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Cytoarchitectural and Axonal Maturation in Human Auditory Cortex

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ABSTRACT

This study followed the maturation of human auditory cortex from the beginning of the second trimester of gestation to young adulthood. Histological and immunohistochemical techniques were used to trace the development of a laminar cytoarchitecture and an adult pattern of axonal neurofilament expression. From the 16th fetal week to the 4th postnatal month, the cortex progresses from a marginal layer and an undifferentiated cortical plate to incipient lamination. Between the 22nd fetal week and the 4th postnatal month, a two-tiered band of neurofilament-immunoreactive axons develops in layer I, but subsequent to the 4th month, the number of immunopositive axons in this layer is greatly reduced. Between the middle of the first year of life and age 3 years, the laminar pattern of cytoarchitecture becomes fully mature and a network of immunostained axons develops in layers VI, V, IV, and IIIc. This axonal plexus in the deep cortical layers continues to increase in density until age 5. Beginning at 5 years of age, a network of neurofilament-positive axons develops in the superficial layers IIIb, IIIa, and II, and by 11–12 years of age, overall axonal density is equivalent to that seen in young adulthood. This extended time span of axonal maturation has implications for the emergence of auditory cortical function.

Keywords: development, neurofilaments, thalamocortical, callosal, transcortical

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INTRODUCTION

The human brainstem auditory pathway is relatively mature by the time of term birth and becomes adult-like in many aspects of its anatomy and physiology by 1–3 years of age. Human auditory cortex, however, might be expected to have a more extended period of development. The present study aimed to trace cortical maturation through the fetal period, infancy, childhood, and young adulthood, using histological and immunohistochemical techniques in postmortem material. A particularly useful technique was the immunostaining of axonal neurofilament protein (NF), which is the most abundant cytoskeletal element in myelinated axons. Monoclonal antibodies can distinguish between the phosphorylated NF found in axons and the nonphosphorylated form found in neuronal somata and dendrites (Sternberger and Sternberger 1983; Marc et al. 1986). Because axonal NF is stabilized by phosphorylation, it is more resistant to proteolysis (Goldstein et al. 1987) and less sensitive to method of postmortem fixation (Sillevis Smitt et al. 1993) than the nonphosphylorated NF epitopes in dendrites.

The composition and function of axonal NF changes over time during development. Neurofilament protein is a polymer with low (NF-L), medium (NF-M), and high (NF-H) molecular weight subunits. In rats, NF-L is expressed in neurons beginning on embryonic day 11 and appears to function in establishing and maintaining neuritic outgrowth (Escurat et al. 1990). NF-M and NF-H are expressed later in the perinatal period, and levels of all three subunits continue to rise postnatally (Plioplys et al. 1986; Guadáno-Ferráz et al. 1990; Schlaepfer and Bruce 1991). In

maturing axons, a direct relationship exists between neurofilament density, axonal diameter, and degree of myelination (Friede and Samorajski 1970; Hoffman et al. 1984). Work in mutant mice with selective disruption of the three NF subunits has shown that axonal caliber is determined by the level of NF-H but not NF-L (Cleveland et al. 1991; Marszalek et al. 1996). Experimental alteration in levels of the NF subunits also demonstrates roles for NF-M and NF-H in axonal conduction, as NF-M and NF-H knockout mice have an abnormally prolonged refractory period and NF-H knockouts show a significant decrease in outward rectification (Kriz et al. 2000). The latter finding suggests that NF-H modulates ion channel function in myelinated axons.

Neurofilament proliferation thus appears to be part of a developmental process in which axons increase in diameter, develop myelin sheaths, and acquire a rapid conduction velocity. This is consistent with our observations in the human brainstem auditory pathway, where an adultlike pattern of NF expression develops just prior to the appearance of myelin and the time of the first recordable auditory brainstem potentials (Moore et al. 1995, 1997a; Ponton et al. 1996). Information on the pattern of neurofilament expression in cortical axons should thus contribute to our understanding of the emergence of cortical function. Some results of the present study have been previously presented in abstract form (Moore et al. 1997b).

METHODS

Tissue collection

Postmortem tissue was obtained at neuropathological autopsy at the University of Southern California–Los Angeles County Hospital, where an approved experimental protocol is on file with the Department of Pathology. All subjects from whom tissue was taken died in hospital of intrinsic causes, i.e., not from external trauma. No tissue was taken from cases with any type of congenital malformation, from cases with a history of HIV infection, including maternal HIV, or from cases in which there had been long-term chemotherapy or radiation therapy. The brains were placed in buffered formalin solution at the time of the primary autopsy and preserved in that solution for several weeks prior to the neuropathological autopsy. Fetal age was determined by the neuropathologist on the basis of the clinical history and the fissural pattern of the cortex, and is considered to be accurate plus or minus one week. The tissue collected at autopsy consisted of one hemisphere in subjects up to 27 fetal weeks and the superior temporal gyrus from one hemisphere in older subjects. For this study, cortical tissue was processed from 20 cases, including 5 fetuses between 16 and 37 weeks of gestation, 3 term infants at 40–42 weeks of gestation, and 12 postnatal subjects ranging in age from 3 weeks to 27 years. The subjects are approximately evenly divided between male and female.

Histological processing

In this laboratory, all tissue blocks were embedded in celloidin. Advantages of celloidin embedding include maintenance of the integrity of fragile fetal and infant tissue, preservation of good neuronal morphology, and minimal tissue shrinkage. In the embedding process, the larger temporal lobe samples were divided into two or three tissue blocks, with orientation maintained by long pins inserted through the tissue segments. Tissue blocks were dehydrated in graded ethanols and embedded in 15% celloidin (Parlodion, Malinkrodt, St. Louis, MO, USA), using ether-absolute alcohol as a solvent. Each block was sectioned in the coronal plane on a sliding microtome at 50 μm, and sections were preserved on numbered papers in 80% ethanol. From each tissue block, a series of every eighth section was stained by the Nissl method (cresylecht violet). Stained sections were dehydrated in 70% and 95% ethanol, but the final dehydration was done in butanol to avoid dissolving the celloidin. Dehydrated sections were cleared in xylene and mounted on glass slides with Permount. After examination of the Nissl series, any tissue samples in which neurons showed evidence of autolytic changes were discarded.

Immunohistochemical method

A complete age series was produced with each of two primary antibodies, namely, MUO73-UC (Biogenex, San Ramon, CA, USA) (monoclonal, used at a dilution of 1:80) and SMI-31 (Sternberger Monoclonals Inc., Lutherville, MD, USA) (monoclonal, used at a dilution of 1:1000). Both antibodies are specific to axonal neurofilaments and were raised against a combination of the low and high molecular-weight subunits. Essentially identical results were obtained with the two primary antibodies. As in our prior brainstem studies, we immunostained celloidin sections according to the protocol developed by Shi et al. (1992). Sections were etched in sodium methanolate (1 part saturated sodium hydroxide in absolute methanol, 2 parts absolute methanol) for 5–20 minutes. Sections were then rinsed for 10 minutes each in absolute methanol, 70% methanol, and tris-buffered saline (TBS). After preincubation for 15 minutes in 0.3% Triton X-100, the sections were incubated in primary antibody, diluted in TBS with 1% of the appropriate normal serum and

0.3% Triton X-100. Incubations were done either overnight at room temperature or for 1–3 days at 4°C. Subsequently, sections were incubated for 1–2 hours in biotinylated secondary antiserum and then in avidin from a Vectastain kit (Vector Labs, Burlingame, CA, USA) or a Sternberger kit (SMI). All incubations were separated by several rinses in TBS. The final chromagen reaction was carried out in 0.05% 3,3′-diaminobenzidine tetrahydrochloride with the addition of 0.08% nickel chloride. To avoid drying the celloidin, reacted sections were handled free-floating while being dehydrated in graded ethanols and absolute butanol, cleared in xylene, and coverslipped with Permount.

