Human-specific Organization of Primary Visual Cortex: Alternating Compartments of Dense Cat-301 and Calbindin Immunoreactivity in Layer 4A

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There is evidence that the cortical anatomy of the magnocellular (M) visual pathway, which carries information about motion and luminance contrast, was modified in human evolution. Recent results indicate that layer 4A of humans contains a meshwork of tissue bands that stain densely for nonphosphorylated neurofilament (NPNF), a protein that is preferentially expressed in elements of the M pathway, whereas apes and monkeys lack a comparable pattern. Here we examined the distribution of staining for Cat-301 – a monoclonal antibody well established to stain M-related structures preferentially – in area V1 of humans, apes (chimpanzees, orangutans), Old World monkeys (macaques) and New World monkeys (spider monkeys, squirrel monkeys). Single-staining experiments, using a peroxidase–tetramethylbenzidine (TMB) reaction, revealed alternating zones of dark and light staining for Cat-301 in layer 4A of humans, similar to those observed with NPNF. Double-staining studies in humans revealed that Cat-301-immunoreactive somas and neuropil were localized within the same tissue bands that stained strongly for NPNF and, furthermore, that these bands alternated with irregularly shaped territories that stained very strongly for calbindin. Nonhuman primates, by contrast to humans, displayed weak Cat-301 and calbindin staining in layer 4A. The co-localization of Cat-301 and NPNF in human layer 4A, and the weak staining for these molecules in layer 4A of other primates, suggests that the cortical representation of the M channel was modified in recent human evolution. The calbindin-rich compartments in human layer 4A cannot be related to a particular geniculostriate pathway on neurochemical grounds; they may constitute an interneuronal population that increased in human evolution.

Introduction

Comparative studies of the mammalian primary visual area (V1; area 17; striate cortex) have revealed some notable phylogenetic variations in cellular, laminar, compartmental and connectional organization. Within the primate order, differences have been documented between prosimians (lemurs, lorises) and anthropoids (monkeys, apes and humans), and between anthropoid groups (Hässler, 1967; Casagrande and Kaas, 1994; Preuss, 1995). There is now evidence that the organization of area V1 in humans differs in certain aspects from that of most other primates, particularly in the organization of layer 4A. In most New World and Old World monkey species that have been examined, layer 4A of the primary visual area is marked by a thin, dense band of cytochrome oxidase (CO) staining that is coincident with a thin layer of terminals arising from the parvocellular layers of the lateral geniculate (Horton, 1984; Wong-Riley, 1994). This sheet is punctuated by columns of apical dendrites and short stacks or cones of pyramidal cell bodies extending upward from layer 4B into 4A (Peters and Sethares, 1991; Hendry and Bhandari, 1992; Peters, 1994). These punctuations give the sheet of CO staining and geniculate terminals a distinctively lattice-like or ‘honeycomb’ appearance in sections cut parallel to the pial surface (Hendrickson et al., 1978). Humans, however, lack a CO-dense band (Horton and Hedley-Whyte, 1984), as do chimpanzees, the animals most closely related to humans (Preuss et al., 1999). The reduction of the CO-dense layer 4A band in the evolution of the hominoid primates (i.e. apes and humans) suggests that there may have been a corresponding reduction of the parvocellular geniculate projection to this layer (Horton and Hedley-Whyte, 1984; Preuss et al., 1999).

Layer 4A apparently underwent further modification during recent human evolution. Preuss et al. (Preuss et al., 1999) stained primary visual cortex from humans and a variety of other nonhuman primate species (including apes, Old World monkeys, and New World monkeys) for nonphosphorylated neurofilaments (NPNF) using monoclonal antibody SM1-32. In layer 4A of humans, they observed a distinctive pattern of NPNF staining in which bands of darkly stained tissue, consisting of dense neuropil with embedded cell bodies, were intermingled with lightly stained territories, giving the layer a mesh-like appearance in coronal sections. In apes and monkeys, by contrast, layer 4A lacked prominent bands of tissue stained for NPNF; instead, layer 4A was generally lightly stained in these species, and most of the stained elements that were observed consisted of apical dendrites of pyramidal cells with somas located in deeper layers. A comparable pattern of differences between humans and nonhuman primates was obtained using an antibody against microtubule-associated protein 2 (MAP 2), which also stains pyramidal cell bodies and dendrites in nonhuman primates, although staining was not as consistent across individuals as with the NPNF antibody. There are other indications that human layer 4A has an unusual pattern of compartmental organization: Hendry and Carder (Hendry and Carder, 1993) reported that human layer 4A exhibited dense but irregular staining for a calcium-binding protein, calbindin D-28k, whereas this layer was more lightly and uniformly stained in the Old World and New World monkeys they examined.

These results raise several important questions about the organization of layer 4A in human primary visual cortex. First, what is the relationship of the different compartments of human layer 4A to the three recognized channels of visual information processing, that is, the magnocellular (M), parvocellular (P) and koniocellular (K) pathways (Livingstone and Hubel, 1988; Casagrande and Kaas, 1994; Callaway, 1998; Hendry and Reid, 2000)? Preuss et al. (Preuss et al., 1999) suggested that the tissue bands that stain darkly for NPNF are related to the M pathway, because NPNF staining is reported to be comparatively dense in the magnocellular layers of the LGN (Gutierrez et al., 1995; Chaudhuri et al., 1996; Vickers, 1997) and in other neuronal populations related to the M pathway (Campbell and Morrison, 1989; Hof and Morrison, 1995; Chaudhuri et al., 1996). There is, however, a molecule that has been even more definitively associated with the M system than NPNF, namely, the antigen labeled by monoclonal antibody Cat-301. Cat-301 stains many different regions of the nervous system, but within the visual system it...
differentially stains structures related to the M pathway, including the magnocellular LGN layers, layer 4B of area V1, the thick stripes of area V2, and area MT (Hendry et al., 1984, 1988; DeYoe et al., 1990). Cat-301 labels an extracellular-matrix proteoglycan (Zaremba et al., 1989; Fryer et al., 1992; Lander et al., 1997) that is concentrated in the so-called ‘perineuronal nets’ that invest certain classes of neurons (Brückner et al., 1996; Celio et al., 1998).

If the interpretation of layer 4A compartmentation suggested by Preuss et al. (Preuss et al., 1999) is correct, we would expect Cat-301 to yield a mesh-like pattern of staining similar to that seen with NPNF staining, and double-staining studies should demonstrate that Cat-301 is localized within the same tissue bands that stain strongly for NPNF. Weighing against this expectation is a study of Cat-301 immunoreactivity in area V1 of humans (Hockfield et al., 1990), which indicated that layer 4A stains lightly in humans, similar to macaques. However, that study was done using diaminobenzidine (DAB) immunocytochemistry, which is less sensitive than the tetramethylbenzidine (TMB) techniques now available (Weinberg and van Eyck, 1991; Llewellyn-Smith et al., 1993).

In the present study, therefore, we revisited the issue of Cat-301 immunoreactivity in area V1 using TMB histochemistry with tissue obtained from humans, apes (chimpanzees and orangutan), Old World macaque monkeys, and New World squirrel monkeys and spider monkeys. We also directly examined the relationship between NPNF and Cat-301 immunoreactivity in human V1 with a sequential double-staining procedure using alternative and readily distinguishable TMB reaction products. In this procedure, the first antibody was localized with TMB stabilized with DAB and cobalt (TMB–DAB–Co), which yields a reddish-brown reaction product, and the second antibody was localized with TMB alone, which yields a blue reaction product. The concept is similar to sequential double-staining techniques using, for example, DAB in combination with benzidine dihydrochloride (Levey et al., 1986), but exploits the greater sensitivity of TMB. This procedure was adopted in preference to a double-immunofluorescence approach, because the high levels of autofluorescing lipofuscin commonly present in human cortex can obscure labeling, and because immunoperoxidase techniques employing TMB are likely to be more sensitive than immunofluorescence and better suited for demonstrating compartmental patterning of neuropil at relatively low magnification. Furthermore, because NPNF is an intracellular protein and the Cat-301 antigen is an extracellular-matrix constituent, it is unlikely that one reaction product would obscure or interfere with the other in any cells that express both molecules.

A second issue investigated here concerns the calbindin-rich territories in human layer 4A reported by Hendry and Carder (Hendry and Carder, 1993). Although Hendry and Carder did not find similar features in macaques and squirrel monkeys, they did not examine apes, so it has yet to be determined whether calbindin-rich territories are a specialization of the ape–human group or a true human specialization. Therefore, we examined calbindin immunoreactivity in apes as well as in humans, macaques, squirrel monkeys and spider monkeys. In addition, we examined in humans the relationship between layer 4A compartments that express calbindin with those that express NPNF or the Cat-301 antigen using the same double-staining technique described above.

