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Histological Asymmetries of Primary Motor Cortex Predict Handedness in Chimpanzees (*Pan troglodytes*)

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Abstract

Like humans, chimpanzees display robust and consistent hand preferences during the performance of certain tasks. Although correlations have been demonstrated between gross anatomic measures of primary motor cortex asymmetry and handedness in captive chimpanzees, the relationship between histological architecture and behavioral lateralization has not yet been investigated. Therefore, we examined interhemispheric asymmetry of several different microstructural characteristics of the primary motor cortex in the region of hand representation from 18 chimpanzees tested on a coordinated bimanual task before death. At the population level our data showed leftward bias for higher layer II/III neuron density. Of note, however, there was no population-level asymmetry in the areal fraction of Nissl-stained cell bodies, a finding that differs from previous studies of this cortical region in humans. Nonetheless, we found that asymmetry in the density of layer II/III parvalbumin-immunoreactive interneurons was the best predictor of individual hand preference. These results suggest that histological asymmetries are related to handedness in chimpanzees, while overall patterns of asymmetry at the population level might differ from humans.

Indexing terms

primary motor cortex; handedness; interneuron; parvalbumin; brain evolution

Once thought to distinguish humans from other animals, behavioral lateralization and neuroanatomical asymmetries have now been shown to be commonplace among other species (Rogers and Andrew, 2002; Halpern et al., 2005). In particular, great apes display many of the same cerebral hemispheric biases that are also present in humans, such as population-level asymmetry in the surface area of the planum temporale (Gannon et al., 1998; Hopkins et al., 1998), the length of the Sylvian fissure (Yeni-Komshian and Benson, 1976; Hopkins et al.,

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2000), and the sulcal anatomy of the inferior frontal cortex (Hopkins and Cantalupo, 2004). Furthermore, humanlike lateralization in chimpanzees (*Pan troglodytes*) for preferential hand use has been documented across a range of behaviors (Hopkins, 2006). Despite the accumulation of data concerning such directional biases in humans and nonhumans alike, however, the functional relationship between neuroanatomical asymmetries and behavioral lateralization is still not clearly understood.

With regard to humans, the expression of handedness shows a complex pattern of association with structural interhemispheric asymmetries of the primary motor cortex (Brodmann's area 4) and language-related cortical areas (Shapleske et al., 1999; Toga and Thompson, 2003; Sun and Walsh, 2006). Although voxel-based morphometry of in vivo magnetic resonance images (MRI) from humans has not revealed an association between brain anatomy and handedness (Good et al., 2001), this technique is not sensitive to subtleties of sulcal contours. In contrast, more focused examinations of asymmetry in the region of hand representation in the primary motor cortex have demonstrated a relationship (Hammond, 2002). In particular, right-handed individuals have a deeper central sulcus in the area of hand representation of the left hemisphere, whereas mixed- and left-hand dominant individuals do not exhibit significant asymmetries of the central sulcus (Foundas et al., 1998; Amunts et al., 2000). This central sulcus asymmetry appears to correlate with handedness only in males (Amunts et al., 2000). Similarly, morphological asymmetries of the central sulcus are also associated with hand preference on a coordinated bimanual task in chimpanzees (Hopkins and Cantalupo, 2004; Dadda et al., 2006) and New World capuchin monkeys (Phillips and Sherwood, 2005). Taken together, these findings suggest that structural asymmetries of the primary motor cortex reflect hemispheric specialization for controlling skilled actions of the dominant hand in primates (Hammond, 2002).

The implicit assumption behind such studies of central sulcus asymmetry is that the observed effects are a consequence of underlying hemispheric differences in the histological composition or overall volume of this region of primary motor cortex. While some authors have suggested that gross anatomical asymmetries are due mainly to interhemispheric differences in total neuron numbers, but not packing density (Galaburda et al., 1990; Rosen et al., 1993), there is actually very little empirical evidence to validate this claim in the human brain and no evidence in nonhuman primate brains.

Furthermore, the direct correspondence between microstructural asymmetries and hand preferences also remains uninvestigated. Currently available data on histological asymmetries in human primary motor cortex derive from studies of postmortem brain samples of unknown handedness (Amunts et al., 1996, 1997). These studies have demonstrated population-level asymmetries in the proportion of space occupied by neuropil in the site of hand representation as measured by the gray level index (GLI) method. Because the overwhelming majority of humans (~90%) are right-handed, it is assumed that these population-level cortical asymmetries relate to hand preferences.

One aim of the present study was to assess whether chimpanzees show population-level microstructural asymmetries in the primary motor cortex. A second aim was to examine the relationship between histological asymmetries of the primary motor cortex and task-specific handedness in chimpanzees. Lastly, because macrostructural asymmetries in the primary motor cortex hand area have been previously documented in chimpanzees, another goal of this study was to examine whether variation in asymmetries in gross morphology correlates with microstructural asymmetries measured from the same region. Our research design takes advantage of the substantial body of data on lateralized hand use in captive chimpanzees that has been amassed over more than a decade (Hopkins et al., 2004). Over time, several of the chimpanzee subjects that had been previously behaviorally characterized for hand preferences

have died of natural causes. We collected brains postmortem from several of these chimpanzees, providing a unique opportunity to study brain-behavioral correlations. Based on previous studies linking handedness with morphological asymmetries in the precentral gyrus (Hopkins and Cantalupo, 2004; Dadda et al., 2006), we hypothesized that histological asymmetries of the primary motor cortex would predict individual differences in hand preferences. To examine this possibility, we measured asymmetry in a panel of histological variables in postmortem brains and determined their contribution to explaining variation in handedness as measured before death on an experimental bimanual coordination task.

MATERIALS AND METHODS

Subjects

Eighteen chimpanzee subjects were used in this study, including 8 females (mean age at death = 36 years, SD = 12.7, range = 13–48) and 10 males (mean age at death = 26 years, SD = 10.7, range = 10–41). Six of the chimpanzee subjects were wild-caught before 1973 and lived in captivity since that time. The remaining 12 chimpanzees were born in captivity. All 18 subjects lived in social groups ranging from 2 to 13 individuals at Yerkes National Primate Research Center in Atlanta, Georgia. All subjects died from natural causes and were not part of any research protocol that may have contributed to their death.

