform gyrus, and anterior cingulate gyrus. The BOLD time course of the FEF, intraparietal sulcus (ips), and lateral sulcus (ls) is also shown (Fig. 2E) as well as the strength of their temporal correlation (Fig. 2F). A very similar functional connectivity pattern was seen in the hemisphere contralateral to the seed and with a seed placed in the right hemisphere (Fig. 3A–B). The positively correlated network closely resembles previously described resting-state connectivity of the FEF in anaesthetized monkeys (Vincent et al. 2007) and saccade-related fMRI maps in monkeys (Koyama et al. 2004; Baker et al. 2005). Group-averaged maps displayed on the individual anatomical images are shown in Supplementary Figure 2. Seed ROIs placed in the central sulcus showed a very different connectivity pattern, consistent with the somatomotor system (Fig. 3C–D). This analysis demonstrates that cortical seeds with a 1.5-mm radius result in robust functional BOLD fMRI connectivity maps in anaesthetized macaques.

**Positively Correlated Cingulate Networks**

The functional connectivity of the complete cingulate cortex was studied using 34 seeds placed throughout the cingulate gyrus, cingulate sulcus, and dorsal convexity (see Materials and Methods). Figure 4 shows the positively and negatively correlated functional connectivity for the left hemisphere on flat maps. Black asterisks indicate the location of the seed regions. The maps show distinct positively and negatively correlated cortical regions that vary both along the rostral/caudal axis as well as the dorsal/ventral axis. Many of the caudal seeds in the cingulate gyrus (i1–i4), cingulate sulcus (ml1–ml4), and dorsal convexity (s1–s4) show positive correlations with somatomotor areas in and around the central sulcus and the parietal cortex and few positive correlations with prefrontal cortex. More rostral seeds have positive correlations with the arcuate sulcus (s5–s8, ml4–ml6) and the prefrontal cortex (s4, s6–s8, ml6–ml8). Positive correlations with the arcuate sulcus are also present for some of the seeds in cingulate gyrus (i1–i4, i6–i8). Subgenual seeds (i9 and i10) show strong positive correlations with anterior insular cortex. The right hemisphere shows similar connectivity patterns (see Supplementary Fig. 2).

**Negatively Correlated Cingulate Networks**

A very prominent feature of the cingulate connectivity maps is the significant anticorrelations among many cortical areas. For example, the subgenual seeds (i9 and i10) are anticorrelated with somatomotor, parietal, and superior temporal areas. There are also several examples where positive correlations shift to negative correlations between neighboring seeds. As an example, seed ml2 is negatively correlated, whereas its neighbor seed ml3 is positively correlated, with insular cortex (Fig. 4).

**Interscan and Intermonkey Reproducibility of Cingulate Networks**

Between-scan whole-brain functional maps showed good overall correspondence (Table 1, $P < 10^{-20}$, $P$ value corrected for multiple comparisons using Bonferroni correction). The group-averaged functional maps of inferior cingulate cortical seeds (i1–i10) for scan 1 (Fig. 5A) and scan 2 (Fig. 5B) are displayed on flattened cortical views of both hemispheres to allow qualitative comparisons of scans. Both positively and negatively correlated brain areas are reproducible across scans for all seeds, though there is some variability. Intermonkey comparison showed greater variability across monkeys than that observed across scans. The functional connectivity maps of each monkey (M1–M6) for left medial–lateral cingulate cortical seeds (ml1–ml8) are displayed on a flattened cortical view of the left hemisphere in Figure 6. The correlation matrices across monkeys for each seed ($P < 10^{-20}$, $P$ value corrected for multiple comparisons using Bonferroni correction) are displayed in the far right column.