Materials and Methods

Subjects and Tissue

We examined occipital lobes from a total of nine humans (Homo sapiens), six chimpanzees (Pan troglodytes), one orangutan (Pongo pygmaeus), five macaque monkeys (representing Macaca mulatta, M. nemestrina and M. fascicularis), six squirrel monkeys (Saimiri sciureus), and three spider monkeys (Ateles geoffroyi). Subsets of these cases were used for each of the different immunohistochemical experiments, as described below. The human material was obtained from the brain bank of the Northwestern University Alzheimer’s Disease Center (NWADC). The human sample included individuals of both sexes ranging in age from 54 to 78 years of age (median = 70 years). Eight of nine brains were rated as normal control brains on neuropathological criteria by NWADC; one brain showed some evidence of Alzheimer’s-related changes. Post-mortem delays ranged from 5 to 25 h (median = 9 h). Five of the chimpanzees, and the one orangutan, came from the University of Louisiana at Lafayette’s New Iberia Research Center. One additional chimpanzee came from the Yerkes Primate Center. All had died of natural causes or were euthanized for humane reasons. Post-mortem delays for the apes ranged from 0 to 7 h (median = 4 h). The Old World and New World monkey brains came from animals at the New Iberia Research Center and Vanderbilt University. All were sacrificed with a lethal dose of barbiturate. Procedures involving nonhumans were carried out in accordance with guidelines established by the institutional animal care and use committees of the New Iberia Research Center, Yerkes Primate Center and Vanderbilt University.

The human brain tissue examined in this study came from the posterior-most part of the occipital lobe, extending no more than ~5 cm anterior to the occipital pole. The region of human area V1 examined, therefore, included the central and parafocal representations of the visual field in area V1 (Horton and Hoyt, 1991), but not the peripheral field representation, located at more anterior levels of the occipital lobe.

The human brains were blocked and fixed by immersion in phosphate-buffered 2–4% paraformaldehyde for 1–4 days. In some cases, larger brain blocks were subdivided in 1–1.5 cm slabs prior to immersion to reduce fixation artifact. Three chimpanzee brains and one orangutan brain were fixed by immersion in aldehydes for 1–4 days. The solutions used for immersion were buffered 2–4% paraformaldehyde solution (two chimpanzees, one orangutan), buffered 4% paraformaldehyde and 0.15% glutaraldehyde (one chimpanzee), and buffered 10% formalin (one chimpanzee). Three additional chimpanzees were fixed by perfusion. One was fixed at the time of death with buffered 4% paraformaldehyde and 0.1% glutaraldehyde, while two others were perfused post-mortem with buffered 2–4% paraformaldehyde. Two macaque brains, removed within 15 min of death, were fixed by immersion in buffered 4% paraformaldehyde. The other Old World and New World monkey brains were fixed by perfusion with buffered 2–4% paraformaldehyde or in a few cases with a mixture of 2–4% paraformaldehyde and 0.08–0.15% glutaraldehyde.

Following fixation, brain blocks were immersed in sucrose or glycerol solutions to prevent freezing artifact, and then stored in an ethylene-glycol-based cryoprotectant solution (Watson et al., 1986) at -20°C. Prior to sectioning, blocks were immersed in buffered 40% sucrose at 4°C for several days to remove cryoprotectant solution. After cutting, tissue sections were stored in cryoprotectant solution at ~20°C.

Single Immunocytochemistry: Cat-301

We examined Cat-301 immunoreactivity in five humans, five chimpanzees, one orangutan, four macaques, six squirrel monkeys and two spider monkeys. Cat-301 staining intensity was found to vary across species, so we ran series of dilution and incubation trials for each species. As discussed in the Results, final primary antibody dilutions ranged between 1:4 and 1:40, depending on the species, with incubation conditions ranging from overnight at room temperature to 7 days at 4°C. The Cat-301 antibody was kindly supplied by Dr Susan Hockfield (Yale University).

Sections were prepared for immunocytochemistry by rinsing them in Tris-buffered saline (TBS; pH 7.4) to remove cryoprotectant and immersing them in a series of methanol–hydrogen peroxide solutions to inactivate endogenous peroxidase. Sections then underwent a treatment to digest chondroitin, which partially masks the Cat-301 epitope situated in the core of a chondroitin sulfate-containing proteoglycan (Fryer et al., 1992): sections were rinsed for 10 min in chondroitinase buffer (0.1% bovine serum albumin, 0.1% CHAPS, 0.04% sodium acetate anhydrous,
after being stabilized with DAB and cobalt, and the reaction product
incubated overnight at room temperature. After digestion and rinsing,
sections were incubated in Cat-301 supernatant diluted in a solution of 5% 
fetal calf serum, 0.1% Triton, and 0.001–0.1% sodium azide made up in
Dulbecco’s modified Eagle medium. Following incubation, sections were
immersed in biotinylated secondary antibody (12.5 µl/ml; Amersham 
Pharmacia Biotech, Piscataway, NJ, USA) for 2 h, rinsed, and immersed
for 1 h in streptavidin–biotin complex (12.5 µl/ml; Amersham). The
sections were then reacted for 8–10 min in TMB solution (0.025% TMB, 0.05% 
ammonium paratungstate, 0.1% glucose oxidase, 0.2% d-glucone and
0.004% ammonium chloride), and the reaction product stabilized for 8
min in DAB–cobalt solution (0.1% DAB, 0.02% cobalt chloride, 0.1% 
glucose oxidase, 0.2% d-glucone and 0.004% ammonium chloride).
Preparation of these solutions is described by Llewellyn-Smith et al. 
(Llewellyn-Smith et al., 1993). Reacted sections were mounted on
gelatin-coated slides. Selected sections were counterstained for
Nissl with thionin to evaluate the laminar distribution of labeling. In all
experiments, control sections were prepared in the same manner as
positive sections, except that the primary antibody was omitted from
the incubation step. Control sections showed no specific staining.

Single Immunocytochemistry: Calbindin, NPNF and MAP 2
We examined immunoreactivity for calbindin D-28k in six humans, five
chimpanzees, one orangutan, three macaques, three squirrel monkeys
and three spider monkeys using the TMB–DAB–Co technique. In all
species, separate series of sections were stained using a monoclonal
antibody, CL 300 (Cello et al., 1990), obtained from Sigma, and using a
polyclonal antibody, AB 1778, obtained from Chemicon (Temecula, CA, 
USA). With both antibodies, staining intensity varied across species, and
final dilutions employed ranged from 1:1000 to 1:4000 for the monoclonal
antibody and 1:1000 to 1:8000 for the polyclonal. At all dilutions, sections
were incubated overnight at room temperature. To visualize the location
of labeling using these antibodies, sections were first rinsed in TBS to
remove cryoprotectant and immersed in a series of methanol–hydrogen
peroxide solutions to inactivate endogenous peroxidase. Sections were
then blocked with 3% normal sheep or donkey serum for 1.5–3 h,
rinsed in TBS, and incubated in primary antibody under conditions described
above. Triton X-100, typically at a concentration of 0.1%, and 0.1% sodium
azide were added to the blocking and incubation solutions. Following
incubation, sections were immersed in biotinylated secondary antibody
followed by streptavidin–biotin complex, and reacted with TMB, DAB
and cobalt as described above for Cat-301 immunocytochemistry. Reacted
sections were mounted on gelatin-subbed slides, and selected sections
were counterstained with thionin. Negative control sections, prepared by
omitting primary antibody from the incubation solution, showed no
specific staining.

In addition to the TMB–DAB–Co material, we examined calbindin
immunoreactivity using DAB enhanced with imidazole (Preuss et al., 
1999) in six humans, three chimpanzees, one orangutan, three
macaques, four squirrel monkeys and three spider monkeys. These
sections were incubated in a 1:1000 dilution of the calbindin monoclonal
antibody. Results obtained with DAB under these conditions were similar
to those obtained with TMB–DAB–Co, and included the same pattern of
species differences in staining intensity.

We also examined staining for NPNF in three humans and MAP 2 in
two humans to confirm that TMB–DAB–Co immunohistochemistry
yielded results consistent with our published results using DAB
histochemistry (Preuss et al., 1999). NPNF immunoreactivity was
examined using monoclonal antibody SM-32 (Sternerberger Monoclonals,
Luthersville, MD, USA) at dilutions of 1:10 000, with sections incubated
overnight at room temperature. MAP 2 immunoreactivity was examined
using clone HM-2 (Sigma) at dilutions of 1:40 000, with sections
incubated overnight at room temperature. Labeling was visualized using
the TMB–DAB–Co procedures described above.

Double Immunocytochemistry
In single-staining procedures, the initial TMB reaction product was a deep
blue or blue–green color. This changed to a dark, reddish-brown color
after being stabilized with DAB and cobalt, and the reaction product
became even lighter and redder during dehydration and coverslipping.
We took advantage of the differences between the TMB reaction product
and the TMB–DAB–Co reaction product in double-staining experiments:
sections were incubated in a first primary antibody, which was visualized
with TMB stabilized with DAB and cobalt, and then incubated in a second
primary antibody, which was visualized with TMB alone.