Behavioral measurements

Hand preference was considered for a task measuring coordinated bimanual actions, referred to as the tube task (Hopkins, 1995). Handedness data from these subjects have been previously reported (Hopkins, 1995; Hopkins et al., 2004). For this task, peanut butter is smeared on the inside edges of polyvinylchloride tubes ≈ 15 cm in length and 2.5 cm in diameter. Each time the subjects reached into the tube with their finger, extracted peanut butter, and brought it to their mouth the hand used was recorded. We used measurements of hand preference on this task because it is stable across the lifespan and the strength of handedness elicited in chimpanzees by the tube task is significantly higher than for other actions, such as bimanual feeding or simple reaching (Hopkins, 2007). Although the number of responses obtained from each subject differed for this task, a minimum of 30 responses were obtained for each individual.

Binomial z -scores were calculated for each subject on the basis of the frequency of left- and right-hand use. Subjects with z -scores greater than 1.95 or less than -1.95 were classified as right- and left-handed, respectively. Subjects with z -scores between -1.95 and 1.95 were classified as ambidextrous. In addition, a handedness index (HI) was derived for each subject by subtracting the number of right-handed responses from the number of left-handed responses and dividing by the total number of responses: $HI = (R - L)/(R + L)$. Positive values reflect right-hand preference and negative values represent left-hand preference. The absolute value of the HI corresponds to the consistency of directional hand preference. Individuals that performed neuroanatomical measurements were blind to the HI score of the subjects.

MRI and measurement

Brains from each subject were obtained after death and were immersion-fixed in 10% formalin. In most cases the precise postmortem interval was not recorded at Yerkes National Primate Research Center; however, it was never greater than 14 hours. All of the brains were scanned postmortem using a T2-weighted protocol with a 1.5 T magnet. Images were collected in the transverse plane using a gradient echo protocol (pulse repetition = 22.0 s, echo time = 78.0 ms, number of signals averaged = 8–12, and a 256×192 matrix reconstructed to 256×256). The “knob” area of the precentral gyrus that corresponds to the location of hand representation was identified in serial 1-mm slices in the axial plane following procedures previously used in

human and ape brains (Yousry et al., 1997; Hopkins and Pilcher, 2001). Quantification of the knob region was obtained in the axial plane because this is the most common approach used in human studies (Hammond, 2002) and it is difficult to reliably quantify this region from other planes in chimpanzees. Nonetheless, a previous study that quantified the hand knob in human brains from the sagittal plane found that these measures of asymmetry correlated with the subjects' handedness (Foundas et al., 1998). Because these results are largely consistent with the findings reported for measures from the axial plane, we reasoned that the axial measurement of the knob was sufficiently representative of the primary motor cortex hand area of chimpanzees.

Morphological measurements of the hand knob were performed using ANALYZE software (ANALYZE, Lenexa, KS). The horizontal epsilon or inverted omega that projected into the postcentral gyrus was traced on each image (Fig. 1). The dorsal and ventral edges of the knob served as the markers for defining the boundaries of the area. For each slice and hemisphere, an area measurement of the region was calculated by use of a mouse-driven pointer that traced the region of interest. The total of area measurements from all slices in which the knob was present were summed and used to derive a volumetric measure for each hemisphere (ranging from 5 to 13 slices in the sample).

Tissue preparation and immunohistochemistry

The region of hand representation in primary motor cortex was dissected from each hemisphere as a block ≈ 4 cm thick containing the pre- and postcentral gyri. To estimate the location of hand representation, the hand knob landmark was viewed on horizontal MRI scans of each brain (Hopkins and Pilcher, 2001) and the corresponding dorsoventral level was noted on the lateral surface. In addition, prior to dissection the central sulcus was spread open to reveal the middle genu, which is anatomically equivalent to the hand knob seen in axial MRI sections in human brains (Yousry et al., 1997). The position of the hand knob landmark generally accords with previous electrophysiological studies of motor maps in this species (Grünbaum and Sherrington, 1903; Leyton and Sherrington, 1917; Hines, 1940; Dusser de Barenne et al., 1941; Bailey et al., 1950). Moreover, a recent functional imaging study on grasping using positron emission tomography (PET) in five chimpanzees found significant activation in the knob region in the hemisphere contralateral to the hand used (Hopkins et al., 2006).

After dissection, tissue blocks were cryoprotected by immersion in buffered sucrose solutions up to 30%, frozen on dry ice, and sectioned at 40 μ m with a sliding microtome perpendicular to the axis of the central sulcus. Every 10th section (400 μ m apart) was stained for Nissl substance with a solution of 0.5% cresyl violet. For each individual, sections from both hemispheres were Nissl-stained together in order to ensure comparable staining conditions for subsequent analyses. Sections not used for immediate staining were cryoprotected in a storage solution consisting of glycerol, ethylene glycol, dH₂O, and phosphate buffer (3:3:3:1 volume/volume) and archived at -20°C .

Immunohistochemistry was performed for each antigen on adjacent 1:20 series of sections. Free-floating sections were stained with mouse monoclonal antibodies to a non-phosphorylated epitope in neurofilament H (SMI-32 antibody; Covance International, Netherlands; dilution 1:2,000) and parvalbumin (Swant, Belinzona, Switzerland, Cat. no. 235; dilution 1:10,000). The SMI-32 mouse monoclonal IgG₁ antibody was raised against the non-phosphorylated epitope of neurofilament H isolated from homogenized hypothalami of Fischer 344 rats. On conventional immunoblots, SMI-32 visualizes two bands (200 and 180 kDa), which merge into a single neurofilament H line on two-dimensional blots (Sternberger and Sternberger, 1983). The antibody reacts with a nonphosphorylated epitope from 200-kD neurofilament heavy chain of most mammalian species. This protein is expressed in cell bodies, dendrites, and some thick axons within a subset of neurons that are mostly pyramidal cells (Campbell

and Morrison, 1989; Hof and Morrison, 1995). Other cells and tissues are unreactive and the antibody does not recognize the phosphorylated 200-kD neurofilament heavy chain. The mouse monoclonal IgG₁ PV antibody was raised against PV from carp muscle. It has been shown to bind with high affinity to the tertiary structure of PV from multiple species including macaque monkeys and humans, with binding eliminated by addition of exogenous PV (Celio et al., 1988). No crossreactivity with other calcium-binding proteins was noted in radioimmunoassay and immunoblotting assays (Celio et al., 1988). In primate brain tissue the pattern of staining with this antibody is consistent with that previously established for other PV antibodies (Conde et al., 1994).