Image processing

Microphotographs were taken on a Nikon Optiphot microscope equipped with a Nikon FX-35WA camera. Photographic negatives were digitized with a Polaroid SprintScan 35 scanner. The digitized images were stored as TIFF files and processed in Adobe Photoshop on a Macintosh Power PC computer. Tissue size indicated in the final composite images is corrected for shrinkage occurring during celloidin embedding, previously estimated as 12% in linear dimension (Moore 1987). Because the tissue is received in a fixed state, no correction can be made for size change which may occur during formalin fixation.

RESULTS

Development of temporal lobe topography

Topographic development of the cerebral hemisphere during the second trimester of gestation can be traced in Figure 1. At the 16th week of gestation (Fig. 1 16 fw), a section through the midpoint of the cerebral

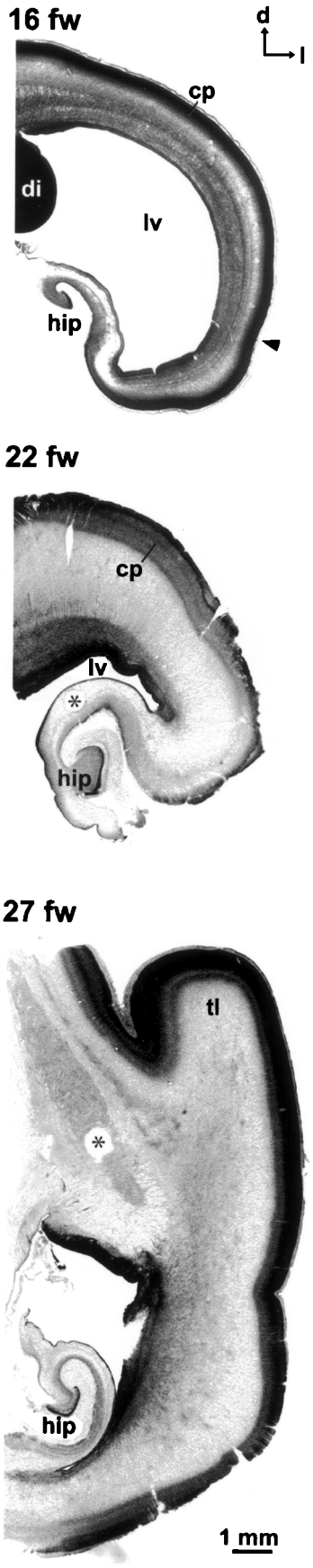

FIG. 1 Topography of the cerebral hemisphere around midgestation. All sections are taken from the approximate midpoint of the cerebral hemisphere. Arrows indicate dorsal (d) and lateral (l). At 16 and 27 fetal weeks (16 fw, 27 fw), arrowhead indicates the boundary between the developing dorsal isocortex and ventral allocortex, including the hippocampal formation (hip). At 22 and 27 fetal weeks, an asterisk indicates the artifact created by pins placed through the tissue block for orientation. **Upper panel.** At the 16th fetal week (16 fw), the cerebral hemisphere consists of a midline diencephalon (di) and a thin-walled telencephalic vesicle enclosing an expanded lateral ventricle (lv). The cortical plate (cp) is a dense cellular layer just beneath the pial surface of the telencephalic wall. **Middle panel.** At the 22nd fetal week (22 fw), the telencephalic wall has thickened and the lateral ventricle is reduced in size. The cortical plate has increased in depth and has developed a cell-dense central layer. **Lower panel.** By the 27th fetal week (27 fw), the temporal lobe (tl) is distinct from the more ventrally located insular cortex.

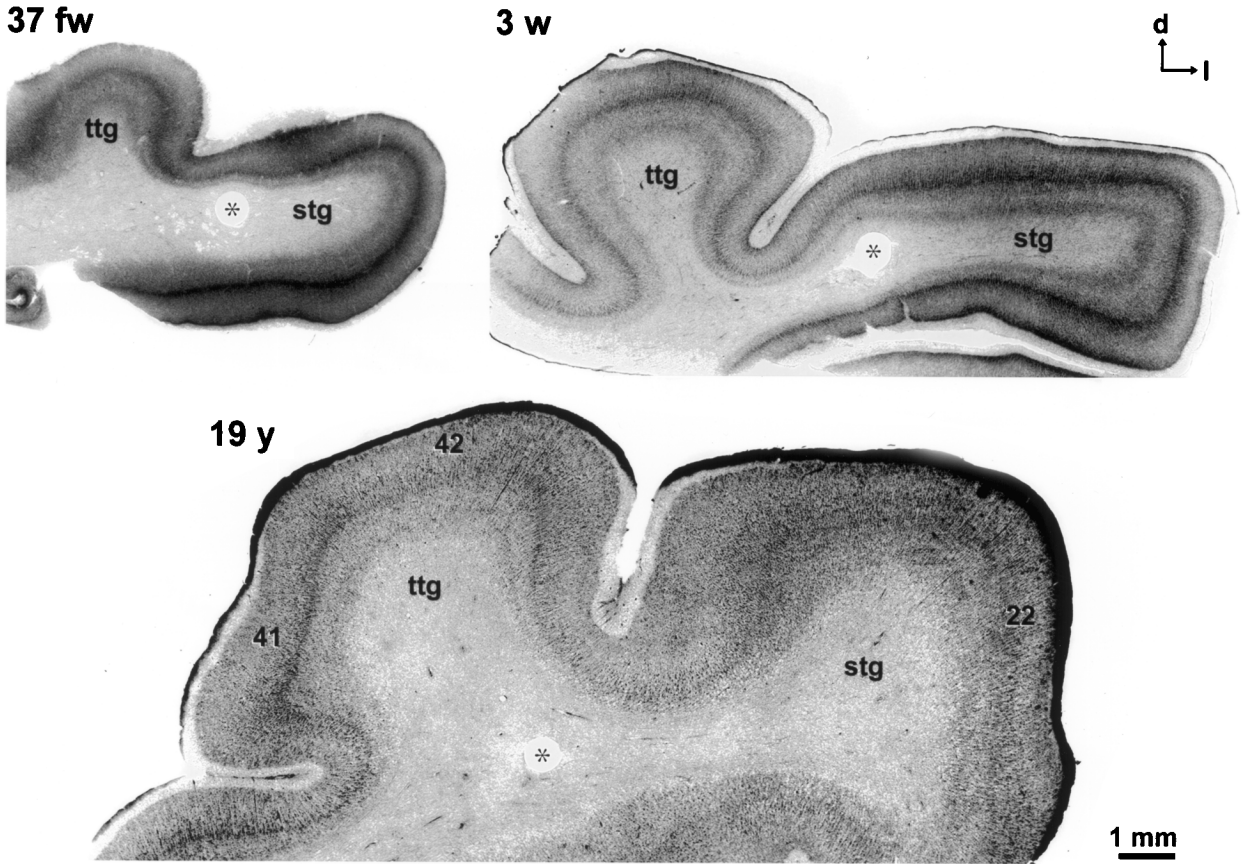

FIG. 2 Topography of the temporal lobe in the late fetal and postnatal periods. Arrows indicate dorsal (d) and lateral (l). Asterisks indicate the artifact created by pins placed through the tissue block for orientation. **Upper-left panel.** At the 37th fetal week (37 fw), the transverse temporal gyrus (ttg) is distinct frm the superior temporal gyrus (stg). **Upper-right panel.** By 3 weeks postnatal age (3 w), both gryi are larger and the transverse temporal gyrus is more distinctly bounded by sulci. Within the cortical plate, the deeper layers have increased in thickness. **Lower panel.** At 19 years (19 y), both gyri have increased in size and mature cortical lamination allows identification of Brodmann areas 41, 42, and 22.

hemisphere demonstrates that the telencephalic wall is about 1.2 mm thick and the ventricular space is still very large. At the outer (pial) surface of the telencephalic wall, a clear marginal layer and a densely cellular cortical plate about 400 μm deep form the rudimentary cortex. By the 22nd fetal week (Fig. 1 22 fw), the telencephalic wall has become thicker and the lateral ventricle is reduced in size. The cortical plate has expanded to a depth of about 800 μm and there is an indication of a central dense layer. By the 27th week of development (Fig. 1, 27 fw), the temporal lobe has begun to form and is clearly separated from the insular cortex ventral to it. Cells in the superficial half of the cortex exhibit dense columnar packing, while cells in the deeper half are relatively dispersed. The central dense layer is more pronounced than at 22 weeks of gestation, giving the cortex a more laminated appearance.