**Cingulate Clusters and Networks**

The complete correlation matrix of all seed ROIs time courses is shown in Figure 7. Following average-linkage hierarchical

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Figure 3. Functional connectivity of FEFs and primary motor cortex (M1) in isoflurane-anesthetized macaques ($n = 6$). Flatten cortical views of both hemispheres are shown for a seed ROI placed in the left (A) and right (D) FEF and the left (C) and right (D) M1 cortical areas. Color overlays represent thresholded z-score maps ($\gamma > 2.3$; cluster significance: $P < 0.05$, corrected for multiple comparisons) normalized to the space of the F99 template (van Essen 2004). Black asterisks indicate the location of the seed ROI. Sulci labels are indicated in Figure 2.
cluster analysis of the cingulate portion of the matrix (outlined in black), a cluster separation at a Euclidean distance of 1.5 was found to distinguish 4 major clusters (Fig. 8). The 4 identified cingulate clusters show a clear spatial separation within the cingulate cortex (Fig. 8, inset). \(k\)-means clustering resulted in a near identical partitioning of the 4 networks although superior seed 7 (s7) was included in the red-colored cluster and the medial right hemisphere seed 4 (mr4) was included in the blue-colored cluster. Given the spatial proximity to the borders of the average-link derived cluster membership, the s7 and mr4 seeds may represent the locations of transition zones between the clusters. The correlation matrix and average-link partitioning were also reproducible across scans with few changes to cluster membership (Supplementary Fig. 5). The average-link partitioning scheme was used for all further analysis. The shared functional connectivity among the seeds of each cluster is shown overlaid on cortical representations for both hemispheres in Figure 9. The left and right hemisphere networks do not include the contralateral medial lateral seeds (m1-8) included in each cluster such that the right hemisphere does not display the connectivity of ml1-8 seeds included in the cluster but only the mr1-8 seeds and vice versa. Functional volume maps of these 4 clusters are shown in Figure 10. Based on their functional connectivity, we have labeled these networks tentatively “somatomotor,” “attention-orienting,” “executive,” and “limbic.”

**Somatomotor Cluster**
This seed cluster (i1–i2, m1–m4, s1–s5) has positive correlations with areas in the precentral and postcentral gyrus, central sulcus, intraparietal sulcus, and supramarginal gyrus. A weaker positive correlation is present within the arcuate sulcus. The cluster is also positively correlated with the posterior part of the caudate nucleus (Fig. 10 at \(-5\) and \(0\)). Anticorrelated areas are found in the anterior insular cortex, inferior occipital sulcus, and occipitotemporal sulcus. At the subcortical level, we found anticorrelated areas in the anterior caudate and putamen (Fig. 10 at \(+5\) and \(+10\)). This network resembles the somatomotor network (Fig. 3C–D) that was previously found with seeds placed in the macaque central sulcus (Vincent et al. 2007).

**Attention-Orienting Cluster**
Adjacent to the somatomotor cluster lies a seed cluster (i3, i4, m5, m6, s6, s7) that we tentatively named attention-orienting because of its positive connectivity with many areas implicated in directing spatial attention and the eyes to sensory stimuli appearing at unexpected locations, including FEF and the lateral intraparietal area (Corbetta et al. 2008). This cluster is also positively correlated with the dorsolateral prefrontal cortex (dorsal bank of principal sulcus), arcuate sulcus, posterior parietal areas including lateral intraparietal and inferior parietal cortex, areas in the insular cortex, superior temporal gyrus, and middle temporal gyrus. Functional connectivity between inferior parietal cortex and cingulate motor areas has been previously reported in macaques (Mars et al. 2011). In addition, this network includes the posterior cingulate cortex. We did not find any clear subcortical activity associated with this cluster. Similar to the somatomotor cluster, this cluster is negatively correlated with subregions in early visual cortex.