Prior to immunostaining, sections were rinsed thoroughly in phosphate-buffered saline (PBS) and pretreated for antigen retrieval by incubation in 10 mM sodium citrate buffer (pH 3.5) at 37°C in an oven for 30 minutes. For the SMI-32 antibody, antigen retrieval used the same buffer with pH 8.5 at 90°C in a water bath to achieve improved staining. Sections were then immersed in a solution of 0.75% hydrogen peroxide in 75% methanol to eliminate endogenous peroxidase activity. After rinsing again, sections were incubated in the primary antibody in a diluent containing PBS with 2% normal horse serum and 0.3% Triton X-100 for ~48 hours on a rotating table at 4°C. After rinsing in PBS, sections were incubated in the secondary antibody (biotinylated antimouse IgG, Vector Laboratories, Burlingame, CA; dilution 1:200) and processed with the avidin-biotin-peroxidase method using a Vectastain ABC kit (Vector Laboratories). Immunoreactivity was revealed using 3,3'-diaminobenzidine (DAB). Sections were counterstained with cresyl violet to visualize nonimmunoreactive neurons and cytoarchitectural boundaries. Specificity of the reaction was confirmed by processing negative control sections as described, but excluding the primary antibody. No immunostaining was observed in control sections. Each set of sections from each hemisphere were stained together to control for interexperiment variation. In this way, because lateral asymmetry was of interest, differences in fixation protocols, duration of fixation, storage conditions, and postmortem delay within each individual case would affect both hemispheres equally. Examples of immunohistochemical staining results are shown in Figure 2.

Histological identification of the region of interest

We identified the region of interest for all subsequent quantitative measurements as primary motor cortex (Brodmann's area 4) based on previous descriptions of the cytoarchitecture of this area in chimpanzees (Bailey et al., 1950; Sherwood et al., 2003, 2004b, 2006). In brief, the primary motor cortex is distinguished by giant Betz cells in the lower portion of layer V, low overall cell density, large average cellular sizes, a poorly defined layer IV, and a diffuse border between layer VI and the subjacent white matter. The border between area 4 and area 3a, which usually occurs close to the fundus of the central sulcus, is recognized by the development of a well-defined granular layer IV. Although the border between area 4 and premotor cortex (area 6) may occur along the convexity of the precentral gyrus, we restricted our analyses to the portion of area 4 on the anterior bank of the central sulcus (Fig. 3).

Measurement of relative layer thickness

Using a portion of the precentral gyrus where cortical layers were most easily distinguishable and not obscured by tangential sectioning, three sections spaced 400 µm apart were selected to measure the relative thickness of cortical layers. Measurement sites were positioned to fall along a part of the anterior bank of the central sulcus that was not folded at the crown of a gyrus or within the depth of a fundus. At each measurement site a reference contour was drawn from the pial surface to the white matter interface following the radial orientation of cortical minicolumns at low magnification (4×) using a computerized stereology and morphometry system consisting of a Zeiss Axioplan 2 photomicroscope equipped with a Ludl XY motorized stage, Heidenhain z-axis encoder, an Optronics MicroFire color videocamera, a Dell PC workstation,

and StereoInvestigator software v. 6 (MBF Bioscience, Williston, VT). The length of each cortical layer was then measured along the radial guideline. We segmented the primary motor cortex along the most obvious layer boundaries: I, II/III, and V/VI (Fig. 3). In general, these laminar subdivisions correspond to functional differences in connectivity patterns. Molecular layer I contains mainly apical dendrites and horizontally oriented axons. Neurons in supragranular cortical layers II/III are involved in corticocortical integrative processes and have axonal projections to other ipsi- and contralateral cortical areas. Infragranular layers V/VI are comprised of neurons participating in corticofugal systems projecting to the spinal cord, brainstem, striatum, and thalamus.

The border between layers III and V was identified by the presence of a poorly developed, yet detectable, inner granular layer IV. In each section measurements were performed at two locations spaced 2 mm apart. At each measurement site the fraction of the total cortical thickness occupied by the width of each laminar segment was calculated as the relative layer thickness. An average relative layer thickness for each hemisphere was obtained from these measurements.

Areal fraction of Nissl-stained tissue

We quantified the areal fraction (AF) of tissue comprised of Nissl-stained cell bodies of neurons, glia, and endothelial cells in layers II/III and V/VI from high-resolution images. The AF represents the proportion of stained cellular profiles that project onto a two-dimensional measuring plane. Using the same three Nissl-stained sections used for analysis of relative layer thickness, digital images were collected using fractionator sampling as implemented by the StereoInvestigator system. First, contours were drawn around layers II/III and V/VI at low magnification. Then a fractionator sampling design (grid spacing of $600 \times 800 \mu\text{m}$ for layer II/III; $800 \times 800 \mu\text{m}$ for layer V/VI) was used to obtain a series of 8-bit grayscale image frames in a systematic random fashion with a $20\times$ (0.50 N.A.) Plan-Neofluar objective lens. Prior to collection of image frames in each section, the exposure of the digital camera was standardized to an average target intensity of 70%. Images covered $440 \times 587 \mu\text{m}$ and were $1,600 \times 1,200$ pixels in size, yielding a resolution of 0.37 pixels per μm . Image frame acquisition was monitored during fractionator sampling and all images that fell outside of the laminar region of interest boundaries were omitted from further processing. On average, 24.3 ± 4.5 (mean $\pm$ SD) image frames representing each laminar region of interest were collected for AF analysis. To measure the AF, images were processed in ImageJ software v. 1.32j with background subtraction using a rolling ball algorithm (Sternberger, 1983), converted to binary by an automated threshold routine based on Rider and Calvard (1978), and dilation and erosion were applied to fill small holes representing light staining of cellular nuclei (Fig. 4). After converting the image to binary, the percentage of the measuring frame area occupied by pixels representing stained elements was calculated. The AF value for each hemisphere is the section-weighted mean of AF measurements calculated from all frames.