In the late fetal period, at 37 weeks of gestation (Fig. 2, 37 fw), a transverse temporal gyrus (Heschl's gyrus) and a superior temporal gyrus are apparent. In the early postnatal period, at 3 weeks after term birth (Fig. 2, 3 w), the transverse temporal gyrus is clearly demarcated by sulci and the deeper layers of the cortex are better developed. By young adulthood (Fig. 2, 19 y), both the transverse temporal and superior temporal gyri have increased in size. The cortex is considerably thicker and its laminar organization is more complex. The development of distinct gyri in the temporal lobe gives the first indication of the location of subareas of auditory cortex. Auditory koniocortex has been designated as areas 41/42 by Brodmann (1908), TC/TB by von Economo (1929), and KAm/KAlt (medial and lateral auditory koniocortex) by Galaburda and Sanides (1980). These two small cortical areas are consistently described as located on and around the transverse temporal gyrus. Though their exact boundaries vary across individuals, area 41/TC/KAm generally lies at the medial aspect of the transverse temporal gyrus and area 42/TB/KAlt is located more laterally on the gyrus. The superior temporal gyrus, with the exception of its rostral pole, has been designated area 22 by Brodmann and TA by von Economo. This extensive area contains several subregions

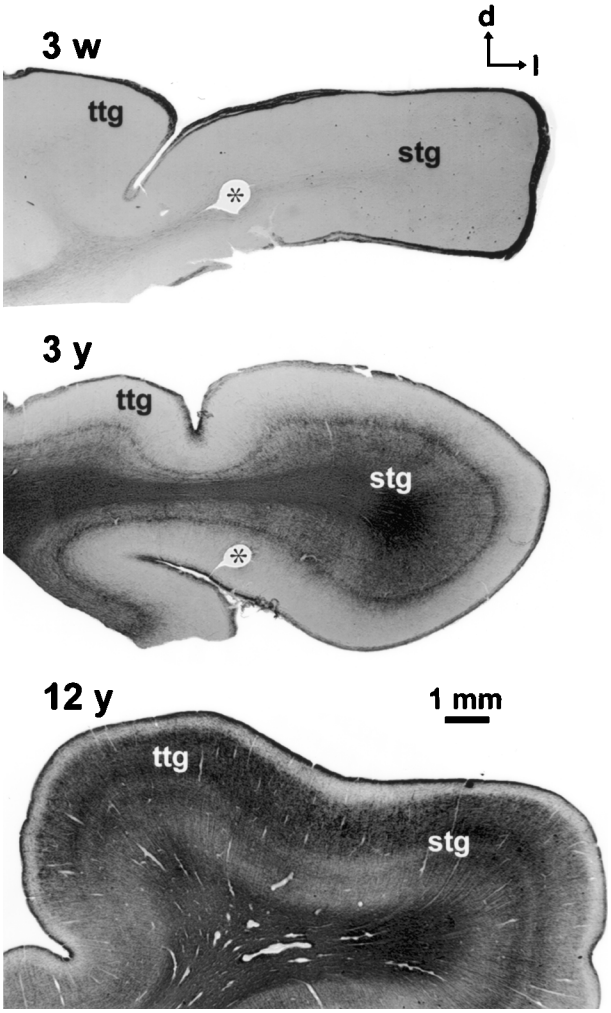

FIG. 3 Overview of NF-immunostaining. Arrows indicate dorsal (d) and lateral (l). Asterisks indicate the artifact created by pins placed through the tissue block for orientation. **Upper panel.** At 3 weeks postnatal age (3 w), immunoreactive axons are concentrated in layer I across the transverse temporal gyrus (ttg) and superior temporal gyrus (stg). **Middle panel.** By age 3 years (3 y), NF-positive axons fill the central white matter and form a network in the deeper cortical layers. NF-positive axons in layer I are less numerous than in the perinatal period and are concentrated near the surface of the layer. **Lower panel.** At age 12 years (12 y), layers VI through II are filled with a dense array of NF-immunoreactive axons. As at 3 years, NF-immunoreactive axons are present at the outer edge of layer I.

in the schema of Galaburda and Sanides (1980). The present study was confined to the transverse temporal gyrus and the portion of the superior temporal gyrus lying directly lateral to it on the superior temporal plane, i.e., the internal auditory parakoniocortex (PaAi) of Galaburda and Sanides (1980).

Like cytoarchitectonic development, the process of axonal maturation is quite uniform across the transverse temporal and superior temporal gyri. Panoramic views of this region in NF-immunostained sections reveal the overall course of neurofilament expression. At 3 weeks postnatal age (Fig. 3, 3 w), NF-positive axons form a dense band extending through the marginal layer of the transverse temporal and superior temporal gyri. By 3 years of age (Fig. 3, 3 y), the band of NF-positive axons in the marginal layer is thinner and mostly confined to the outer edge of the layer. At this age, NF-immunostained axons are present in the central core of white matter of the temporal lobe and form a network of axons which is restricted to the deeper cortical layers. By 12 years of age (Fig. 3, 12 y), a dense array of neurofilament-positive axons is present in layers II through VI of both gyri.

A more detailed view of the changes in cytoarchitectural organization and neurofilament expression is shown in the following higher-power illustrations. In the illustrations of cortex in the second trimester (Fig. 4), microphotographs are taken from the approximate midpoint of the cerebral hemisphere. In older subjects, the curvature of the transverse temporal gyrus often results in oblique sections through its cortex, even when the tissue block is sectioned orthogonal to the pial surface. In order to obtain comparable sections through the full depth of the cortex, the microphotographs in the older subjects (Figs. 5, 7, and 8) are taken from cortex of the superior temporal plane lateral to the transverse temporal gyrus, i.e., from area 22/TA/PaAi.

Cytoarchitecture and neurofilament expression in the second trimester

Cortical specimens from the second trimester are from the 16th, 22nd, 24th, and 27th fetal weeks. At the earliest time point, the 16th week of development (Fig. 4, 16 fw, N), the Nissl-stained material shows a well-defined marginal layer. The cortical plate is densely cellular, particularly in its upper half, and has a total depth of about 400 μm. By the 22nd week (Fig. 4, 22 fw, N), the cortical plate has doubled in depth to about 800 μm and is composed of cells in parallel vertical columns. Cells are less densely packed in the deeper half of the plate, but there is no definitive lamination. By the 27th week of development, the cortex is about 1 mm thick and, with the development of a central layer of greater cell density, exhibits some evidence of lamination.