In one set of experiments, we used the NPNF antibody (SMI-32) as the
first primary antibody and Cat-301 as the second primary antibody,
staining a total of five humans and three macaques. Sections were
incubated in NPNF (diluted at 1:10 000–1:12 000) overnight at room
temperature, reacted with TMB–DAB–Co, and then incubated in Cat-301
(at dilutions of 1:10–1:40 for humans and 1:10–1:40 for macaques) and
reacted for TMB only. Sections were incubated in Cat-301 for 48 h at
room temperature in one series of experiments, and for 7 days at 4°C in
another series; human and macaque cases were included in both series so
there was no confounding of species and incubation condition. We carried out
chondroitin digestion to unmask the Cat-301 antigen (as described above)
at the beginning of the experiment in most cases, prior to staining for
NPNF, although in some cases sections were digested immediately after
the NPNF step. Similar results were obtained with both procedures.

In a second set of experiments, we stained sections for NPNF and
calbindin, or for Cat-301 and calbindin, using the same polyclonal
calbindin antibody employed in single-staining studies. Sections from
three humans and two macaques were stained for NPNF followed by
calbindin. Three humans and three macaques were stained for Cat-301
followed by calbindin, and three humans and two macaques were stained
for calbindin followed by Cat-301. Similar results were obtained with
either order of staining. NPNF antibody was diluted by 1:12 000 for both
humans and macaques, and sections were incubated overnight at room
temperature. Cat-301 dilutions ranged from 1:10 to 1:20 in humans and
1:20 to 1:40 in macaques, with sections incubated for 2 days at room
temperature. Calbindin antibody was diluted by 1:3000 or 1:4000 for
humans and by 1:4000 for macaques, with sections incubated
overnight at room temperature. Higher concentrations of calbindin antibody
were used in macaques than in humans because in single-staining
experiments, macaques were found to stain more weakly for calbindin than humans
at a given concentration, as described in the Results.

For each case in the double-labeling experiments we prepared
sections under three control conditions. Sections in the positive/negative
condition were stained for the first antibody and then carried through all
the steps of the second reaction, except that the second primary antibody
was omitted from its incubation solution. Sections in the negative/
positive condition were exposed to either of the two primary antibodies.
Sections in the negative/negative condition were exposed to neither of
the primary antibodies. Specific staining in the positive/negative sections
was observed only with the reddish-brown TMB–DAB–Co reaction
product; there was no additional, specific staining with the blue TMB
reaction product. This was true even when the identical species-specific
secondary antibody was used in the first and second reactions, as in the
case of experiments with NPNF and Cat-301, which are both mouse
monoclonals. This is consistent with the report of Levey et al., using a
similar double-staining protocol (Levey et al., 1986). Likewise, negative/
positive sections evinced only specific staining with the blue TMB
reaction product. Negative/positive sections showed no reddish-brown
or blue staining of any kind other than minor and readily identifiable
peroxidase artifacts.

Microscopy, Photography and Image Processing
Sections were examined under brightfield illumination with an Olympus
BX-50 microscope. Digital images were acquired with a Microluma
scanning camera (Leaf Systems, Bedford, MA, USA) and a SPOT digital
camera (Diagnostic Instruments, Sterling Heights, MI, USA). Photoshop
software (Versions 5 and 6, Adobe Systems, Mountainview, CA, USA) was
used to subtract background images and to adjust brightness, contrast
and sharpness. We also used Photoshop to represent separately the
red–brown and blue elements present in double-immunostained sections.
The resulting figures were particularly useful for showing the laminar and
compartamental distributions of different antibodies in the same sections
at relatively low magnification. In a previous publication (Preuss et al.,
1999), we noted that Nissl staining and DAB labeling in Nissl-
counterstained sections can be examined separately by using Photo-
shop’s tools for splitting color channels. This simple technique proved inadequate for separating elements in our double-immunostained material, however, because the red–brown of the TMB–DAB–Co reaction product and the blue of the TMB product were relatively unsaturated, consisting of mixtures of red, green and blue hues (which varied somewhat from experiment to experiment), rather than pure hues.

In the present study, therefore, we used Photoshop’s Eyedropper tool to extract unique subsets of pixels representing the TMB–DAB–Co and TMB reaction products, respectively. To take an example, we began by selecting with the Eyedropper tool a pixel from a cell or neurite that was clearly stained with TMB–DAB–Co only (i.e. a red-brown pixel). We then used the Color Range function to select pixels with RGB values similar to those of the seed pixel, adjusting the size of the envelope of selected values with the Fuzziness slider, and then copied and pasted the resulting pixel set into a new image space. We repeated this process several times, choosing seed pixels that spanned the range of RGB values representing the TMB–DAB–Co labeling. After building up an image of the red–brown labeling, we selected blue pixel sets in the same manner, building up by iteration an image of the neural elements labeled with the blue TMB reaction product. At each step in building the blue image, we chose only pixels that were not present in the extracted set of red-brown pixels.

Similar results were obtained by reversing the order of selection, extracting a blue pixel subset followed by a red-brown subset. Results were improved by doing many iterations and choosing pixels covering a narrow range of RGB values at each iteration, rather than by doing few iterations that each covered broad ranges of RGB values.

### Laminar Analysis

We examined the laminar distribution of Cat-301 and calbindin labeling in single-immunostained sections counterstained for Nissl substance with thionin. Selected sections were photographed and Photoshop was employed to extract the red pixels representing the TMB–DAB–Co reaction product from the blue pixels representing thionin-stained elements, using either the Eyedropper tool (as described above) or by splitting color channels (Preuss et al., 1999).

Several different schemes exist for numbering the layers of area V1 in anthropoid primates (Brodmann, 1909; Hässler and Wagner, 1965; Braak, 1976; Casagrande and Kaas, 1994; Kalix, 1994). Most modern studies, however, follow a system based on Brodmann (Brodmann, 1909), and elaborated for macaques by Lund (Lund, 1973) (see especially Fig. 1 of that paper), and we follow that system here.

How layer 4 is subdivided in the Brodmann–Lund system is especially pertinent to this study. Brodmann distinguished three subdivisions of this layer. The deepest stratum, 4C, is recognizable by its extremely dense cell packing. Packing density is somewhat less in the upper part of 4C than in the deeper part, so that separate upper (4Ca) and lower (4Cb) subdivisions of this stratum can be recognized (Polyak, 1937). Layer 4B is relatively cell sparse compared to 4A and 4C, and contains scattered, rather large cells (the outer solitary cells of Meynert) in addition to granule cells. Layer 4A is more densely packed with small cells than 4B and lacks the prominent outer Meynert cells. The border between 4B and 4A can usually be identified with confidence. The upper border of layer 4A, however, is often not very distinct, as the deep part of layer 3 may appear only slightly less dense than layer 4A (Peters, 1994). Layer 4A has been considered especially indistinct in humans (Wong-Riley et al., 1993), and perhaps even absent (Horton and Hedley-Whyte, 1984). Nevertheless, 4A was recognized in humans by Brodmann (Brodmann, 1909), and modern workers have largely followed suit (Braak, 1976; Hendry and Corder, 1993; Wong-Riley et al., 1993; Jones et al., 1994; Yoshioka and Hendry, 1995). In our thionin-stained material, layer 4A was perhaps less distinct in humans than in macaques, but could usually be delineated.

While published descriptions of macaque and human cytoarchitectonic organization provide a useful foundation for comparative studies of anthropoid primate area V1, there is at least one noteworthy variation. Le Gros Clark observed that in some New World monkey species, the solitary cells of layer 4B are not really so solitary, but aggregate to form a well-defined band (Le Gros Clark, 1942). Our observations of the New World monkey Ateles were consistent with this, although layer 4B in the New World monkey Saimiri was usually cell sparse. Some individual chimpanzees (an Old World anthropoid species) also exhibited aggregations of large cells in layer 4B.

### Results

**Cat-301 Immunoreactivity in Humans**

In humans, immunocytochemistry with Cat-301 produced dense staining in the primary visual area and in neighboring cortical areas (Fig. 1). Area V1 was distinguished in Cat-301-stained sections by its well-stratified appearance, with a prominent band of very dark staining in the middle cortical layers. The superficial border of this band had an irregular appearance, with alternating territories of light and dark immunoreactivity. These territories were present at all levels of area V1 we examined, which spanned approximately the posterior half of the area, corresponding to both the central 10° of the visual field (Horton and Hoyt, 1991). We did not observe any systematic variation in the pattern of layer 4A staining over this extent of V1, although the irregular nature of the pattern could make subtle variations difficult to detect without quantitative analysis.

Examination of Cat-301-stained sections counterstained for Nissl (Fig. 2) confirmed that the irregularly shaped territories of dark and light Cat-301 immunoreactivity were located in layer 4A. The dark-staining bands of tissue consisted of very fine neuropil, along with some larger, individually distinguishable neurites, and cell bodies with nonpyramidal morphologies (Fig. 3). Most Cat-301-immunoreactive (Cat-301+) cell bodies were small, ∼10–20 µm in diameter, although scattered larger neurons, on the order of 20–25 µm, were also observed. The neuropil bands commonly extended through the thickness of layer 4A, giving off lateral branches that encapsulated territories of light staining. In some instances, vertical stacks of two or more small capsules were observed to span layer 4A. The lightly stained territories were extremely variable in size and shape. The most well-defined, capsule-like territories were typically on the order of 80–100 µm in cross-sectional diameter, although some territories were much larger.