Stereologic analyses

Quantification of numerical densities of cells and their volumes within layer II/III was performed using the StereoInvestigator stereology system. We focused on layer II/III for these analyses because many prior studies concerning histological asymmetry in the human neocortex have identified significant hemispheric differences specifically in the superficial cortical layers (Hayes and Lewis, 1995, 1996; Buxhoeveden et al., 2001; Hutsler, 2003; Garcia et al., 2004), suggesting that the functional role of these layers may be particularly relevant for hemispheric specialization.

Strict stereological quantification of total cell numbers is not feasible within the restricted region of hand representation of primary motor cortex because distinct boundaries cannot be

established to separate this area from the motor representation of adjacent body parts. Nonetheless, asymmetries of cell-type-specific numerical densities can provide a useful reflection of hemispheric specialization. In this way, our data characterize asymmetries in the cellular composition per unit of tissue. Densities of neurons and glial cells were estimated from sections stained for Nissl substance. The density of PV-immunoreactive (ir) interneurons was also estimated. Cell densities in layer II/III were obtained using the optical disector with fractionator sampling following methods described in a previous study (Sherwood et al., 2007). The same three sections used for AF analysis, or those in adjacent series, were quantified. After outlining the boundaries of layer II/III at low magnification, a set of optical disector frames ($30 \times 30 \mu\text{m}$ for neurons and glia; $90 \times 90 \mu\text{m}$ for PV-ir interneurons) were placed in a systematic random fashion to yield ≈ 30 frames per section. Disector analysis was performed under Koehler illumination using a $63\times$ objective (Zeiss Plan-Apochromat, N.A. 1.4). The thickness of optical disectors was set to $6 \mu\text{m}$ to allow for a minimum $2\text{-}\mu\text{m}$ guard zone on either side of the section after z-axis collapse from histological processing. Cellular densities (N_V) were derived from these stereologic counts and corrected for shrinkage from histological processing by the number-weighted mean section thickness. On average, the coefficient of error (Schmitz and Hof, 2000) of measurements was 0.07 ± 0.03 for neurons, 0.08 ± 0.03 for glial cells, and 0.11 ± 0.02 for PV-ir interneurons. These coefficients of error are somewhat larger than is common in stereologic studies because we restricted our counts to the part of the precentral gyrus where cortical layers were most easy to define. This amount of measurement error would be expected to reduce the probability of rejecting the null hypothesis in statistical tests; however, findings of statistical significance in spite of this error can be considered reliable.

Cellular volumes of neurons immunostained for non-phosphorylated neurofilament protein (NPNFP) and PV were estimated using the nucleator with a vertical design (Gundersen, 1988). Neurons were selected for volume measurement systematic randomly by applying optical fractionator sampling in two sections. In this way, the distribution of cell volumes obtained comprises an unbiased representation of the total population. The vertical axis of the probe was a line running superior-to-inferior to the pial surface. The centroids of neurons within the inclusion boundaries of optical disectors were marked and two transect lines from randomly selected directions were centered at the marker and superimposed over the neuron. The intersection of each line with the outer surface of the neuronal soma was marked and cellular volume was measured based on the nucleator principle. Because it was not feasible to perform isotropic-uniform-random sectioning in these rare behaviorally characterized chimpanzee materials, our mean cellular volume estimates contain a degree of bias due to a preferred sectioning orientation. However, due to normal variations in the orientation of the tissue in our preparations, not all cells were cut along the same axis, thereby generating a degree of randomness in the sample. Furthermore, coronal and sagittal sections have been shown to yield comparable results to isotropic-uniform-random sections using this probe (Schmitz et al., 1999). In each hemisphere for each individual, 73.1 ± 13.6 cell soma volumes were sampled for NPNFP-ir neurons and 43.6 ± 16.6 for PV-ir interneurons. Mean cell volume was calculated for each hemisphere.

Data analysis

Lateral asymmetries in the various anatomical measurements were quantified by calculating an asymmetry quotient (AQ) using the formula: $AQ = (R - L) / ((R + L) \times 0.5)$. Positive AQ values signify right hemisphere dominance, negative values signify left hemisphere dominance, and zero denotes symmetry. The absolute value of the AQ indicates the degree of asymmetry. Table 1 provides AQ values for all neuroanatomical variables in each chimpanzee subject. Mann–Whitney *U*-tests did not reveal significant differences between sexes for HI score or any neuroanatomical AQ measurement; therefore, sexes were pooled in subsequent

analyses. Furthermore, because none of the behavioral or neuroanatomical measurements showed a correlation with age, it was also not considered in the analysis. Nonparametric Spearman rank order correlations were used to examine associations with handedness because coordinated bimanual HI data was not normally distributed. We applied a sequential Bonferroni adjustment on a per hypothesis basis to adjust α for multiple comparisons in correlation and *t*-test analyses. However, because type II error is increased by this method, we report statistical significance at $\alpha' = 0.1$.

Forward stepwise multiple regression analysis was used to examine whether the combination of various anatomical AQ variables could predict variation in HI score. The forward stepwise approach sequentially selects the most highly correlated independent variable, removes the associated variance in the dependent, then enters further independents into the model which most correlate with the remaining variance in the dependent until selection of an additional independent does not increase r^2 by a significant amount ($P > 0.05$). The final model includes a reduced number of predictor variables which collectively make the strongest, uncorrelated contributions to explaining variation in the dependent. Assumptions of multiple regression analysis were checked and each independent predictor variable was normally distributed as determined by Shapiro–Wilk's *W*-tests. Furthermore, after multiple regressions, plots of studentized residuals versus unstandardized predicted values did not show nonlinearity or heteroscedasticity. Finally, binomial logistic regression was used to explore relationships between anatomical predictors and categorical handedness classification. For analyses that did not involve Bonferroni correction, statistical significance is reported at $\alpha = 0.05$ (two-tailed).