In the NF-immunoreacted material, there is essentially no staining at the 16th fetal week (Fig. 4, 16 fw, NF). By the 22nd fetal week, a few NF-immunoreactive axons are present in the center of the marginal layer (Fig. 4, 22 fw, NF). These axons are relatively thick, varicose, and meandering in their course. At the 24th and 27th fetal weeks, still only a limited number of NF-immunostained axons are present within the marginal layer. There are no axons expressing NF in the cortical plate during this period, but by the 27th fetal week a

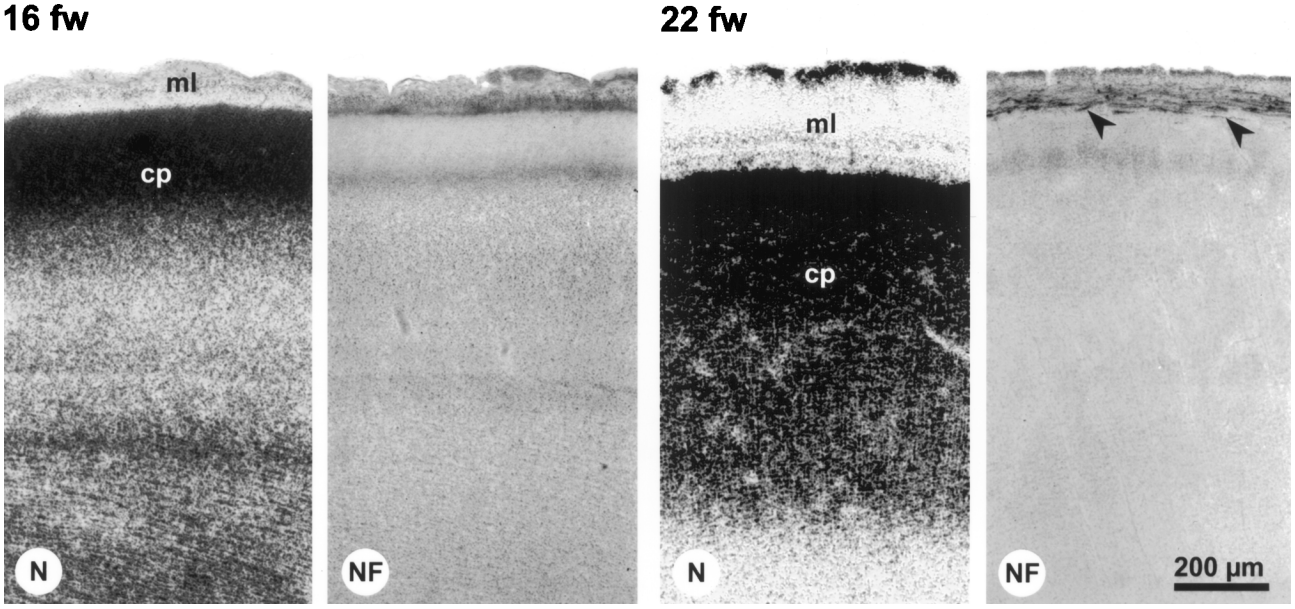

FIG. 4 Cytoarchitecture and axonal maturation in the second trimester. At the 16th fetal week (16 fw), Nissl staining (N) reveals a relatively acellular marginal layer (ml) and a densely cellular cortical plate (cp). In neurofilament material (NF), there are no immunopositive structures in the telencephalic wall. At the 22nd fetal week (22 fw), Nissl staining shows that the cortical plate has roughly doubled in depth and the cell density has decreased in the deeper part of the plate. In NF-immunostained material, a few axons are present in the marginal layer (arrowheads).

few thin NF-positive axons are present in the white matter core of the temporal lobe.

Cytoarchitecture and neurofilament expression in the perinatal period

Observations of development in the perinatal period are based on specimens from the 37th, 40th, and 42nd weeks of gestation, and from postnatal ages 3 weeks and 4.5 months. By the end of the fetal period, at 37–42 weeks of gestation, the cortex is about 1.2 mm deep (Fig.5, 37 fw, N). There is incipient lamination in that presumptive layers II and IV are visible because of their dense concentration of small, darkly stained neurons. By the 3rd postnatal week, increasing cell size and differentiation allows layers II through VI to be distinguished. By 4.5 months, cortical depth has increased to 1.4–1.6 mm and the array of small neurons in presumptive layers II and IV is denser (Fig. 5, 4.5 m, N).

By the 37th and 40th fetal weeks, the number of NF-immunostained axons in layer I has markedly increased from the few isolated axons seen at midgestation, so that NF-positive axons form a dense band within the layer (Fig. 5, 37 fw, NF). By 3 weeks postnatal age, the band of immunoreactive axons has become two-tiered. The lower tier consists of closely packed axons, while the upper tier contains axons that are more dispersed (Fig. 6, 3 w, upper panel). The cortical plate-derived layers at this stage of development contain only isolated axons running with vertical, oblique, or horizontal courses. However, a moderately dense stream of NF-positive axons is present in the white matter core of the temporal lobe (Fig. 6, 3w, middle panel). At 4.5 months (Fig. 5, 4.5 m, NF), the density and configuration of the band of immunostained axons in layer I remains very similar to that seen at 3 weeks of age. As at earlier ages, only isolated NF-positive axons are present within the cortical plate.

Cytoarchitecture and neurofilament expression in early childhood

The observations of this period are based on specimens from subjects at 1, 2, and 3 years of age. The Nissl material shows that significant cytoarchitectural maturation occurs between 4.5 months and 1 year of age. By 1 year (Fig. 7, 1 y, N), overall cortical depth is adultlike at 1.8–2.0 mm and the cortex has a mature laminar organization. Cell packing density is lower than in the perinatal period, presumably indicating development of a greater volume of neuropil. Layers IV and II are less dense, but still contain a concentration of small granular neurons. Layer III has increased in depth and shows a surface-to-deep gradient in cell size, with large, darkly staining pyramidal cells in the deeper part of the layer, making it possible for the first time to define layers IIIa, IIIb, and IIIc. Layers VI and V are broader, and cells in VI are somewhat larger than those in layer V. At ages 2 and 3 years, the cortex shows no marked change in overall depth and organization, but the large, darkly staining neurons in layers