The dark-staining territories of layer 4A were continuous with the tissue of layer 4B, which showed very strong cell-body and neuropil labeling with Cat-301 (Fig. 3). Layer 4B contained cells similar in morphology and size to those in layer 4A, although larger neurons (20–25 µm) were more numerous. Layer 4Ca also contained numerous labeled nonpyramidal cells, although neuropil labeling was lighter than in layer 4B. Layer 4Cb contained some small, lightly stained neurons and little neuropil staining. In the superficial strata, Cat-301+ cell bodies were particularly numerous in the deep part of layer 3. In addition, there was a group of large cells present in upper layer 3 and layer 2 that stained relatively completely with Cat-301, revealing elaborate, multipolar dendritic arbors (see especially Fig. 5A). In some sections, patchy or columnar aggregations of stained cells and neuropil were observed in layer 3 (Figs 1 and 2C), consistent with reports that Cat-301 stains blobs (puffs) in area V1 of primates (Hockfield and McKay, 1983; Hendry et al., 1984, 1988). There was an additional band of Cat-301+ cells and neuropil located deep in the cortex, corresponding to layers 5 and 6. Within this band was a line of very large, well-stained cells in the deepest part of layer 5 (Figs 2A–C and 4A), corresponding to the giant Meynert–Cajal cells (Braak, 1976).

The network of dark, Cat-301+ tissue bands in layer 4A was very similar in form to the pattern of staining produced with NPNF or MAP 2 antibodies, as illustrated in Figure 4. The distribution of staining for NPNF and MAP 2 we obtained using the TMB–DAB–Co technique in the present study closely matched
Cat-301 Immunoreactivity in Humans Compared with Nonhuman Primates

In nonhuman primates, as in humans, immunostaining with Cat-301 yielded separate main bands of dark label in the middle and deep strata of the cortex, corresponding to layer 4B (in some cases extending into layer 4Ca, also), and in layers 5 and 6 (Fig. 5). However, none of the nonhuman primate species we examined showed the mesh-like pattern of darkly stained tissue bands surrounding lightly stained territories that was present in layer 4A of humans. Rather, in all nonhuman species, layer 4A appeared as a relatively light stratum, with a modest number of stained cells and dendrites.

Additional phyletic differences were noted, including strong variations in the apparent strength of Cat-301 immunoreactivity. Of the taxa examined, macaques and spider monkeys required the shortest incubation periods (1 day) and the lowest antibody concentrations (1:20–1:40) to yield strong immunostaining. In humans, strong staining required somewhat higher antibody concentrations (1:10–1:20) than those optimal for macaques; longer incubation times also resulted in enhanced staining, although strong staining was obtained in many cases with 1 day incubation periods. Squirrel monkeys showed the weakest labeling of the taxa examined, requiring long incubations (7 days) in high concentrations of primary antibody (1:4–1:8) to yield consistent staining, and even under those conditions we observed less specific staining of V1 than in humans, macaques, or spider monkeys. The great apes (chimpanzees, orangutan) also showed generally weak Cat-301 immunoreactivity, even with long incubation periods and high antibody concentrations, although individual neurons or classes of neurons were very well stained in some cases that otherwise exhibited weak staining. Strong species differences in Cat-301 staining intensity have been noted in other investigations (Hendry et al., 1988; Jain et al., 1994; Preuss et al., 1998). In addition to these general species differences in immunoreactivity, there were several marked differences in the specific laminar distribution of Cat-301 staining, particularly in the deepest layers of cortex. In macaques, and in the one orangutan examined, layer 6 was the most densely stained stratum of the cortex, and labeled cells were especially numerous in the deepest part of layer 6. In humans and chimpanzees, by contrast, layer 4B was the most densely stained layer.

Relationship of Cat-301 and NPNF Staining in Humans

As noted above, Cat-301 immunostaining yielded intermingled territories of dark and light staining in human layer 4A that resembled the pattern of labeling observed with NPNF and MAP 2 immunostaining. Double-staining studies indicated that the dark and light territories of Cat-301 immunoreactivity were coincident with dark and light zones of NPNF immunoreactivity. This is readily apparent in low-magnification photomicrographs of double-stained sections with color-processing separation of NPNF+ and Cat-301+ elements (Fig. 6A–C). At higher magnification, we observed bands of tissue containing numerous, intermingled neurites and somas that were positive for Cat-301.
or NPNF elements, separated by territories that contained very few stained elements (Fig. 6D,E). Within the darkly stained territories, many Cat-301+ neurites appeared to be closely apposed to NPNF+ neurites, although we observed no unambiguously double-labeled neuronal elements in layer 4A. Double-stained cell bodies were observed in other layers, however, including some of the giant Meynert–Cajal cells in deepest layer 5 (not illustrated). The appearance of individual cell bodies and neurites stained for Cat-301 was consistent with that observed in single-stained preparations (Fig. 6C,D): Cat-301 stained primarily small- to medium-sized multipolar, nonpyramidal neurons and fine, diffuse neuropil in 4A, while NPNF-immunoreactive cell bodies appeared to be somewhat larger on average and included both nonpyramidal and small pyramidal profiles. The neurite complement that stained for NPNF included dendrites extending into layer 4A from deep layer 3 and from 4B; the latter included thick apical dendrites arising from large pyramidal cells, as well as the finer processes of smaller cells.

Figure 2. Laminar analysis of Cat-301 immunoreactivity in human area V1. (A) shows a photomicrograph of a section immunostained for Cat-301 and counterstained for Nissl. From this image, separate representations of Nissl staining (B) and Cat-301 immunoreactivity (C) have been extracted by selecting arrays of blue pixels and red–brown pixels, respectively. (D) shows a higher-magnification photomicrograph of a section stained for Cat-301 and for Nissl. The locations of light territories outlined by dark Cat-301 staining are marked with stars. (E) shows the location of blue pixels, representing Nissl-stained elements extracted from (D), along with stars marking the locations of territories that stain lightly for Cat-301. The mesh-like pattern of Cat-301 staining is located within layer 4A. Scales: 250 µm.

Calbindin Immunoreactivity in Humans Compared with Other Primates
As a first step in elucidating the relationship of calbindin distribution in human layer 4A to that of Cat-301 and NPNF, we examined an extensive series of sections from humans and other primates stained with a monoclonal and a polyclonal antibody for calbindin. In all taxa examined, calbindin antibodies stained large numbers of small neurons in layers 2–4, and smaller numbers of cells in layers 5 and 6 (Fig. 7). Extensive staining of fine neuropil was also observed, especially in the upper layers, and in some taxa, dense neuropil staining outlined more lightly stained territories that presumably correspond to blobs, as reported previously (Blümcke and Celio, 1992; Hendry and Carder, 1993). Among the primates examined, however, only humans displayed irregularly shaped territories of dense immunoreactivity in layer 4A that stood out against the more moderately stained layers 5 and 4B (Figs 7A,B and 8A). These territories consisted of very darkly stained, small (~10 µm in diameter), round somas surrounded by very fine neuropil. The
lightly stained territories surrounding the dark zones also contained calbindin+ cell bodies and neuropil, although these elements were less numerous and more lightly stained than in the dark-staining zones. In apes, calbindin staining in layer 4A was more uniformly distributed than in humans, and of more moderate density. Staining of chimpanzee layer 4A was...
comparable in density to layer 4B (Figs 7C and 8B). The single orangutan we examined was similar to the chimpanzees, although layer 4A appeared slightly less densely stained than 3 and 4B in most sections (Fig. 7D). The Old World and New World monkeys examined all exhibited a prominent, relatively uniform, label-sparse band in layer 4A, sandwiched between well-stained layers 3 and 4B (Figs 7E-H and 8C). In all species examined, the calbindin+ neurons in layer 4A had small, round cell bodies, which resemble calbindin-containing GABAergic interneurons described in other layers of area V1 (Van Brederode et al., 1990; Jones et al., 1994).

As with Cat-301, we noted substantial differences between species in the apparent strength of staining for calbindin. Pan and Saimiri yielded the most robust staining, and Macaca the weakest, such that relatively high concentrations of the polyclonal primary antibody (1:1000–1:2000) were required to produce levels of cell staining in Macaca comparable to those obtained with lower concentrations (1:6000–1:8000) in Pan and Saimiri, and even at these higher concentrations, neuropil staining in Macaca was relatively weak. A similar relationship between species and staining intensity was observed with the monoclonal antibody.

**Relationship of Calbindin Immunoreactivity to NPNF and Cat-301 Immunoreactivity in Humans and Macaques**

Double-staining experiments revealed a complementary relationship between calbindin immunoreactivity and staining for
NPNF and Cat-301 in human layer 4A. In all human cases examined, tissue zones that stained densely for calbindin corresponded to territories that stained lightly for NPNF or for Cat-301 (Figs 9 and 10A–C). As in our single-stained material, the dense-staining calbindin+ zones were filled with small, intensely immunoreactive neurons and neuropil. The tissue bands that stained strongly for NPNF and Cat-301 also contained some calbindin+ neurons. We observed no unambiguously double-stained elements in layer 4A, although individual cell bodies that stained for both calbindin and Cat-301 were observed in other layers, and were especially prominent in the upper part of layer 3.