Photomicrography

Photomicrographs were obtained using an Optronics MicroFire digital camera mounted on a Zeiss Axioplan 2 microscope. Brightness and contrast of images were adjusted using Adobe Photoshop 6.0 software (San Jose, CA). Adobe Illustrator 8.0 was used for assembling and labeling figures.

RESULTS

Population-level asymmetry

In the current sample of 18 chimpanzees, there was significant rightward dominance of hand preference for the coordinated bimanual task (mean HI = 0.30, SD = 0.58; single-sample *t*-test: $t_{17} = 2.24$, $P = 0.04$). Based on *z* scores, 14 chimpanzees were classified as right-handed, 3 were left-handed, and 1 was ambidextrous (4 were classified as nonright-handed). In addition, this group of chimpanzees displayed a significantly larger hand knob in the left hemisphere (mean AQ = -0.19, SD = 0.37; single-sample *t*-test: $t_{17} = -2.15$, $P = 0.05$). In a previous study of a larger cohort of captive chimpanzees, population-level right-hand dominance for the coordinated bimanual task was also demonstrated, although the hand knob region of the primary motor cortex did not show significant asymmetry (Hopkins and Cantalupo, 2004).

Next, single-sample *t*-tests were performed on the AQ of each histological variable to test for a significant deviation from symmetry, i.e., a reference constant of zero (Table 2). Figure 5 shows mean AQs for all neuroanatomical measurements. Of all the histological measures, only layer II/III neuron density showed a significant population-level bias (mean AQ = -0.20, SD = 0.29, $t_{17} = -3.01$, $P = 0.008$, corrected $P = 0.08$). The left hemisphere tended to contain a higher density of neurons than the right.

Test of correlation between external morphology and histology

A series of Spearman rank order correlations were used to examine the relationship between asymmetry in the external morphology of the primary motor cortex and its underlying

microstructural organization. Only the correlation between the hand knob AQ and layer II/III PV-ir cell volume AQ was significant ($r = -0.61$, $n = 18$, $P = 0.008$, corrected $P = 0.08$).

Prediction of the direction and strength of hand preference

We calculated a Spearman correlation matrix to examine the bivariate relationships between each neuroanatomical AQ value and coordinated bimanual HI separately. The hand knob AQ ($r = -0.50$, $P = 0.04$, $n = 18$) showed an association with the HI score; however, it was not significant after adjusting α for multiple comparisons. No other correlations were significant.

Next we performed forward stepwise multiple regression analysis to evaluate whether a linear combination of independent variables could predict HI scores on the coordinated bimanual task. The AQ values for all neuroanatomical measures were entered as independent variables. This analysis yielded a model that explained a significant proportion of variance in handedness (adjusted $r^2 = 0.51$, $P = 0.02$). In this analysis, the standard partial regression coefficient (β) represents a measure of the unique contribution of each independent variable. It is the average amount the dependent variable changes when the independent varies by one standard deviation and other independent variables are held constant. Listed in order of the strength of the standard partial regression coefficient, the predictors that contributed to this model included layer II/III PV-ir interneuron density AQ ($\beta = -0.62$, $t_{11} = -2.84$, $P = 0.02$), hand knob AQ ($\beta = -0.45$, $t_{11} = -2.09$, $P = 0.06$), relative thickness of layer V/VI AQ ($\beta = 0.44$, $t_{11} = 2.15$, $P = 0.05$), AF in layer II/III AQ ($\beta = -0.40$, $t_{11} = -1.73$, $P = 0.11$), layer II/III neuron density AQ ($\beta = 0.39$, $t_{11} = 2.21$, $P = 0.05$), and layer II/III NPNFP-ir mean cell volume AQ ($\beta = -0.37$, $t_{11} = -1.98$, $P = 0.07$). Bivariate plots of these predictor variables and coordinated bimanual HI score are shown in Figure 6.

Test of differences between right-handed and nonright-handed chimpanzees

Visual inspection of Figure 6 makes it clear that data from the nonright-handed subjects (i.e., left-handed and ambidextrous) exerted strong leverage on the regression analysis. These results prompted us to also test for differences among subjects that were categorized dichotomously as either right- or nonright-handed. First, we used a series of Mann–Whitney U -tests to examine whether neuroanatomical AQ values differed between handedness groups. Of all the comparisons, only layer II/III PV-ir interneuron density displayed a difference between handedness categories ($U_{4,14} = 8$, $z = 2.12$, $P = 0.03$); however, it was not significant after adjusting α for multiple comparisons. We also used binomial logistic regression analysis to examine whether the combination of neuroanatomical AQ values could differentiate individuals that were categorized dichotomously as either right-handed or nonright-handed. The logistic regression approached significance ($\chi^2 = 18.13$, $df = 11$, $P = 0.08$).

DISCUSSION

Our findings indicate that the region of hand representation of the primary motor cortex of chimpanzees exhibits population-level asymmetry of histological architecture, although in a manner that differs from humans. Furthermore, our data reveal an association between individual hand preferences and microstructural asymmetries of this cortical area. This study is of special significance because it represents one of the first characterizations of histological asymmetry within any region of the chimpanzee neocortex and therefore provides an essential comparative context for understanding the evolutionary history of cortical asymmetries in humans.