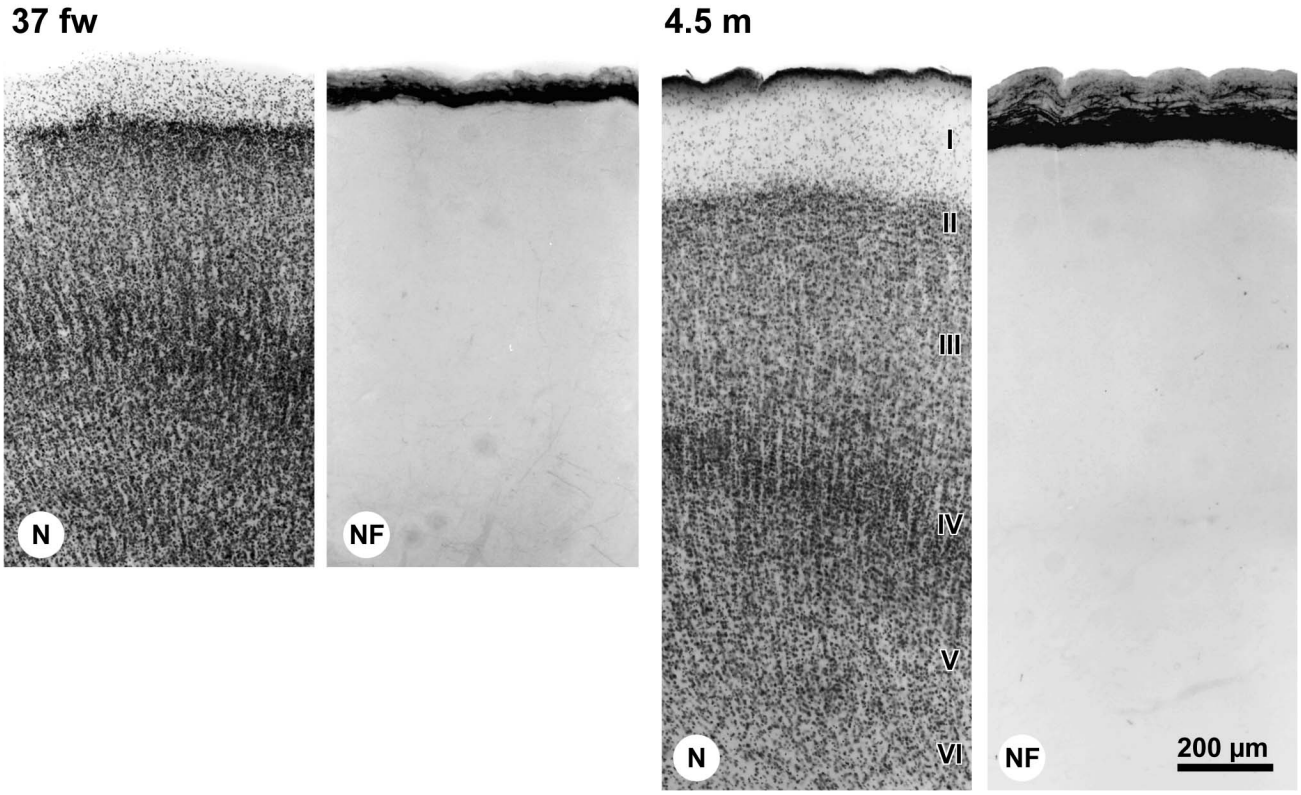

FIG. 5 Cytoarchitecture and axonal maturation in the perinatal period: At the 37th fetal week (37 fw), the Nissl material (N) shows a concentration of small neurons in prospective layers IV and II. In the immunostained material at this age (NF), a thick band of immunopositive axons is present in layer I. At 4.5 months (4.5 m) in Nissl-stained material (N), cortical depth has increased and there is incipient differentiation of cortical layers II through VI. In the immunostained material (NF), the band of NF-positive axons in layer I now consists of a lower tier of closely packed axons and an upper tier of more dispersed axons.

III and VI are more prominent than at age 1 (Fig. 7, 3 y, N).

In the neurofilament material, the appearance of the marginal layer at 1–3 years is very different from that of the perinatal period. Within the marginal layer, the number of NF-immunoreactive axons in the deeper tier is reduced, while superficial axons remain numerous (Fig. 6, lower panel). In the deeper cortical layers at this age, sparse NF-positive axons are present in layers VI, V, and IV (Fig. 7, 1 y, NF). These axons are oriented mostly horizontally. By 2 and 3 years of age, overall axonal density in the deeper layers has increased and many vertical axons are clearly continuous into the white matter core of the gyrus. The vertical axons give rise to angular branching formations in layer IV and to thin branches extending into the deepest part of layer III. Both the angular branchings and the vertical branches into layer III are more prominent at age 3 than at 1 or 2 years (Fig. 7, 3 y, NF). Thus, by age 3, layers VI, V, IV, and the lowest portion of layer III are filled with a moderately dense grid of vertical and horizontal axons. A few NF-positive axons course through the superficial layers; these axons vary in diameter from thin to thick and often run with a spiraling trajectory (Fig 6, lower panel). The axons in the upper cortical layers are somewhat more numerous at ages 2 and 3 than at 1 year, but their overall distribution is still sparse.

Cytoarchitecture and neurofilament expression in later childhood

Observations of this developmental stage are based on cortical specimens from subjects 5, 11, and 12 years of age, with comparison specimens from 17, 19, 20, and 27 years. The Nissl material illustrates that cortical depth at 5, 11, and 12 years is unchanged from 1–3 years and the pattern of cytoarchitecture is also basically similar to that seen at earlier ages (Fig. 8, 5 y and 12 y, N). In the present study, we do not observe any change in the cytoarchitectural organization of auditory cortex between 5–12 years and young adulthood (17–27 years of age).

In contrast to the stable cytoarchitecture, marked changes occur in axonal maturation in later childhood. In the NF-immunostained material at 5 years (Fig. 8, 5 y, NF), the meshwork of axons in layers VI to IIIc has become denser and shows a greater prominence of vertical axons. There is no obvious change in axonal density in the deeper cortical layers

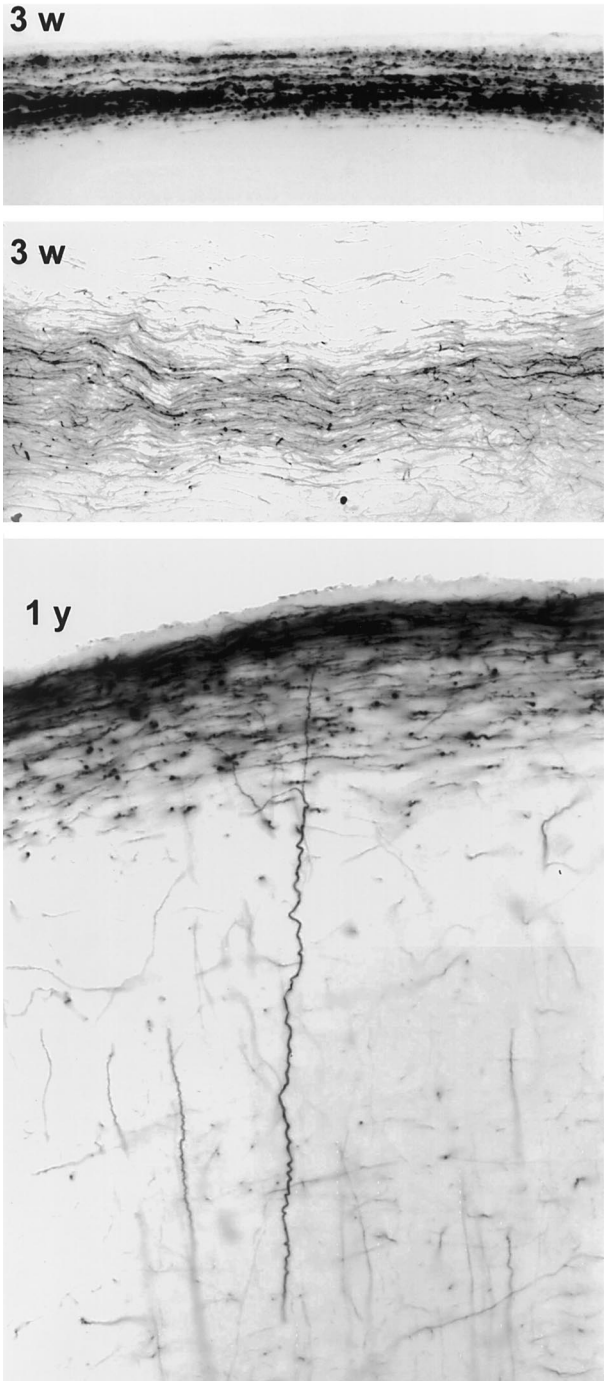

FIG. 6 Axonal morphology. **Upper panel.** NF-positive axons in layer I at 3 weeks postnatal age form a dense lower tier and a more dispersed upper tier. **Middle panel.** At 3 weeks, NF-immunoreactive axons are present in the white matter core of the temporal lobe. **Lower panel.** At 1 year of age, NF-positive axons in the superficial layers run vertically and horizontally and often have a spiralling trajectory. Within layer I, the density of the deeper tier of NF-positive axons is greatly reduced.

between 5 and 11–12 years. However, during these later childhood years, a network of horizontal and vertical NF-immunostained axons appears in layers IIIb, IIIa, and II (Fig. 8, 12 y, NF). With the development of this NF-positive axonal grid in the superficial layers, the pattern of immunostaining at ages 11 and 12 becomes essentially identical to that seen at 17–27 years of age.