Comparison of human and macaque sections double-stained for Cat-301 and calbindin (Fig. 10D,E) highlighted the differences between these taxa noted in single-stained sections. In humans, layer 4A was filled with intermingled territories of Cat-301 and calbindin immunoreactivity. In macaques, by contrast, layer 4A was a label-sparse band, the dominant elements being dendrites extending through this layer from somas residing in neighboring layers. Similar results were obtained

Figure 6. Photographs of double-immunostained sections from human area V1, illustrating the localization of NPNF and Cat-301 within the same tissue compartments of layer 4A. (A) A section stained for NPNF with TMB–DAB–Co, which yields a reddish reaction product, followed by incubation with Cat-301 and reaction with TMB only, which yields a blue reaction product. (B) An array of red–brown pixels selected from the image in (A), indicating the location of NPNF immunoreactivity. (C) An array of blue pixels, extracted from the image in (A), indicating the location of Cat-301 immunoreactivity. (D,E) Higher-magnification photomicrographs showing layers 4A and 4B in sections double-stained for NPNF (red) and Cat-301 (blue). (D) shows the region enclosed within the box in (A); (E) is from a different case. Scales: (A–C) = 250 µm; (D, E) = 100 µm.
comparing human and macaque sections double-stained for NPNF and calbindin (not illustrated).

**Discussion**

**Evidence for Two Alternating, Neurochemically Distinct Tissue Compartments in Human Layer 4A**

Immunostaining for Cat-301 in human area V1 revealed a mesh-like pattern of staining, with dark-staining tissue bands surrounding territories of light staining, very similar to the pattern observed after staining for NPNF or MAP 2. The immunoreactive bands consisted of fine neuropil, along with some larger, more distinct dendritic branches and small-to-medium-sized nonpyramidal neurons. In double-staining experiments, Cat-301 and NPNF labeled the same tissue compartments within layer 4A. Of the primate taxa we examined, which included apes, Old World monkeys, and New World monkeys, only humans exhibited substantial Cat-301 immunoreactivity in layer 4A, and only humans showed a mesh-like distribution of staining in layer 4A.

Immunostaining for calbindin also revealed an irregular pattern of darkly stained territories in layer 4A of humans, but not in great apes, Old World monkeys, or New World monkeys. The dark zones consisted of numerous small, round cells and neuropil; lighter calbindin staining was observed outside the dark zones. Double-staining experiments indicated that the zones of dense calbindin immunoreactivity were located in the territories within layer 4A that stained lightly for NPNF and Cat-301.

Our results thus indicate that human layer 4A is composed of two alternating types of tissue compartments, one compartment being a meshwork of tissue bands that strongly express the Cat-301 antigen and NPNF, and the other consisting of calbindin-dense territories set in the interstitial spaces of the meshwork. The organization of layer 4A in human area V1 is distinctly different from that of the other primate species we examined, including chimpanzees (Pan), the animals most closely related to humans, and macaque monkeys, the primates that have been most intensively studied, as models of the human visual system. Compared to other primates, human layer 4A shows increased staining for molecules related to the M pathway.

**Comparison with Previous Studies of Cat-301 Immunoreactivity in Primate V1**

Most studies of Cat-301 immunoreactivity in area V1 of primates have focused on macaque monkeys (Hockfield et al., 1983; Hendry et al., 1984, 1988; DeYoe et al., 1990; Hockfield and Sur, 1990; Hockfield et al., 1990; Jain et al., 1994), although there are also reports on humans (Hockfield et al., 1990) and prosimian primates (Hendry et al., 1988; Jain et al., 1994). There are no previous published accounts of Cat-301 immunoreactivity in the visual cortex of apes or New World monkeys.

Published studies of Cat-301 immunoreactivity in macaques indicate these animals display two distinct strata of strong cell and neuropil staining, corresponding to layer 4B (or 4B plus 4Cα) and layer 6, the latter being most strongly stained. Staining of individual neurons or small clusters of neurons is observed in other layers. The majority of stained cells have nonpyramidal
morphologies. Hockfield et al. reported that humans display a similar distribution of staining, with a very prominent upper band of staining corresponding to 4B/4Ca, and a deep band corresponding to layer 6, although staining of the deep stratum is weaker in humans than macaques, so that 4B/4Ca is the most prominent stratum in humans (Hockfield et al., 1990). They did not note substantial Cat-301 immunoreactivity in layer 4A of humans, nor any difference between humans and macaques in the appearance of 4A. Our observations of Cat-301 staining in macaques are consistent with published accounts. In humans, furthermore, consistent with Hockfield et al., we observed that staining of layer 6 was weak compared to staining of layers 4B/4Ca. Unlike Hockfield et al., however, we also observed territories of strong Cat-301 immunoreactivity in layer 4A of humans, which distinguished humans from the other primates we examined.

We suggest that the disparity between the present results and previously published studies with Cat-301 can be attributed primarily to the greater sensitivity of the TMB–DAB–Co technique compared to the DAB methods used in previous studies. The difference is exemplified by the greater number of stained cells visible at low magnification and the more extensive staining of dendritic trees achieved in the present study compared with other published studies using Cat-301. TMB replaced DAB as the preferred chromagen in studies of connectivity with horseradish peroxidase about two decades ago, owing to the much greater sensitivity of TMB (Mesulam and Rosene, 1979), but successful adaptation of TMB for use with immunocytochemistry was not achieved until more recently, when techniques were developed to stabilize the reaction product at the relatively high pH required for immunocytochemistry (Liang and Wan, 1989; Norgren and Lehman, 1989; Weinberg and van Eyck, 1991; Llewellyn-Smith et al., 1993). TMB also has the merit of being readily adapted for use in sequential double-labeling experiments, such as those presented here. There are, to be sure, disadvantages to TMB compared to DAB. We have found it more difficult to control nonspecific background staining with TMB than with DAB, and it sometimes produces granular artifacts. These problems are most acute when TMB is used without DAB–Co stabilization, as for instance in the second stage of double staining, because the stabilization procedure tends to clear the background. Nevertheless, for many purposes, the benefits of TMB immunocytochemistry outweigh its shortcomings.

Comparison with Previous Studies of Calbindin Immunoreactivity in Primate V1

In all the primate taxa we examined, we observed numerous calbindin+ neurons in layers 2, 3, 4B, 4Ca and 4Cb, with scattered neurons present in layers 5 and 6. Neuronal labeling was also prominent in the upper layers of most taxa, although not in macaques. Previous studies of calbindin distribution in area V1 of primates all demonstrated cell-body labeling in layers 2 and 3, but the strength of neuropil staining and cell-body staining in layers 4–6 has varied. The laminar patterns of staining we observed are very similar to patterns observed in studies which demonstrated staining of the deeper cortical layers, in addition to layers 2 and 3 (Homo: Hendry and Carder, 1993; Yoshioka and Hendry, 1995; Yan et al., 1997; Letinic and Kostovic, 1998); Macaca: (Van Brederode et al., 1990; Blümcke et al., 1994; Yoshioka and Hendry, 1995; Peters and Sethares, 1997); Saimiri: (Blümcke and Celio, 1992; Hendry and Carder, 1993); Callithrix: (Goodchild and Martin, 1998).

It is noteworthy that in all the Old World and New World monkeys that we examined layer 4A stood out as a relatively light band between more darkly stained layers 3 and 4B. This is a common feature of published reports for Old World and New World monkeys. There is one discrepant report, for the New World monkey Callithrix by Goodchild and Martin (Goodchild and Martin, 1998), who demonstrated a laminar pattern of...
labeling very similar to other New World and Old World monkeys, but who designated the cell-sparse band as layer 4B rather than 4A.

Hendry and Carder observed that calbindin staining of layer 4A in humans differs markedly from that of Old World and New World monkeys: layer 4A is label sparse in monkeys, while humans possess irregularly shaped zones of dense calbindin staining (Hendry and Carder, 1993). Results of the present study confirm these observations, as have other reports for humans (Yoshioka and Hendry, 1995; Yan et al., 1997; Letinic and Kostovic, 1998). The clear difference between humans, on the one hand, and Old World and New World monkeys, on the other, raises the question of whether apes are human-like or monkey-like. Our observations indicate that apes differ from both humans and monkeys: apes display more staining of layer 4A than do monkeys, and thus lack a prominent label-sparse band, but the staining is weaker than in humans (being comparable only to the level of staining in layer 4B) and is not segregated into label-rich and label-poor territories. This suggests that the compartmental pattern observed in humans is a true human specialization, which evolved from a condition similar to that found in living chimpanzees.