Methodological considerations

In discussing our results, it is important to keep in mind that the AF value represents the Nissl-stained cellular volume fraction, which is not the same as the numerical density of the

constituent cells. The AF is based on both the number of cells and their sizes. Furthermore, the AF is calculated from all Nissl-stained cellular profiles and hence represents the contribution of different classes of neurons, various types of glia, and endothelial tissue. In the present study, we used AF for two reasons. First, the AF is inversely proportional to the amount of neuropil space available for synapses, dendrites, and axons. Therefore, it provides a useful indirect measure of the fraction of tissue involved in interconnectivity among neurons. Second, for the purpose of analyzing hemispheric asymmetry in histological architecture, our measurement of AF was designed to be comparable to the GLI as reported in previous studies of human cortex (Amunts et al., 1996, 1997, 1999, 2003). The GLI technique is used for multidimensional characterization of cytoarchitecture and definition of cortical area boundaries. In plotting vertical changes in cell volume densities, the GLI is measured from many small fields within the image frame across a series of transects that run from the pial surface to the white matter interface. Our method differs in that we used fractionator sampling to collect image frames and extracted an AF measurement from the entire image. Nonetheless, one application of the GLI method in prior studies of asymmetry of human neocortex has been to calculate average layer-wise or whole cortex GLI values. As discussed below, this particular application of the GLI method, which provides data that are similar to our technique for measuring AF, has consistently found asymmetries in humans.

Population-level effects and comparison to humans

Population-level asymmetries of histological organization have been described for areas of the human cerebral cortex that are known to be functionally lateralized such as the primary motor cortex, Broca's area, and the planum temporale (Hayes and Lewis, 1995; Amunts et al., 1996, 1999; Anderson et al., 1999; Buxhoeveden et al., 2001; Hutsler, 2003; Uylings et al., 2006). Interpretation of the relationship between functional lateralization and anatomical asymmetry is complicated in these postmortem studies, however, because information is generally not available for subjects regarding hemispheric dominance for language and handedness. Nonetheless, conclusions about such an association are often drawn under the assumption that any robust anatomical asymmetries relate to the high incidence of right-handedness and left hemisphere language dominance in humans (Toga and Thompson, 2003).

In this context, the most direct comparison of our results with findings from postmortem human brain studies concerns population-level asymmetry. To make our analyses comparable to existing human data, we measured relative layer thicknesses, neuron density, AF, and NPNFP-ir pyramidal cell volumes. Additional measurements of glia density, PV-ir interneuron density, and PV-ir cell volumes were performed to extend beyond currently documented asymmetries in the human cerebral cortex. To our knowledge, the sample size of behaviorally characterized chimpanzee brains that were available for the current study ($n = 18$) exceeds the sample size from nearly all studies of adult humans, with the exception of one study of pyramidal cell size asymmetry in area 45 (Hayes and Lewis, 1995). It is also important to note that right-handedness occurs in a smaller proportion of captive chimpanzees than in humans. Approximately two-thirds of all captive chimpanzees examined are right-handed on the bimanual coordination task (Hopkins, 2006). Of the chimpanzees in the current study, 78% (14 out of 18) were classified as right-handed. Thus, it might be expected that any population-level tendencies for histological asymmetries will be more difficult to detect in chimpanzees than in humans based on small sample sizes. Nonetheless, we found that neuron density in layer II/III was significantly higher in the left hemisphere primary motor cortex across this sample of chimpanzees. Neuron densities have not yet been examined for asymmetries in the primary motor cortex of humans using stereologic methods. However, asymmetries of neuronal numerical density in other cortical areas of humans have been reported. In normal humans, neuron density distributions do not show asymmetries within the planum temporale (Anderson

et al., 1999) or Broca's area (areas 44 and 45) (Garcia et al., 2004; Uylings et al., 2006), whereas dorsolateral prefrontal cortex (area 9) has greater overall neuron densities in the left hemisphere (Cullen et al., 2006). This pattern of results in humans raises the question of whether neuron density asymmetries relates to functional lateralization.

Besides neuron density, we did not find evidence of population-level asymmetry of any other histological feature, including AF. In fact, the AF AQ values in layers II/III and V/VI were among the least asymmetric of any of the variables analyzed (Table 1, Fig. 5). These results contrast with findings from humans, where significantly lower GLI (i.e., a greater amount of neuropil space) was found in the left hemisphere primary motor cortex in a sample of 12 adult individuals of unknown handedness (Amunts et al., 1996). A later study by Amunts et al. (1997) showed that neuropil asymmetry of human primary motor cortex develops within the infragranular cortical layers in childhood, whereas the subsequent appearance of neuropil asymmetry does not occur in supragranular layers until adulthood. Furthermore, analyses of Broca's area (areas 44 and 45) (Amunts et al., 1999), the cortex of the planum temporale (area Tpt) (Anderson et al., 1999; Buxhoeveden et al., 2001), as well as areas V1, V2, and V5/MT+ (Amunts et al., 2007) in humans have all reported left dominance of the neuropil volume fraction. Interestingly, neuropil asymmetry has not been found in area Tpt of chimpanzees or rhesus macaques (Buxhoeveden et al., 2001). Although caution should be exercised when interpreting results that fail to show asymmetry in nonhuman species based on small sample sizes, it is notable that several different cortical regions in humans appear to exhibit relatively greater neuropil space in the left hemisphere at the population level, whereas no such asymmetry has yet been detected in chimpanzees or other primates.

Taken together with these previous studies, our findings suggest that the human cortical phenotype differs from chimpanzees in showing a fundamental structural asymmetry in the space occupied by neuropil versus cell somata. Such divergence in the microstructural architecture of homotopic cortical areas might be, at least in part, determined by initial asymmetries in neuronal connectivity occurring early in development and guided by a combination of intrinsic and extrinsic signals (Stephan et al., 2007). Indeed, several genes that show differential expression between the cerebral hemispheres during early fetal development of humans also exhibit elevated rates of coding-sequence and regulatory evolution in humans compared to chimpanzees (Sun et al., 2006). This evidence suggests that some of the genes associated with brain asymmetry in humans have been the target of natural selection, since chimpanzees and humans diverged from a common ancestor 6 – 8 million years ago. Of the genes that are both upregulated and show higher levels of expression in the left hemisphere of human fetal cortex, those comprising the upper 10% of fold-differences between species include genes involved in functions such as protein assembly, intracellular transport, and transcription (*PINI*), *c-fos* signaling pathways (*CROC4*), enzymatic regulation of glycolysis (*PFKP*), and assembly of the cytoskeleton (*CKAP1*).