DISCUSSION

Sequence of cortical development

Our initial expectations were that development would proceed from primary to secondary and tertiary areas of auditory cortex, i.e., that development at all stages would be more advanced in areas 41/42 than in area 22. This did not prove to be the case. Instead, the course of cytoarchitectural development was uniform across the superior temporal lobe in terms of both cytoarchitectural and neurofilament maturation. In particular, changes in neurofilament expression occurred in horizontal systems of axons across the entire region, with different time courses observed for axons in the marginal layer, axons in the deeper cortical layers, and axons in the superficial layers.

The observations of very early maturation of axons in layer I are in accord with the generally precocious development of the marginal layer. Layer I in the human brain is formed beginning around the 8th fetal week when the primordial plexiform layer is split by the developing cortical plate into a marginal layer at the pial surface and a deeper-lying subplate (Marin–Padilla 1970; Zecevic et al. 1999). Cells of layer I, including Cajal–Retzius cells, are among the earliest-born cortical neurons and can be identified in human cortex as early as the 6th–8th fetal week by their expression of acetylcholinesterase (AChE), the calcium-binding proteins calbindin and calretinin, and the glycoprotein reelin (Meyer and Gonzáles–Hernández 1993; Zecevic et al. 1999). The earliest-forming synapses are also located in the marginal layer, being present at the 6th–7th fetal weeks, prior to the formation of the cortical plate (Molliver et al. 1973; Zecevic 1998).

Within the laminae derived from the cortical plate, we observe axonal maturation first in the deep layers and later in the superficial layers. This reflects the well-known phenomenon of "inside-out" formation of the cortical plate, in which newly generated cells migrate through the layers of older neurons to the surface of the plate during early development (Rakic 1972). The deep-to-surface sequence of maturation of cortical neurons has been described in Golgi studies of human fetal cortex (Rabinowitz 1964; Marin–Padilla 1970). A deep-to-surface gradient is also observed in neuronal expression of MAP2 (Honig et al. 1996) and

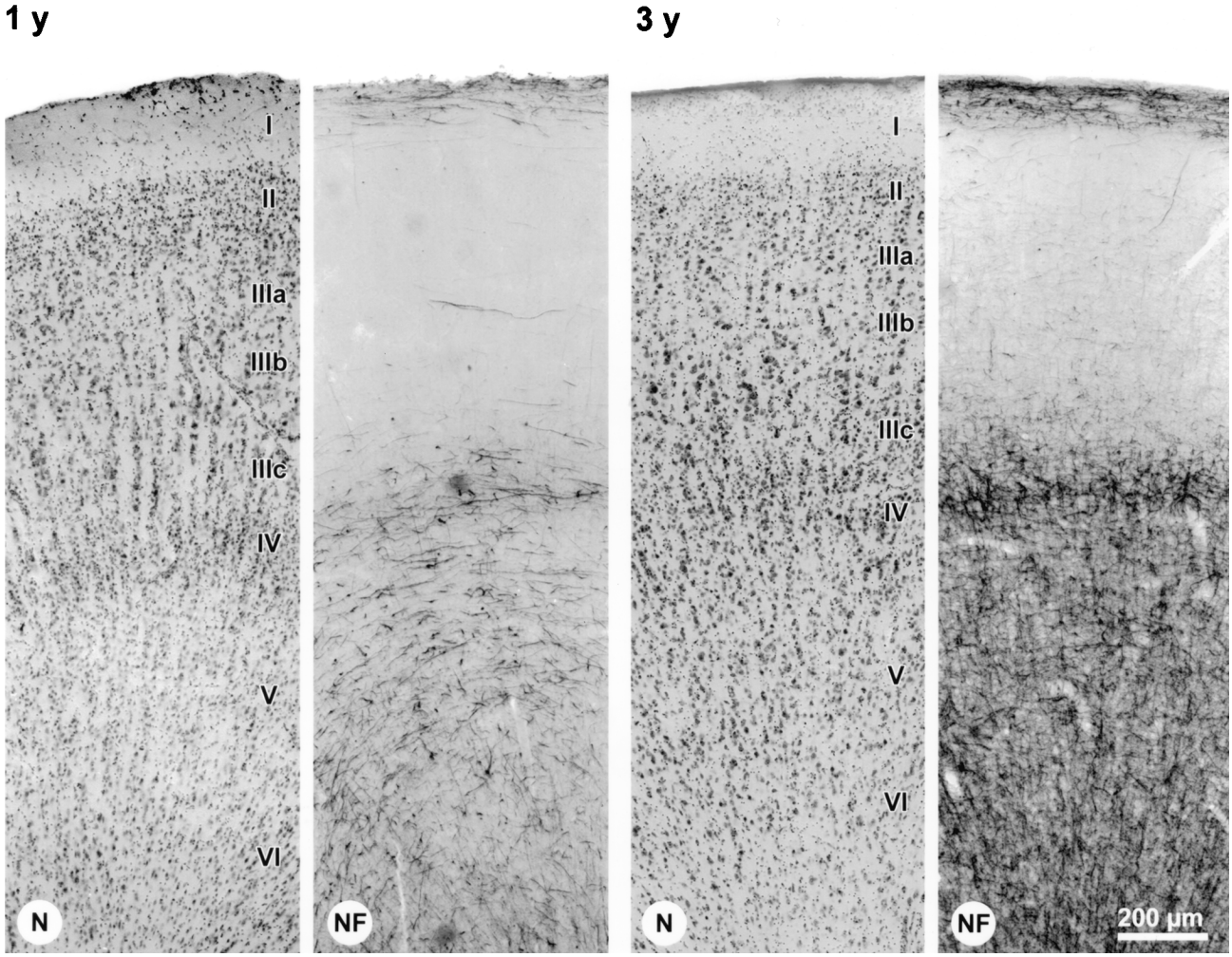

FIG. 7 Cytoarchitecture and axonal maturation in early childhood: Roman numerals indicate cortical layers I–VI. At 1 year (1 y), the Nissl material (N) shows good definition of all six cortical layers and a gradient of cell size within layer III. NF-positive axons run mostly horizontally through layers VI–IV, though isolated NF-positive axons are present in the superficial layers. At age 3 years (3 y), cytoarchitecture (N) is similar to that seen at 1 year, but the large neurons in layers III and VI are more prominent than at earlier ages. In NF-immunostained material (NF), the deeper cortical layers now contain many vertically oriented axons which end in branched formations within layer IV and fine terminals in layer IIIc. At both 1 and 3 years of age, immunostained axons in layer I are less numerous than in the perinatal period and are concentrated near the surface of the layer.

calbindin (Yan et al. 1997) within the developing cortical plate of human visual cortex. With some phenomena, the maturational gradient extends beyond the perinatal period into childhood and teen years. As examples, the superficial layers of human visual cortex do not attain an adultlike pattern of expression of dendritic neurofilament protein (Ang et al. 1991) and of calmodulin and parvalbumin (Letinic and Kostovic 1998 until about 15 years of age. Similarly, neurons in those layers do not begin to express the glycoprotein, chromogranin A, until 9 years of age and do not show an adult pattern of expression until age 25 (Ang et al. 1992).

Given this general pattern of cortical development, it is not surprising that the sequence of axonal neurofilament expression described in cat visual cortex (Liu et al. 1994) is very similar to the present observations in human auditory cortex. In cat, there is heavy NF-immunostaining of horizontal fibers in the marginal layer by postnatal day 20. From postnatal day 20 to postnatal day 75, immunoreactive axons appear progressively in cortical layers VI, V, and IV, with fine fibers extending into the deeper part of layer III. NF-immunostaining of significant numbers of axons in the superficial layers does not begin in cat until the third postnatal month and continues to increase past the fourth postnatal month.