*Figure 4.* Functional connectivity of the left cingulate cortex in isoflurane-anesthetized macaques (\(N = 6\)). Cortical flat maps of the left hemisphere are overlaid with thresholded \(z\)-score maps for statistically significant voxels for each inferior (left column), medial–lateral (middle column), and superior (right column) seed indicated with a black asterisk (\(z > 2.3\); cluster significance: \(P < 0.05\), corrected for multiple comparisons). Bottom right corner: locations of 26 spherical seed ROIs (\(r = 1.5\) mm) displayed to scale on midsagittal and coronal slices of the F99 atlas (van Essen 2004). Parallel rows of seeds were placed along the superior (s1–s8), inferior (i1–i10), and medial–lateral (m1–m8) bank of the cingulate sulcus. The arrow shows the level of the genu of the arcuate sulcus on the lateral surface of the hemisphere. Main: Sulci labels are indicated in Figure 2.
Executive Cluster

The seeds forming this cluster are located rostral and ventral to the attention-orienting cluster (i5–i8, m7–m8, s8). The most prominent feature of this cluster is strong positive correlations with the lateral prefrontal cortex, especially its anterior regions. The cluster also has positive correlations with areas...

Figure 5. Interscan comparison of the functional connectivity in isoflurane-anesthetized macaques (N = 6) for inferior cingulate cortex seeds (i1–i10). Flattened cortical views of both hemispheres are shown for both scan 1 (A) and scan 2 (B). Color overlays represent thresholded z-score maps z > 2.3; cluster significance: P < 0.05, corrected for multiple comparisons) normalized to the space of the F99 template (van Essen 2004). Black asterisks indicate the location of the seed ROI. Correlation values between interscan connectivity maps are shown in Table 1. Sulci labels are indicated in Figure 2.
in the superior temporal sulcus and visual areas. It is anticorrelated with somatomotor areas in the central sulcus and areas in the intraparietal sulcus and with a large area in the insular cortex. At the subcortical level, this cluster is positively correlated with the anterior part of the caudate nucleus (Fig. 10 at +5 and +10), mediodorsal thalamus (Fig. 10 at –10), and superior colliculus (Fig. 10 at –15 and –20). This is particularly evident in the posterior parietal cortex that has been previously shown to be a component of the macaque limbic network (Margulies et al. 2009). Lesion studies have also implicated the frontal ACC gyrus areas in social valuation (Rudebeck et al. 2006).

Resting-state investigations of the macaque have not typically reported connectivity with the cerebellum, possibly attributable to poor signal quality resulting from the constraints of the head RF coil placement. No positively correlated functional connectivity was observed for any clusters with the cerebellum, however, anticorrelations were found between the somatomotor, attention-orienting, and executive cluster and the cerebellum. This is in agreement with human resting-state investigations that also only showed anticorrelated cerebellar areas for seeds placed in the cingulate (Margulies et al. 2007). No correlations were found between the limbic cluster and the cerebellum.

The spatial overlap of the thresholded cluster networks (z > 2.3) for positively (Fig. 12A,C) and negatively (Fig. 12C,D) functionally connected regions of both hemispheres are displayed on cortical flat maps. The spatial separation of networks is indicated by the lack of primary color overlap in the maps.

**Figure 6.** Intermonkey comparison of the functional connectivity in isoflurane-anesthetized macaques (N = 6) for left medial-lateral cingulate cortex seeds (m1–m8). A flattened cortical view of the left hemisphere is shown for each monkey (M1–M6). Color overlays represent thresholded z-score maps (z > 2.3; cluster significance: P < 0.05, corrected for multiple comparisons) normalized to the space of the F99 template (van Essen 2004). Black asterisks indicate the location of the seed ROI. The far right column displays the correlation matrix between the thresholded individual connectivity maps (P < 10–20). P value corrected for multiple comparisons using Bonferroni correction. Sulci labels are indicated in Figure 2.
Spatial Smoothing

The cluster network connectivity maps resulting from data that was smoothed (Fig. 11, row 1) and unsmoothed (Fig. 11, row 2) share the same positively and negatively connected brain regions, and other than the blurring effects caused by smoothing, had little to no effect on the identified networks.