Histological relationships with handedness

Gross anatomical asymmetries of the region of hand representation of the primary motor cortex have been shown to correlate with hand preferences in humans, chimpanzees, and capuchin monkeys (Amunts et al., 2000; Hopkins and Cantalupo, 2004; Phillips and Sherwood, 2005; Dadda et al., 2006), suggesting that handedness is facilitated by a greater total mass of neural tissue devoted to controlling the dominant hand. This hypothesis is further supported by evidence from magnetoencephalography in humans (Volkmann et al., 1998) and intracortical microstimulation in New World squirrel monkeys (Nudo et al., 1992) indicating that the size of forelimb movement representation is significantly increased in the primary motor cortex opposite to the preferred hand. However, beyond these large-scale interhemispheric biases in the cortical tissue dedicated to hand movement representation, do changes to the internal

histological wiring of primary motor cortex also subserve the expression of handedness? As discussed previously, current data from postmortem human brain studies cannot address this question directly.

Our results demonstrate that variation in the direction and magnitude of hand preference in chimpanzees can be predicted by asymmetries of certain histological features of the primary motor cortex. It is unlikely that the neuroanatomical asymmetries observed among the nonright-handed chimpanzees in our sample can be explained by other variables aside from hand preference, such as age or sex (see Table 1). The four nonright-handed chimpanzees were equally balanced for sex and their age did not significantly differ from the right-handed subsample (Mann–Whitney $U_{8,10} = 11$, $z = -1.3$, $P = 0.21$).

Among the various statistical approaches that we used to examine these associations, asymmetries in layer II/III PV-ir interneuron density and the knob showed the strongest relationship with hand preference. Thus, asymmetries at both macro- and microstructural levels may be involved in organizing lateralized deployment of the hands. Interestingly, PV-ir interneuron density AQ made a stronger unique contribution than knob AQ in explaining handedness on the coordinated bimanual task in the final reduced multiple regression model. Our multiple regression analysis also identified several other variables that made a unique contribution to explaining hand preferences. However, none of these variables showed a significant relationship with handedness in simple bivariate correlations or comparisons between handedness categories, indicating that there is a complex interaction among these histological features that may account for lateralized behavior better than any single one of these variables in isolation.

Layer II/III PV-ir interneurons tended to be denser in the hemisphere opposite to the direction of hand preference on the coordinated bimanual task. Asymmetries of PV-ir interneurons may correspond to hemispheric specializations for processing of complex temporal sequences. Interneurons that express the calcium-binding protein PV are characterized by fast-spiking physiological properties and comprise chandelier and basket cell phenotypes (Markram et al., 2004). These interneuron types have the capacity to exert strong inhibitory influence on pyramidal cells via synapses on the soma and axon initial segment. In the dorsolateral prefrontal cortex of macaque monkeys, for example, PV-ir interneurons are involved in maintaining sustained firing in neuronal ensembles during the delay phase of working memory (Wilson et al., 1994; Rao et al., 1999). Thus, this class of interneuron can shape the temporal pattern of activation in neuronal populations, a function that may be especially important in the processing of complex sequences inherent to dexterous manual behaviors. In addition, this finding complements neuro-physiological data from squirrel monkeys that shows that forelimb movement maps display a higher degree of fragmentation and spatial complexity within the side of primary motor cortex opposite to the dominant hand (Nudo et al., 1992). In this context, relatively greater numbers of PV-ir interneurons, with their horizontally directed axonal domains, may be important in coordinating pyramidal cell firing across more spatially dispersed movement representations.

It is also interesting to note that this class of interneuron shows phylogenetic variation that might relate to species differences in motor control. PV-ir interneurons are proportionally more frequent in the orofacial representation of primary motor cortex in hominids (humans and great apes) as compared with Old World monkeys (Sherwood et al., 2004a), whereas visual cortex does not show this relative increase (Sherwood et al., 2007). Because hominids display a greater degree of dexterous motor control of the orofacial muscles, these comparative data, along with the current findings, suggest that variation in the distribution of PV-ir interneurons may comprise an important microanatomical substrate for fine motor coordination in the cerebral cortex.

CONCLUSION

We examined histological asymmetry in the region of hand representation of the primary motor cortex in chimpanzees. Our analyses revealed an association between hand preference and layer II/III PV-ir interneuron density asymmetry that is distinct from the previously documented relationship to gross anatomical asymmetries of the precentral gyrus. Whereas a correlation between morphological asymmetry of the precentral gyrus and handedness has also been demonstrated in humans, there are no data available concerning hemispheric bias in the distribution of interneurons. Because of the close phylogenetic relationship between humans and chimpanzees, the present findings provide important insight into the evolution of brain and behavioral asymmetries within the human lineage. In the future, it will be essential to determine whether asymmetry of PV-ir interneurons represents a common neuroanatomical substrate for hand preferences that is shared between these species due to homology. Based on our data, it is evident that AF asymmetry does not display a strong association with hand preference in chimpanzees. These results in chimpanzees give cause to reevaluate the proposed functional relationship between AF asymmetry and handedness in humans. Instead, the population-wide bias toward greater neuropil space in the left hemisphere of primary motor cortex and other cortical areas in humans might represent a novel trait that has emerged recently in evolution since the split from the last common ancestor shared with chimpanzees.

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Fig. 1.

The region of the hand knob landmark as seen in four axial MRI sections of a chimpanzee brain from dorsal to ventral (A–D). Blue lines indicate the tracing of the central sulcus (CS). The horizontal epsilon or inverted omega that projects into the precentral gyrus was traced on each image, shown here in yellow. The dorsal and ventral edges of the knob defined the boundaries of the area measured.

Fig. 2. Examples of histological staining in chimpanzee primary motor cortex (Brodmann's area 4). Staining for Nissl substance with cresyl violet (**A**), nonphosphorylated neurofilament protein with cresyl violet counterstain (**B**), and parvalbumin with cresyl violet counterstain (**C**) are shown. Scale bar = 50 μ m.

Fig. 3.