Development of axons in the marginal layer

The first axons displaying neurofilament expression are seen in the marginal at layer around midgestation, at the 22nd fetal week. By the late fetal period and

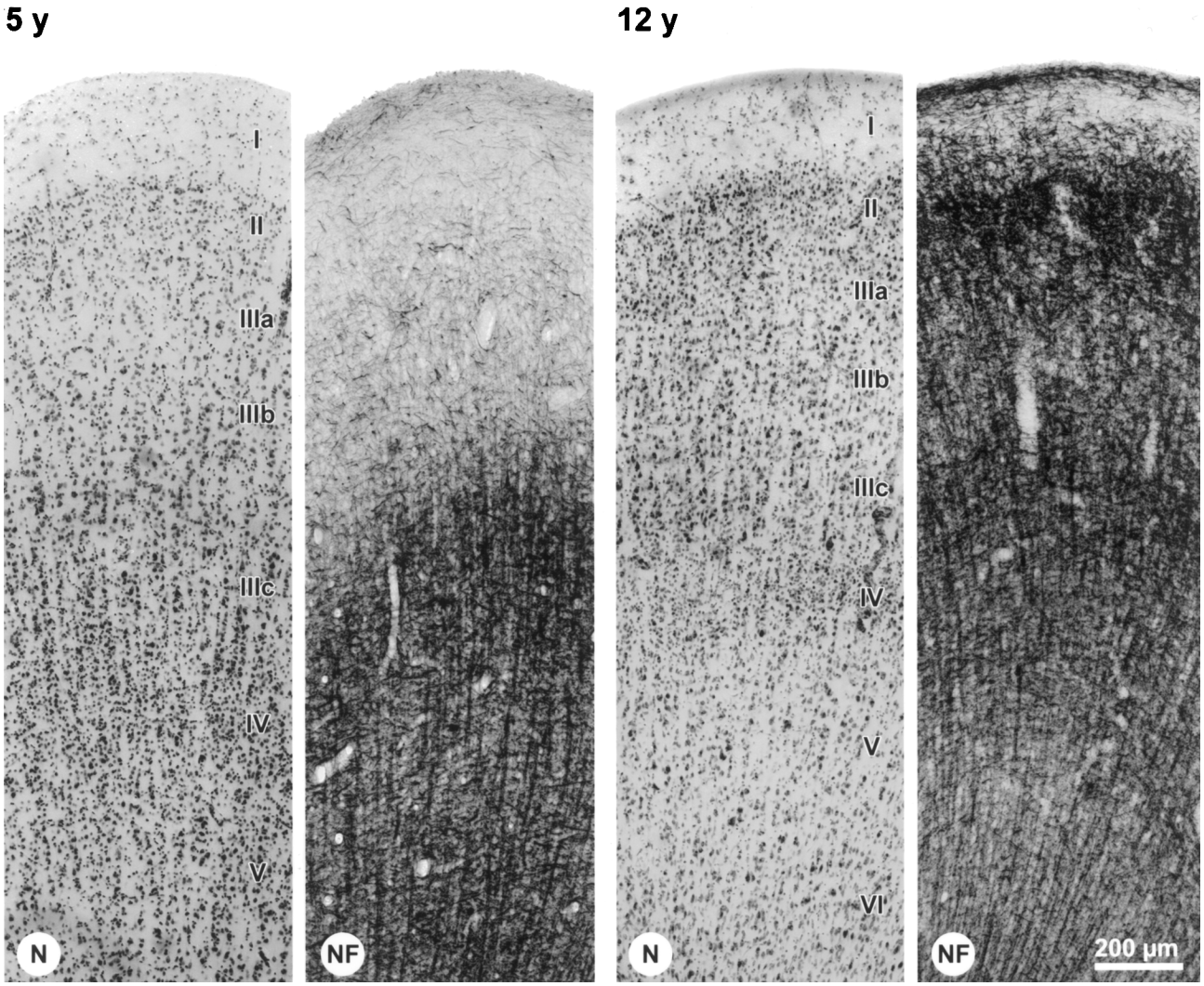

FIG. 8 Cytoarchitecture and axonal maturation in later childhood: Roman numerals indicate cortical layers I–VI. At ages 5 and 12 years (5 y, 12 y), the Nissl material (N) indicates that cortical depth and cytoarchitecture are similar to that seen at ages 1–3 years. NF immunostaining at 5 years (5 y, NF) shows that axonal density in layers VI–IIIc is greater than at ages 1 to 3 years. By 12 years of age, layers IIIb, IIIa, and II have become filled with a grid of horizontal and vertical immunostained axons (12 y, NF). At both 5 and 12 years, a limited number of NF-positive axons are present near the surface of the marginal layer.

the time of term birth, NF-positive axons form a prominent band in the center of layer I. In the months after birth, the band becomes differentiated into upper and lower tiers that differ in packing density. This two-tiered band of axons remains prominent to at least 4.5 months of age, but subsequently there is a reduction in number, especially of the deeper-lying axons. With this regression, the only axons that remain in the marginal layer into adulthood are a sparse population concentrated near the pial surface.

Layer I axons are not a homogeneous population. One identified subgroup consists of the axons of Cajal–Retzius cells, neurons that are located within the marginal layer and are most numerous during prenatal life. Axons arising from these intrinsic neurons have been identified in perinatal rats by biocytin injection (Hestrin and Armstrong 1996; Zhou and Hablitz 1996), by the immunoreactivity of both somata and axons for GABA (Imamoto et al. 1994), and by their immunostaining for calretinin and glutamate (del Rio et al. 1995). In fetal and perinatal human cortex, Cajal–Retzius cells and their axons have been demonstrated by Golgi impregnation (Marin–Padilla and Marin–Padilla 1982), by parvalbumin immunostaining (Ding et al. 2000), and by Di-I injection (Meyer and Gonzáles–Hernández 1993). These intrinsic axons are generally described as thick fibers that form a plexus deep to the neurons from which they arise.

Other layer I axons originate from outside sources, such as the auditory thalamus. In lower mammals, axons from the ventral division of the medial geniculate, which terminate mainly in layer IV, have been

shown to give off collateral branches to layer I (Cetas et al. 1999). In addition, the medial division of the medial geniculate sends axons directly to layer I (Herkenham 1980; Hashikawa et al. 1995). Extrinsic axons also include the "backward" transcortical projections from higher sensory processing areas within the same hemisphere. Such recurrent projections into layer I have been shown to run from S2 to S1 in somatosensory cortex (Cauller et al. 1998) and from V3 to V2 to V1 in visual cortex (Wong–Riley 1978; Rockland and Pandya 1979). In monkey auditory cortex, projections from the secondary cortex of the superior temporal gyrus form a continuous band on the surface of layer I of the primary area (Galaburda and Pandya 1983; Panda and Rosene 1993). Golgi studies of the human marginal layer have described extrinsic axons as thin fibers lying superficial to the plexus of Cajal–Retzius cell axons (Marin–Padilla and Marin–Padilla 1982). This raises the question as to whether the lower tier of axons observed in the NF-immunostained material represents Cajal–Retzius cell axons, and the upper tier of extrinsic axons from the thalamus and adjacent cortical areas. If so, it is possible that the superficial axons that remain into adult life are thalamic and/or cortical in origin.