Although we confirmed the previous results of Hendry and Carder (Hendry and Carder, 1993) regarding the distinctive appearance of calbindin staining in human layer 4A, the results of our double-staining experiments suggest a different interpretation of human layer 4A organization than that proposed by Yoshioka and Hendry (Yoshioka and Hendry 1995). They stained...
adjacent tangential (flattened) sections separately for NPNF and for calbindin, and concluded that NPNF and calbindin occupy complementary compartments, which is consistent with our observations of coronal sections double stained for these proteins. They maintained, however, that human layer 4A has an essentially macaque-like, honeycomb organization, whereas our results suggest there are substantial differences between humans and macaques in the spatial arrangement of neurons in layer 4A, as well as in their neurochemical phenotypes. Yoshioka and Hendry also indicated that the calcium-binding protein parvalbumin is distributed in a non-homogeneous manner in layer 4A, and localizes within the same compartments as calbindin. We have not examined parvalbumin immunoreactivity in flattened sections, but parvalbumin has a relatively uniform appearance in coronal sections through layer 4A of humans (Blümcke et al., 1990; Jones et al., 1994; Yoshioka and Hendry, 1995; Letinic and Kostovic, 1998), quite different from the irregular distribution of calbindin which can be readily seen in coronal sections.

**Organization and Evolution of Layer 4A in Old World and New World Monkeys**

The present results highlight the existence of substantial differences in the cellular architecture of layer 4A in humans and nonhuman primates, as summarized in Figure 11. The organization of macaque layer 4A has been very well characterized. It receives a direct geniculate projection, arising from the parvocellular layers (Lund, 1973; Hendrickson et al., 1978; Blasdel and Lund, 1983; Horton, 1984). Macaque 4A also

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**Figure 10.** Double-immunostaining for Cat-301 and calbindin in area V1 in humans and macaques, illustrating the complementary distribution of these antigens in layer 4A. Cat-301 is labeled with the reddish-brown TMB–DAB–Co reaction product, calbindin with the blue TMB reaction product. (A) A double-stained section from human V1, from which reddish-brown and blue pixels have been extracted, as shown in (B) and (C), respectively. (D,E) Higher-magnification photomicrographs of the middle cortical layers of V1 in Homo and Macaca. Human layer 4A contains dense Cat-301 and calbindin immunoreactivity, concentrated in alternating compartments. Macaque layer 4A, by contrast, stands out as a relatively light band, lacking either strong Cat-301 or calbindin staining. Scales: (A–C) = 250 µm; (D,E) = 100 µm.
receives afferents from layer 4C (Fitzpatrick et al., 1985; Yoshioka et al., 1994), itself a target of P-geniculate projections. Examination of sections cut tangential to the pial surface indicates that the sheet of geniculate-recipient tissue in 4A is punctuated by regular gaps, resulting in a honeycomb-like appearance (Hendrickson et al., 1978). The honeycomb can also be seen in tangential sections stained for CO (Horton, 1984; Fitzpatrick et al., 1985; Hevner and Wong-Riley, 1990), which presumably reflects the high level of activity of geniculostriate afferents. The pyramidal cell bodies and apical dendrites that extend upward from layer 4B into 4A, filling the gaps in the honeycomb walls, can also be visualized histochemically, using antibodies that preferentially label pyramidal cells, such as the MAP 2 (Peters and Sathares, 1991; Hendry and Bhandari, 1992) and NPNF antibodies (Hof and Morrison, 1995; Yoshioka and Hendry, 1995; Chaudhuri et al., 1996; Hof et al., 1996; Preuss et al., 1999). The affinity of these pyramidal-cell ‘cones’ (Peters and Sathares, 1991) or ‘cores’ (Hendry and Bhandari, 1992) to the M, P and K pathways has been less clear than that of the honeycomb walls, which are clearly P related. Peters and Sathares (Peters and Sathares, 1991) suggested that since these clusters of cells and dendrites are surrounded by P-geniculate afferents, they are well placed to receive P inputs. Hendry and Bhandari (Hendry and Bhandari, 1992) indicated that the clusters should be related to the M pathway, because they are rooted in layer 4B, and 4B receives input from layer 4Ca, which is a major target of projections from the magnocellular geniculate layers. Recent physiological evidence, however, suggests these cells receive inputs from both the M and the P pathways (Yabuta et al., 2001).

Layer 4A of macaque monkeys is thus composed of two separate compartments, one related to the P pathway (the honeycomb walls) and a second that is probably related to both the M and P pathways (the pyramidal-cell cores). Although other primate species have not been studied in as much detail as have macaques, there is considerable information available about layer 4A from a variety of species, information that suggests the honeycomb architecture is present in the majority of New World and Old World monkeys. This evidence comes from studies of thalamocortical connectivity (Wiesel et al., 1974; Hendrickson et al., 1978; DeBruyn and Casagrande, 1981; Diamond et al., 1985; Florence et al., 1986; Spatz, 1989), CO staining (Carroll and Wong-Riley, 1984; Horton and Hedley-Whyte, 1984; Hess and Edwards, 1987; Spatz et al., 1994; Chaudhuri et al., 1995; Preuss et al., 1999) and NPNF immunohistochemistry (Hof and Morrison, 1995, Chaudhuri et al., 1996; Hof et al., 1996; Preuss et al., 1999). The broad representation of anatomical features associated with the honeycomb architecture among New World and Old World anthropoids indicates that ancestral anthropoids probably had a honeycomb-like layer 4A (Preuss et al., 1999).

There is at least one group of monkeys, however, that lacks a honeycomb organization: the New World monkeys of the genus Aotus, the owl monkeys. Owl monkeys lack a direct P-geniculate projection to layer 4A (Horton, 1984) and also lack the dense band of CO staining that accompanies that projection in other monkeys (Kaas et al., 1976; Horton, 1984; Diamond et al., 1985). These changes may reflect the owl monkey’s unusual activity pattern: owl monkeys are the only nocturnal anthropoids, and presumably are less dependent than other anthropoids on the P system, the function of which is best suited to photopic conditions.

Organization and Evolution of Layer 4A in Hominoid Primates

There is another group of primates that departs from the ancestral, honeycomb-like organization of layer 4A: the ape–human group. There is evidence for at least two sets of evolutionary modifications in this group, one that left its mark on both apes and humans, and a second that affected only humans (Fig. 11). The first set of modifications is indicated by changes in CO and calbindin staining. As originally reported by Horton and Hedley-Whyte (Horton and Hedley-Whyte, 1984), there is no CO-dense band in layer 4A of humans (Hendry and Carder, 1993; Wong-Riley et al., 1993; Clarke, 1994; Yoshioka and Hendry, 1995; Preuss et al., 1999), nor is there in the apes (chimpanzees, orangutan) that have been examined (Preuss et al., 1999). This result is unlikely to be an artifact of poor post-mortem preservation, as the band was found to be absent even in chimpanzees fixed by perfusion, but was present in macaques fixed by immersion (Preuss et al., 1999). By analogy to owl monkeys, it has been suggested that the absence of a CO-dense 4A band in humans and chimpanzees may indicate that the direct P-geniculate projection to layer 4A present in most monkeys was reduced or lost in apes and humans (Horton and Hedley-Whyte, 1984; Wong-Riley et al., 1993; Preuss et al., 1999).

Figure 11. A reconstruction of layer 4A evolution in anthropoid primates. The photomicrograph set illustrates the condition of layer 4A and adjacent layers in sections stained for CO, calbindin, NPNF and Cat-301 in a New World monkey (Saimiri), an Old World monkey (Macaca) and two hominoids (Pan and Homo). The phylogenetic diagram illustrates the branching tree of relationships among anthropoid primates as currently understood (Purvis, 1998). The letter code beneath the name of each genus represents the kinds of data published for that genus, as follows: (a) the presence or absence of direct projections from LGN to 4A, (b) the presence or absence of a CO-dense band, (c) level of expression of calbindin in 4A, (d) pattern of expression of NPNF, and (e) the pattern of expression of Cat-301. Data are available for 13 of the 36 anthropoid genera recognized by Purvis (Purvis, 1995). Some genera that genus, as follows: (a) the presence or absence of direct projections from LGN to 4A, (b) the presence or absence of a CO-dense band, (c) level of expression of calbindin in 4A, (d) pattern of expression of NPNF, and (e) the pattern of expression of Cat-301. Data are available for 13 of the 36 anthropoid genera recognized by Purvis (Purvis, 1995). Some genera (Saimiri, Macaca) have been evaluated in all these aspects of organization, whereas for others (e.g. Erythrocebus) much more limited data are available. Citations are presented in the text. The reconstruction was generated using MacClade software (Maddison and Maddison, 1992), which is routinely used in studies of molecular and morphological evolution. The software takes as inputs the branching pattern of relationships among taxa and the state of each character for each taxon; missing data are permitted. The software then determines the sequence of changes that yields the observed distribution of character states with the smallest number of changes (the maximum parsimony method). As an integral part of the analysis, the software reconstructs the ancestral set of character states and identifies branches in which transformations occurred. The reconstruction indicates that the last common ancestor of living anthropoid primates probably had a honeycomb-like layer 4A organization, similar to that present in modern macaque monkeys and most other Old World and New World monkeys that have been examined. The hallmark of ancestral organization are direct LGN inputs, a CO-dense band, low expression of calbindin, limited expression of NPNF (largely restricted to pyramidal-cell cones and associated apical dendrites), and low expression of the Cat-301 antigen. This ancestral organization was modified at no fewer than two points in human phylogeny. The first set of changes (denoted by filled arrows in both the photograph set and the phylogenetic diagram), which occurred early in hominoid evolution, prior to the divergence of the Pongo, Pan and Homo lineages, entailed loss of the CO-dense band and increased expression of calbindin. The reduction of CO may have occurred in conjunction with the reduction or loss of the direct LGN afferents, as occurred in the evolution of owl monkeys (Aotus). Definitive connectional evidence for apes and humans is lacking, however. Also associated with the evolution of hominoids was a conspicuous increase in expression of NPNF in layer 3 (denoted with the asterisk), as discussed in Preuss et al. (Preuss et al., 1999). The second set of changes (denoted by open arrows), occurred more recently in human evolution, after the divergence of the Pan and Homo lineages, and were associated with the appearance of a mesh-like pattern of NPNF and Cat-301 expression, and the dense expression of calbindin in the interstitial tissue zones. Vernacular names for primate genera are as follows: Saimiri – squirrel monkeys, Cebus – capuchin monkeys, Callithrix – marmosets, Aotus – owl monkeys, Atelis – spider monkeys, Macaca – macaque monkeys, Papio – baboons, Cercopithecus – guenons (including green monkeys), Erythrocebus – patas monkeys, Mipitecicus – talapoin monkeys, Pongo – orangutans, Pan – chimpanzees, Homo – humans.
Alternatively, the P-geniculate projection may be more dispersed in apes and humans, spanning layers 4B and 4A, rather than occupying a narrow band, as it does in most monkeys (Preuss et al., 1999). This possibility is suggested by the observation that CO staining tapers off rather gradually in the cortex superficial to layer 4C in apes and humans, in contrast to monkeys, where the CO-dense bands of layers 4C and 4A are very sharply delimited (Fig. 11, top right).