Correlational Mapping

The strength of the temporal dependencies between the cingulate and the noncingulate cortical areas of the 4 cluster networks are illustrated in Figure 11 (row 3). The maps represent the average correlation maps across all seeds ROIs of each cluster with a lower bound thresholded of $r = 0.15$. The

Figure 7. Average pairwise correlation matrix of resting-state BOLD time courses of the isoflurane-anesthetized macaque ($N = 6$) from 34 cingulate seed ROIs (outlined with thick black border) and 18 additional cortical and subcortical areas. The upper triangular part of the graph displays the coefficient values according to the color bar on the right and the lower triangular part (mirror image) of the graph displays the corresponding absolute numerical correlation coefficient values.
results show strongly correlated activity in most areas identified by multiple regression analysis though the boundaries between cluster networks is less pronounced. Weak to moderate correlational overlap is most apparent between the somatomotor and the attention-orienting networks.

**Intermonkey Reproducibility of Cluster Networks**

Supplementary Figure 6 displays each cluster network for individual monkeys (M1–M6) derived using the group average-link clustering partitioning scheme (Fig. 8) on a flattened cortical view of the left hemisphere. The pairwise correlation matrices across monkeys for each cluster network ($P < 10^{-20}$, $P$ value corrected for multiple comparisons using Bonferroni correction) are displayed in bottom row of the figure.

**Discussion**

The primate cingulate cortex is known to be anatomically and functionally diverse. It varies in its cytoarchitecture (Vogt et al. 1987, 2005) and these cytological regions have different afferent and efferent connections (Pandya et al. 1981; Vogt and Pandya 1987; Barbas et al. 1999). Functional differences between cingulate regions in monkeys have been demonstrated by a few electrophysiological recording studies (Shima et al. 1991; Morecraft and Tanji 2009) and more recently by task-based fMRI studies (Ford et al. 2009; Stoewer et al. 2010). Here, we present the first attempt to map the large-scale functional connectivity of the cingulate cortex using high-field resting-state high-field fMRI data collected from a group of macaque monkeys.

We found that the functional connectivity of cingulate cortex varied systematically along the rostral/caudal and dorsal/ventral axis, thereby confirming previous anatomical tracer and lesion studies in monkeys (Pandya et al. 1981; Vogt and Pandya 1987; Barbas et al. 1999; Rudebeck et al. 2006). The present results extend a study using resting-state fMRI in humans (Margulies et al. 2007), in which the functional connectivity was constructed from 16 seed ROIs in the cingulate cortex. Consistent with our results and structural
knowledge, Margulies et al. (2007) described the basic rostral/caudal distinctions with the caudal cingulate being correlated with sensorimotor areas and the rostral cingulate cortex being correlated with prefrontal cortex. Between the rostral and the caudal zones, Margulies et al. (2007) observed "transition zones" that were correlated with both ventral and dorsal brain regions and hypothesized to be involved in the integration of cognitive and affective signals necessary for conflict (Botvinick et al. 2001) and error monitoring (Gehring et al. 1995). Here, a fine-grained seed analysis allowed us to identify segregated subdivisions of the cingulate cortex, whose specific connectivity patterns suggest a separable functional involvement, which goes beyond previous insights about transition zones (see below).

Recently, the popular model that proposes a distinction between a dorsal cognitive and a ventral affective ACC subdivision (Bush et al. 2000) has been questioned by Etkin et al. (2011). According to the latter model, both ACC subdivisions make contributions to affective processing. Although our data cannot directly address this controversy, our findings show that both the subgenual seeds (i9, i10) and the dorsal seeds (s4, s6, m4–m6) are positively correlated with the anterior insular cortex that has been implicated in affective processing and subjective feelings (Craig 2009). This finding supports the hypothesis that subregions of the dorsal ACC are also involved in affective processes (Etkin et al. 2011).