A generally recognized function of layer I neurons is a regulatory role in guiding migrating neuroblasts to their correct laminar position. It is believed that reelin and other substances produced by Cajal–Retzius cells act as an attractant to the neuroblasts which drives their continuous migration to the surface (del Rio et al. 1995; Meyer and Goffinet 1998). This notion is supported by the failure of normal migration in reeler mutants whose layer I neurons lack reelin (Frotscher 1998). Though Cajal–Retzius cells almost certainly play a role in cortical migration, neuronal migration in human cortex occurs mainly within the first half of gestation. This means that there is little, if any, temporal overlap between cortical plate migration and NF expression in layer I axons. An alternative suggested function for these axons, which would coincide with their time course of NF expression, is stimulation of neurons in the deeper cortical layers. All layer I axons, both intrinsic and extrinsic, are described as running tangentially for long distances, often up to several millimeters, across the cortical surface. As they traverse the layer, they contact and synapse with large numbers of apical dendritic tufts of deeper-lying pyramidal cells (Baloyannis 1993; Spreafico et al. 1999). It has been suggested (Marin–Padilla and Marin–Padilla 1982) that nonspecific stimulation of cortical plate neurons through their apical dendrites plays a vital role in their structural and functional maturation. In support of this view, the time when the maximum number of NF-expressing axons is present in the marginal layer is the late fetal period into the first year of postnatal life. This is the period during which neurons in the cortical plate-derived layers undergo major anatomical maturation, with the result that the cortex reaches its adultlike laminar organization by 1 year of age. Such broadly activating influences might not be limited to the developmental period, as electrophysiological investigations in adult animals have shown that layer I stimulation can evoke a widespread and long-lasting excitatory postsynaptic potential in layer V pyramidal cells (Cauller and Connors 1994). A continuing excitatory influence exerted by layer I axons could account for the superficially located axons that remain into adulthood.

Development of axons in the deeper layers

NF-positive axons are not present in the deeper layers until sometime between 4.5 months and 1 year of age. By age 3 years, NF-positive axons in the deeper layers form an open meshwork of vertical and horizontal axons. This network of NF-positive axons continues to increase in density to age 5. The observations of late development of NF expression in the deeper cortical layers are in agreement with early studies of cortical myelination. Those studies observed an increased in the number of myelinated axons in cortical layers VI through IIIc at 1 and 2 years of age but they also noted that axonal density at age 2 was still considerably less than in subjects 4 and 6 years of age (Conel 1955, 1959, 1963, 1967).

The morphology of these deep-layer axons strongly suggests that they are, or at least include, thalamocortical afferents. In mammals, medial geniculate axons, labeled with such tracers as HRP, PHA-L, and biocytin, are seen to terminate in auditory cortex in discrete patches within layer IV, with some branching into lower-layer III and upper-layer V (Jacobsen and Trojanowski 1975; McMullen and de Venecia 1993; Pandya and Rosene 1993; Hashikawa et al. 1995; Cetas et al. 1999). Vertical fibers in the deeper layers could also include cortical efferent axons that originate predominantly from layers V and VI (Jacobson and Trojanowski 1975; Games and Winer 1988; Prieto and Winer 1999). During development, efferent axons emerge from the deep cortical layers at the time of entrance of thalamocortical afferents. In rat visual cortex, thalamocortical connections are established on embryonic days 17–19, at which time axons from layers 5 and 6 extend toward the thalamus (de Carlos and O'Leary 1992; Kageyama and Robertson 1993). In hamster visual cortex, deep layer afferent and efferent axons form synchronously during the days preceding and following birth (Lent et al. 1990; Miller et al. 1993). Human thalamocortical axons, visualized by GAP-43 labeling in visual cortex (Honig et al. 1996) and by

AChE in auditory cortex (Krmpotic–Nemancic 1983), first enter the cortical plate around midgestation, at the 22nd fetal week. At this same period, efferent neurons in the subplate and deep layers of visual cortex can be labeled retrogradely by Di-I (Hevner 2000).

Though afferent and efferent axons are present in the deep layers of human cortex from midgestation, neurofilament expression does not begin in these axons until after the middle of the first year of life. At 1 and 2 years of age, the plexus of NF-positive axons in the deep layers is relatively light, but it increases in density by ages 3 and 5. This extended course of neurofilament maturation is in agreement with the relatively late and prolonged myelination of the auditory thalamocortical radiations and deep white matter. Early studies of forebrain myelination (Yakovlev and Lecour 1967; Kinney et al. 1988) noted that the visual thalamocortical radiations begin to myelinate in the weeks before birth and attain mature myelin by the 4th postnatal month. In contrast, they observed only incipient myelination in the auditory radiations at 1 year of age, with an adult level of myelin not achieved until around age 4.

Maturation of axons in the superficial layers

Isolated NF-positive axons are present in layers II through IIIb in early childhood, but a true axonal plexus first develops in these layers after 5 years of age. Between 5 and 11–12 years of age a meshwork of vertical and horizontal axons, equivalent to that seen in young adults, develops in the superficial layers. As in the deep layers, the time of proliferation of neurofilaments coincides with the time of appearance of myelinated axons, as the first significant numbers of myelinated axons in layers II through IIIb are noted at age 6 (Conel 1967).

Axons in the superficial layers should represent primarily corticocortical projections. One basis for this assumption is the fact that thalamic afferents and cortical efferents are restricted to layers IIIc through VI. Another basis is the fact that layers II–IIIb contain many of the cells giving rise to commissural axons (Winer 1984a, 1984b) and association projections (Winguth and Winer 1986). However, neurons in the superficial layers do not account for the entire system of corticocortical projections. Commissural and association projections have been shown to be bilaminar, originating from neurons in both the superficial and the deeper cortical layers. In the commissural system, neurons with axons projecting through the corpus callosum form two bands of neurons, one deep, usually described as layer V, and one superficial, usually described as being in layer III (Jacobson and Trojanowski 1974; Kelly and Wong 1981; Feng and Brugge 1983; Jouandet et al. 1985; Auladell et al. 1995). The same bilaminar pattern of origin is seen in association projections within the same hemisphere (Winguth and Winer 1986; Lent et al. 1990).

The two laminae of transcortical axons are not identical. Cells in the superficial layers are more numerous and have, when mature, a clustered or patchy distribution, while the cells in the deeper layers are more sparsely and irregularly scattered (Kelly and Wong 1981; Feng and Brugge 1983; Winguth and Winer 1986). The two laminae also differ in their developmental time course. In hamster, retrogradely labeled neurons adjacent to an HRP injection site are seen in layer V on postnatal day 1 but are not seen until postnatal day 8 in the supragranular layers (Lent et al. 1990). Maturation of callosal neurons in the superficial lamina continues longer into the postnatal period, up to postnatal day 25 in hamsters (Hedin–Pereira et al. 1999) and the 3rd postnatal month in kittens (Feng and Brugge 1983). In human visual cortex, Di-I diffusion reveals horizontal connections between areas V1 and V2 (Burkhalter et al. 1993). The association projections in layers IV and V emerge at the 37th fetal week and increase in density to the 7th postnatal week. The system of axons in layers II and III does not form until after the 16th postnatal week and reaches its mature form later in the first year of life.

Though intrinsic cortical axons are present in both the deep and superficial cortical layers, the present study provides no basis for distinguishing transcortical axons from subcortical afferents and efferents in the deeper cortical layers. This means that the process of neurofilament maturation we observe in layers II–IIIb presumably represents only the later-developing superficial connections. It also raises the possibility that commissural and association projections are present in the deeper layers before age 5 and, thus, could provide a basis for corticocortical information transfer during early childhood years. In any case, the extended maturation of commissural and association projections must be a factor in the long-term increase in size of intracortical axonal pathways. Radiologic studies show that volume of the corpus callosum increases steadily through childhood and teen years (Pujol et al. 1993; Rauch and Jinkins 1994; Giedd et al. 1996). Similarly, a steady increase in white matter density is seen between ages 4 and 16–17 in the arcuate fasciculus, a temporofrontal association pathway (Paus et al. 1999). The late onset of neurofilament maturation and myelination in the superficial layers of human auditory cortex, along with the continued expansion of commissural and association pathways, are indicators of anatomical development which can provide a basis for increasing complexity in cortical

processing of auditory information during later childhood years.

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