The connectional evidence bearing on the hypothesis that P-geniculate projections were modified in hominoid evolution is meager, and is apparently limited to a transneuronal transport experiment in a single chimpanzee (Tigges and Tigges, 1979) and an analysis of degenerating fibers in one human with a lesion involving the geniculostriate projection (Miklossy, 1992). In both studies, there was evidence of strong geniculate projections to 4C, but no clear evidence of a second, more superficial band of projections corresponding to layer 4A. There is some additional, albeit indirect, evidence regarding the
possible modification of geniculostriate afferent distribution. In macaques, the calcium-binding protein parvalbumin is present in geniculostriate projection neurons (Jones and Hendry, 1989). Blümcke et al. indicate that while both macaques and humans have a dense plexus of parvalbumin-immunoreactive fibers in layer 4C, which presumably are geniculostriate afferents, only macaques have an additional dense plexus in layer 4A (Blümcke et al., 1990). This difference is disputed by Yoshioka and Hendry (Yoshioka and Hendry, 1995), however. Additionally, in macaques, GABA-containing terminals and GABA receptors are enriched in layers that receive direct geniculate inputs, including layer 4A, and are distributed in that layer in a honeycomb pattern similar to geniculo cortical afferents (Shaw and Cyonader, 1986; Fitzpatrick et al., 1987; Hendry et al., 1990, 1994; Jones et al., 1994). In humans, by contrast, Albin et al. reported the lack of a distinct band of GABAβ binding corresponding to layer 4A (Albin et al., 1991). Similar results have been reported from immunocytochemical studies of GABAβ receptor subunit distribution by Jones et al. (Jones et al., 1994). Muñoz et al. reported that macaques display strong immunostaining for GABAβ1a,b receptors in layers 4A, 4Cβ and upper layer 6; all layers that receive direct P-geniculate projections (Muñoz et al., 2001). They found humans to have strong staining of layers 4Cβ and 6, but immunoreactive neurons in layer 4A were less numerous and less intensely stained than in macaque 4A. The balance of available evidence thus suggests that the laminar distribution of geniculostriate projections in apes and humans differs from that of macaques and most other monkeys, and more specifically, that there is a decreased influence of the P geniculate laminae on layer 4A.

Another difference that distinguishes apes and humans from Old World and New World monkeys is an increase in calbindin staining of neurons and neuropil in layer 4A. The calbindin-stained neurons are small, round, multipolar cells, which are staining of neurons and neuropil layer 4A. The calbindin-staining of neurons and neuropil layer 4A (Blümcke et al., 1990). This difference is disputed by Yoshioka and Hendry (Yoshioka and Hendry, 1995), however. Additionally, in macaques, GABA-containing terminals and GABA receptors are enriched in layers that receive direct geniculate inputs, including layer 4A, and are distributed in that layer in a honeycomb pattern similar to geniculo cortical afferents (Shaw and Cyonader, 1986; Fitzpatrick et al., 1987; Hendry et al., 1990, 1994; Jones et al., 1994). In humans, by contrast, Albin et al. reported the lack of a distinct band of GABAβ binding corresponding to layer 4A (Albin et al., 1991). Similar results have been reported from immunocytochemical studies of GABAβ receptor subunit distribution by Jones et al. (Jones et al., 1994). Muñoz et al. reported that macaques display strong immunostaining for GABAβ1a,b receptors in layers 4A, 4Cβ and upper layer 6; all layers that receive direct P-geniculate projections (Muñoz et al., 2001). They found humans to have strong staining of layers 4Cβ and 6, but immunoreactive neurons in layer 4A were less numerous and less intensely stained than in macaque 4A. The balance of available evidence thus suggests that the laminar distribution of geniculostriate projections in apes and humans differs from that of macaques and most other monkeys, and more specifically, that there is a decreased influence of the P geniculate laminae on layer 4A.

Another difference that distinguishes apes and humans from Old World and New World monkeys is an increase in calbindin staining of neurons and neuropil in layer 4A. The calbindin-stained neurons are small, round, multipolar cells, which are likely to be GABAergic interneurons (Van Brederode et al., 1990; Jones et al., 1994). The increase in neurons that express calbindin in hominoid layer 4A may reflect either the modification of a pre-existing cell phenotype (a neuron that did not originally express calbindin or did so at low levels), or the proliferation of a cell type that occurs only in small numbers in monkeys, or the evolution of a new class of interneuron. Although the increase in calbindin staining in hominoids is coincident with the decrease in CO activity, it need not be the case that the two changes have any direct causal relationship.

Species-specific Organization of Human Layer 4A

While human layer 4A shares some features of organization with that of apes, humans have several additional modifications. Human layer 4A is characterized by a system of alternating, interlaced compartments, one consisting of a meshwork of neuropil bands that strongly express the Cat-301 antigen and NPNF, the other consisting of irregularly shaped territories of calbindin-dense tissue set in the interstices of the meshwork. No other primate that has been examined shows such strong staining for Cat-301, NPNF, or calbindin in layer 4A, and furthermore, the way these molecules are compartmentalized in humans is highly unusual.

The neurochemistry and compartmentation of human layer 4A is so unusual it is difficult to determine what relationship there is, if any, between the human pattern and the ancestral honeycomb architecture. The simplest interpretation is that each type of human compartment represents a modified version of one of the compartments of the honeycomb. Under this view, the system of pyramidal-cell cores (related to the M and P pathways) would be homologous to the meshwork of Cat-301+ and NPNF+ tissue bands of humans (which have neurochemical affinities to the M pathway), while the honeycomb walls (related to the P pathway) would be homologous to the calbindin-rich interstitial territories of humans. Alternatively, it might be the case that humans actually retain an ancestral pyramidal-core system, but it is obscured by the superimposition of a new system of mesh-like neuropil bands. Other, more elaborate, interpretations are possible. Any interpretation of human layer 4A organization, however, must take into account the impressive variety of changes that occurred in human evolution. These include changes in the geometry of neural assemblies, from the essentially vertical orientation of the pyramidal-cell cones to the more convoluted arrangement of the mesh bands, as well as changes in neuronal phenotypes, including the novel (or greatly enhanced) expression of the Cat-301 antigen and NPNF non-pyramidal cells and neurites in the mesh bands. In addition, there was evidently enhanced expression of calbindin by small nonpyramidal cells even beyond that seen in apes.

Although the evolutionary transformations undergone by human area V1 were considerable, the kinds of changes that occurred are by no means unprecedented. Differences between humans (or hominoids) and other primates in the neuronal morphologies and neurochemical phenotypes of other cortical areas have been documented (Campbell and Morrison, 1989; Gebhard et al., 1995; del Rio and DeFelipe, 1997; Nimchinsky et al., 1999; Gonzalez-Albo and DeFelipe, 2000; Hof et al., 2001). There are examples from nonprimate mammalian groups as well (DeFelipe, 1993; Hof et al., 1999, 2000; Preuss, 2000).