One noteworthy observation for the cingulate cortex in monkeys at single and group levels were apparent anticorrelations between cingulate regions and other cortical and subcortical areas. Indeed, in many cases positive correlations with a brain area switched to negative correlations between neighboring seed ROIs. Anticorrelations between networks in human fMRI studies have been described between attentional and default-mode networks by Fox et al. (2005). Margulies et al. (2007) also found strong negative networks for many seed ROIs in the human ACC. At a finer scale, areas directly adjacent to the strong local seed ROI connectivity often showed significant anticorrelations (for e.g., see Fig. 4, ml7) suggesting possible lateral inhibition or modulation. Little is known about the physiological basis of anticorrelations measured by resting-state fMRI. Some authors have argued that they represent an artifact due to global signal regression, a common preprocessing stage to remove global variations of the BOLD signal (Murphy et al. 2009). Margulies and colleagues removed the global mean as a nuisance covariable undermining the existence of the negative ACC networks. Fox et al. (2009), however, recently showed that anticorrelations are not attributable to global signal removal alone and suggested an anatomical basis supported by several studies.

Our major result concerns the identification of 4 networks within the cingulate cortex, each comprising distinct functional connectivity patterns with cortical and subcortical regions. The finding of functionally distinct clusters in the monkey cingulate cortex is reminiscent of a recent DTI study in humans that identified distinctive subregions in the cingulate cortex based on their connections with cortical and subcortical areas (Beckmann et al. 2009). The results also parallel previously identified functional subdivisions of the macaque and human prefrontal that found connectivity patterns suggesting sensorimotor, cognitive, and visual domains (Margulies et al. 2009). Based on the functional connectivity patterns of the cluster, we have tentatively labeled these networks somatomotor,
attention-orienting, executive, and limbic. Although these clusters map to the known cingulate subdivisions in the nonhuman primate, there are also a number of notable differences.

The limbic cluster (seeds i9 and i10) corresponds well to areas 32 and 25. Also, the border between the attention-orienting cluster and the executive cluster in the cingulate gyrus corresponds to the border between 24a’/b’ and 24a/b. Likewise, the border between the somatomotor cluster and the attention-orienting cluster in the cingulate gyrus corresponds to the border between areas 24c’ and 24c. Our cluster analysis, however, suggests that area 24c is functionally heterogeneous. The posterior two-thirds (seeds m5 and m6) grouped with the attention-orientation cluster, whereas the anterior part of area 24c was associated with the executive cluster. Therefore, the results from the cluster analysis indicate a functionally similar connectivity pattern for area 24a’/b’ and the posterior third of 24c while the anterior part of 24c belongs functionally to area 24a/b. Moreover, ROIs in the superior frontal gyrus anterior to the posterior tip of the principal sulcus (s6 and s7) clustered together with seeds in area 24a’/b’ and posterior two-third of area 24c.