The part of primary motor cortex (Brodmann's area 4) that was sampled for quantitative measurements. Only the intrasulcal portion of the precentral gyrus was analyzed (**A**). Arrowheads illustrate the typical borders used in defining the region of interest. A higher magnification view of the cytoarchitecture from the segment of primary motor cortex indicated by the dashed line box in **A** is shown in **B**. The layers boundaries that were defined for various quantitative measurements are shown in **B**. PoG, postcentral gyrus; PrG, precentral gyrus; wm, white matter. Scale bar = 500 μ m in **B**.

Fig. 4.

Image conversion method for measurement of areal fraction (AF). An image frame of Nissl-staining in layer II/III is shown (**A**). Images were processed with background subtraction and conversion to binary to measure the proportion of space occupied by stained cells (**B**). Scale bar = 50 μm .

Fig. 5.

The population-level mean asymmetry quotient (AQ) for neuroanatomical measures in the sample of 18 chimpanzees. Positive AQs represent rightward bias and negative AQs represent leftward bias. Bars indicate mean, error bars indicate $\pm$ standard deviation.

Fig. 6. Bivariate plots of predictors of coordinated bimanual handedness index from multiple regression analysis, presented in the order of the strength of the relationship (A–F). Open circles indicate right-handed subjects; closed circles indicate nonright-handed subjects.

TABLE 1

Individual Subject Data

Yerkes Code	Sex	Age at Death	HI	HC	Hand Knob	Neuron Density in Layer II/III	Glia Density in Layer II/III	AF in Layer II/III	AF in Layer V/VI	Relative Thickness of Layer I	Relative Thickness of Layer II/III	Relative Thickness of Layer V/VI	PV-ir Interneuron Density in Layer II/III	PV-ir Interneuron Mean Cell Volume in Layer II/III	NP/NFP- ir Mean Cell Volume in Layer II/III
C0630	F	13	0.51	R	-0.06	0.01	0.00	0.03	0.03	-0.06	-0.06	0.05	-0.06	-0.07	-0.17
C0576	F	20	0.34	R	0.22	-0.16	-0.03	0.05	0.05	-0.04	0.04	0.10	0.25	-0.14	-0.08
C0342	F	35	0.36	R	-0.28	-0.41	-0.41	0.01	0.12	-0.05	-0.05	0.04	-0.38	-0.29	-0.24
C0320	F	39	-0.69	L	-0.06	-0.78	-0.01	-0.21	-0.21	-0.07	0.08	-0.06	0.24	-0.35	-0.14
C0406	F	41	0.91	R	-0.76	0.17	0.16	-0.11	-0.09	0.31	0.08	-0.12	-0.21	0.14	0.41
C0336	F	44	-0.16	A	-0.44	0.04	0.22	0.19	0.20	-0.07	0.04	-0.03	0.07	-0.01	-0.28
C0408	F	45	1.00	R	-0.29	0.06	-0.16	-0.16	-0.03	-0.09	-0.02	0.03	-0.13	-0.04	-0.06
C0242	F	48	0.68	R	-0.13	-0.88	-0.50	0.01	0.08	-0.21	-0.06	0.10	-0.53	0.04	-0.02
C0645	M	10	0.45	R	-0.09	-0.03	0.00	-0.06	-0.04	-0.04	-0.01	0.02	-0.63	0.04	0.57
C0573	M	15	0.83	R	-0.51	-0.15	0.02	-0.08	-0.20	-0.09	-0.16	0.09	0.29	-0.02	-0.30
C0507	M	17	0.35	R	-0.78	-0.10	0.09	0.22	0.19	0.04	0.05	-0.06	-0.24	0.14	0.12
C0491	M	18	0.34	R	-0.08	-0.21	-0.29	0.02	0.00	-0.06	-0.04	0.05	-0.41	0.23	-0.13
C0429	M	24	0.50	R	-0.06	0.17	-0.05	0.03	-0.02	0.26	0.15	-0.15	-0.30	-0.63	-0.40
C0423	M	25	0.36	R	0.09	-0.22	-0.05	-0.06	-0.12	0.20	0.01	-0.03	0.14	-0.02	0.40
C0369	M	32	0.77	R	-0.59	-0.40	-0.22	0.00	0.04	0.13	0.17	-0.05	-0.27	0.12	0.01
C0301	M	35	-0.71	L	0.81	-0.18	-0.34	-0.33	-0.29	0.41	0.16	0.14	0.62	-0.47	0.69
C0273	M	40	0.65	R	-0.23	-0.21	-0.09	-0.13	-0.13	-0.40	-0.17	0.17	0.16	0.03	0.29
C0367	M	41	-1.00	L	-0.15	-0.37	-0.02	-0.04	-0.06	0.08	-0.04	0.03	0.20	0.10	0.83

Neuroanatomical data are reported as asymmetry quotients (AQs).

F, female; M, male; HI, coordinated bimanual handedness index; HC, handedness classification; R, right; L, left; A, ambidextrous; AF, areal fraction of Nissl-stained tissue.

TABLE 2

Descriptive Statistics of Handedness Index (HI) and Asymmetry Quotient (AQ) Values and Results of One-Sample *T*-tests Using a Reference Constant of Zero

	Mean AQ	SD	<i>t</i>	<i>P</i>
Coordinated bimanual HI	0.30	0.58	2.24	0.04
Hand knob	−0.19	0.37	−2.15	0.05
Neuron density in layer II/III	−0.20	0.29	−3.01	0.01
Glia density in layer II/III	−0.09	0.19	−2.05	0.06
AF in layer II/III	−0.03	0.13	−1.12	0.28
AF in layer V/VI	−0.03	0.13	−0.85	0.40
Relative thickness of layer I	0.02	0.19	0.34	0.74
Relative thickness of layer II/III	0.01	0.10	0.42	0.68
Relative thickness of layer V/VI	0.02	0.09	0.85	0.41
PV-ir interneuron density in layer II/III	−0.07	0.33	−0.85	0.40
PV-ir interneuron mean cell volume in layer II/III	−0.07	0.23	−1.22	0.24
NPNFP-ir mean cell volume in layer II/III	0.08	0.36	0.97	0.35

P values are uncorrected.