A definitive account of the relationship of human layer 4A and its compartments to the M, P and K pathways will have to await direct investigations of geniculostriate and intralaminar connectivity, investigations that have not yet been undertaken owing to the difficulty of studying connections in humans (see below). Notwithstanding the lack of direct evidence regarding connectivity in humans, however, the increased staining for Cat-301 and NPNF observed in human layer 4A compared with monkeys and apes suggests that the neural apparatus of this layer in humans is more strongly related to the M pathway than in other primates. Additionally, the changes in CO staining that humans share with at least some other hominoids, and the differences in the distribution of GABA receptors discussed in the previous section, suggest that P geniculate projections were modified in ape and human evolution, and that layer 4A is perhaps less strongly related to the P pathway in hominoids than it is in monkeys. This need not imply a general decrease in the influence of the P pathway on hominoid V1, however, as the pattern of CO staining suggests that the P geniculate afferents were redistributed within ape and human V1, rather than simply lost. The affinities of the calbindin-rich interstitial zones of human layer 4A cannot be interpreted on the basis of current histochemical evidence, because calbindin does not appear to be preferentially related to any particular pathway. It is true that in the lateral geniculate of nonhuman primates, calbindin is expressed preferentially or exclusively in the K layers (Jones and Hendry, 1989; Blümcke et al., 1994; Johnson and Casagrande, 1995; Goodchild and Martin, 1998). In the cortex, however, calbindin staining is relatively light within the main projection targets of the K layers (the CO blobs of layer 3) and dense in the interblob regions, which are targets of projections from cortical layers that receive both M- and P-geniculate afferents (Lachica et al., 1992; Hendry and Carder, 1993; Johnson and Casagrande, 1995; Callaway, 1998). If the calbindin-rich compartments of layer 4A are homologous to the tissue of the honeycomb walls,
as discussed above, they could be related to the P pathway (Yoshioka and Hendry, 1995). This is a defensible proposition, even if direct projections from the P-geniculate to 4A were lost in evolution: owl monkey 4A, which lacks direct LGN projections, nonetheless receives afferents from the parvocellular-recipient layer 4Cβ (Boyd et al., 2000). Such considerations aside, however, there is presently no experimental evidence that clearly links the calbindin-rich compartments of human layer 4A (or any other part of human 4A) to the P pathway.

**Implications for Human Vision Research**

The existence of substantial differences in the organization of primary visual cortex between humans and nonhuman primates (including the commonly studied macaque monkeys) may come as a surprise, given how widely held is the conviction that the human visual system is basically or essentially similar to that of our close relatives. This idea is so deeply entrenched that it is noteworthy when the possibility of non-trivial differences in the neural substrates of vision in humans and other primates is given serious consideration (Crick and Jones, 1993; Sereno, 1998; Heeger, 1999). Yet there is no lack of evidence that humans differ from macaques and other primates. For example, in the retina, short wavelength cones (S-cones) are distributed throughout the fovea in macaques, while humans have an S-cone-free foveola (Bumsted and Hendrickson, 1999). The ratio of long-wavelength to medium-wavelength cones is ~1.1 in macaques, whereas the ratio in humans, although individually variable, averages ~2:1 (Jacobs and Deegan, 1997). Differences in the spectral sensitivities of humans and macaques, measured psychophysically (Harwerth and Smith, 1985; Dobkins et al., 2000), are consistent with the photoreceptor differences. Parasol retinal ganglion cells (which project to the magnocellular LGN layers) have larger dendrites at a given eccentricity in humans than those of macaques, although the dimensions of human and macaque midget cells (which project to the parvocellular LGN) are similar (Dacey and Petersen, 1992). Human ON-center ganglion cells have larger dendritic fields than OFF-center cells at a given eccentricity; no comparable asymmetry has been noted in macaques (Dacey and Petersen, 1992). Human–macaque differences have also been reported in other structures. In the LGN, there are differences in the laminar distribution of calbindin expression (Leuba and Saini, 1996; Münkle et al., 2000). In the cortex, there are differences in the configurations and dimensions of blobs and ocular dominance columns in V1 (Horton and Stryker, 1993; Tootell and Taylor, 1995), and in the neurochemical characteristics of stripes in V2 (Tootell and Taylor, 1995).

There is evidence for functional differences as well. Humans are reported to show greater temporal and spatial luminance contrast sensitivity than macaques at photopic luminances (De Valois et al., 1974; Merigan, 1980; Harwerth and Smith, 1985). There is evidence that center-surround organization differs between humans and macaques, humans having substantially larger inhibitory surrounds at a given eccentricity (Spillmann et al., 1987). Humans typically process global features of a compound stimulus faster than local features, while Old World monkeys show a local advantage (Fagot and Derruele, 1997). Results from functional-imaging studies suggest that attention-related modulation of activity in V1 and extrastriate cortex may be greater in humans than in macaques (Heeger, 1999). Tootell et al. report that human area V3A shows greater motion and contrast sensitivity than one would expect from studies of macaque V3A, while human area V3 is less motion selective that its macaque counterpart (Tootell et al., 1997). The interpretation of possible human–nonhuman differences in cortical activity is complicated by the fact that results have been obtained with different techniques [functional magnetic resonance imaging (fMRI) in humans; microelectrode recording in monkeys]. The increased application of functional imaging techniques in nonhuman primates should allow for more accurate assessments of the ways in which humans differ from, and resemble, other primates (Takechi et al., 1997; Disbrow et al., 1999; Logothetis et al., 1999; Dubowitz et al., 2001).

It would be premature to attempt a synthesis of human visual system specializations based on the candidate differences noted above, given that so few aspects of structure and function have been the subject of comprehensive comparative study—the general lack of information about apes, in particular, is a significant obstacle to understanding human specializations. It is noteworthy, nonetheless, that several of the human–macaque differences cited above are consonant with the view that the M system was modified in human evolution. These include differences in parasol cell size, in temporal and spatial modulation sensitivity, and differences in the responsiveness of areas V3 and V3A to motion and contrast. Differences in attention-related modulation of activity in V1 and other visual areas between humans and macaques could reflect the influence of changes in the M pathway on the parietal cortex, which receives its predominant visual input from the M system (Livingstone and Hubel, 1988; Merigan and Maunsell, 1993; Vidyasagar, 1999).

The abundance of evidence for visual system differences between humans and macaques places the claim for basic or essential similarity in jeopardy. It does not, however, call into question the utility of vision research in macaques or other nonhuman primates. There are, after all, numerous similarities between humans and other primates, and there is no question that the study of nonhuman primates has contributed fundamentally to our understanding of the human visual system. Nonhuman research is indispensable for investigating certain important aspects of brain organization, particularly long connections and the physiological characteristics of individual neurons, that require invasive procedures. Nevertheless, the importance of nonhuman research has been emphasized so strongly that opportunities for directly investigating the structure of the human visual system have not been fully exploited. For despite the limitations of human research, techniques exist for examining many aspects of the histological and neurochemical organization of humans in detail, as the research reviewed in the present paper illustrates, and techniques are available that could be used to examine connectivity over a range of several millimeters (Burkhalter and Bernardo, 1989; Kenan-Vaknin et al., 1992; Galuske et al., 2000). The wider application of available anatomical techniques, along with functional and behavioral methods suitable for use in both humans and nonhuman primates, could contribute greatly to our understanding of what the human visual system shares with macaques and other primates, and how evolution modified the visual system to support specifically human cognitive processes and behaviors.

Evolutionary modifications of the human visual system are likely to be especially pertinent to understanding disease processes. For example, an account of how the M pathway was modified in human evolution would seem to have an important bearing on theories of developmental dyslexia, as there is now abundant evidence that M-pathway pathology and dysfunction are common features of this condition, which affects 5–10% of the population (Livingstone et al., 1991; Stein and Walsh, 1997; Demb et al., 1998; Vidyasagar, 1999; Stein, 2001). The deficits
noted in dyslexia include reduced control of spatial attention, which has prompted the suggestion that M-stream pathology may be present in other disorders in which attention is compromised (Vidyasagar, 1999; Stein, 2001). In macaques, structures related to the M pathway have large numbers of neurons that strongly express NPNP (Hof and Morrison, 1995; Chaudhuri et al., 1996), and NPNP+ neurons are even more numerous in the cortex of apes and humans than in macaques (Campbell and Morrison, 1989; Preuss et al., 1999). It is of interest, therefore, that NPNP-containing neurons are especially susceptible to damage in neurodegenerative conditions such as glaucoma and Alzheimer’s disease (Morrison et al., 1998). The potential biomedical significance of human evolutionary specializations is not limited to the M pathway or to structures enriched in NPNP, moreover. Persons who suffer migraine with visual aura show long-lasting changes in visual sensitivity, even during aura-free periods. It has been hypothesized that these changes result from repeated episodes of ischemia in area V1 during aura, causing damage to GABAergic interneurons (which are thought to be especially vulnerable to hypoxia) and a consequent reduction in intracortical inhibition (Chronicle and Mulleners, 1994; Palmer et al., 2000). In attempting to understand the pathophysiology of migraine, it may be significant that humans—unlike macaques and most other primates—possess a prominent population of small, calbindin-rich, multipolar neurons in layer 4A of area V1, which are most likely GABAergic inhibitory interneurons.

Notes

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