The 4 cingulate clusters differed in their correlation pattern with other cortical and subcortical areas. To some degree, these different functional connectivity patterns can be explained by anatomical afferent and efferent connections of these cingulate areas as identified by tracer injection studies. The somatomotor cluster is reminiscent of cingulate areas that are known to be interconnected with motor and premotor cortex (Morecraft and van Hoesen 1992; Picard and Strick 1996; Dum and Strick 2005; Morecraft and Tanji 2009). The attention-orienting cluster encompasses cingulate areas that connected with the FEFs and dorsolateral prefrontal cortex (Bates and Goldman-Rakic 1993; Morecraft et al. 1993; Wang et al. 2004) and with posterior cingulate cortex (Pandya et al. 1981; Vogt and Pandya 1987). The executive cluster corresponds to an area known to be connected with the prefrontal cortex (Pandya et al. 1981; Vogt and Pandya 1987; Bates and Goldman-Rakic 1993; Lu et al. 1994). Lastly, the limbic cluster is a cingulate region that is most strongly connected with the amygdala and hippocampus (Vogt and Pandya 1987; van Hoesen et al. 1993). It should be emphasized that fMRI connectivity analysis, despite their close correspondence to structural connectivity analysis (Margulies et al. 2009; Honey et al. 2009), complements, but does not replace anatomical tracer studies (Modha and Singh 2010). Our finding of correlated activity in the visual cortex for several seeds despite the absence of direct efferent or afferent connections between the ACC and the visual cortex suggests that the networks found in this study are partly polysynaptic (Fox and Raichle 2007). The cluster-averaged voxelwise correlational analysis of seed time courses revealed overlap of brain areas with weak to moderate (0.15–0.3) temporal correlations shared between cluster networks, particularly the somatomotor and attention-orienting networks (Fig. 11, row 3). Temporal correlations between seeds of different cluster networks are also evident in the cross-correlation matrix from which the cluster analysis was derived (Fig. 7). These results suggest that the cingulate seeds, and the cluster networks that they form, are not completely independent of one another providing support for a hierarchical cingulate network organization (Fig. 8) in which higher levels represent greater integration (Meunier et al. 2010) though they show weaker temporal dependencies (Doucet et al. 2011).
Our data may provide important guidance for future electrophysiological studies in nonhuman studies specifically designed to explore performance and conflict-related signals during oculomotor tasks. Based on our cluster analysis, we propose that the best candidate for conflict-related signals for oculomotor tasks in the macaque may be the cluster that we have labeled executive. This cingulate cluster has strong positive correlations with the prefrontal cortex and the FEFs in the anterior bank of the arcuate sulcus. Moreover, it is the only cluster that is positively correlated with the superior colliculus, a major saccadic motor area in the primate (Johnston and Everling 2008), and the mediodorsal thalamus. It has been shown that the mediodorsal thalamus relays a corollary discharge signal from the superior colliculus to the frontal cortex (Sommer and Wurtz 2002). Such a signal may not only play an important role in error monitoring but may also inform the ACC of an intended motor response which may conflict with sensory signals. The positive correlations of this cluster with visual cortex support the idea that this cluster also has access to visual information.

The attention-orienting cluster also comprises saccade-related brain areas (dorsolateral prefrontal cortex, FEF, lateral intraparietal area), but it lacks thalamic and collicular correlations. Moreover, the executive, but not the attention-orienting network cluster, is positively correlated with the head of the caudate nucleus, a structure thought to be involved in reward-dependent modulation of saccades (Ding and Hikosaka 2006; Nakamura and Hikosaka 2006).

Single neuron activity in the executive cluster is largely unexplored. Ito et al. (2003) and Nakamura et al. (2005), recorded primarily in an area that corresponds to our seed ROIs m5 and m6, which are part of the attention-orienting cluster. A few of Nakamura et al.’s recordings were in the area that corresponds to our seed ROIs m7 (ml7 and mr7), but the more anterior portion of this cluster has not been explored with electrophysiological techniques in a conflict task. In addition, and perhaps more importantly, the activity of neurons in the primate anterior cingulate gyrus has not been characterized. Our data show that several of the seed ROIs in the cingulate gyrus are positively correlated with the FEF. Unfortunately, the cingulate gyrus is in close proximity to the pericallosal artery, making electrode recordings hazardous.

Overall, our data supports RS-fMRI as a valuable approach for the comparative mapping of brain networks in humans and nonhuman primates (Vincent et al. 2007; Margulies et al. 2009). Though comparisons revealed relatively stable large-scale cingulate networks, the variability of functional connectivity maps observed between monkeys also supports RS-fMRI as an important tool to characterize individual network differences for studies reliant on stereotaxic measures and assumptions of homologous organization. The connectivity differences may reflect variability of both functional and structural anatomy that is valuable when interpreting electrophysiological results. In addition, the fMRI technique allows us to assess the strength in functional connectivity for the entire living brain in vivo and also the dynamical changes with time. In this way, fMRI would also complement electrophysiological techniques in the study of integrated neuronal network activity.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/
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Notes
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References


