Glial cells develop a laminar pattern before neuronal cells in the lateral geniculate nucleus

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ABSTRACT The lateral geniculate nucleus, which lies between the retina and the striate cortex in the visual pathway of mammals, is often made up of several distinctive cell layers or laminae. We have used immunohistochemical methods to localize two glial cell intermediate filament proteins, glial fibrillary acidic protein and vimentin, and have found that the presence of glial cell layers develop in the lateral geniculate nucleus. The correlation between glial cell lamination and neuronal lamination is consistent with the suggestion that glia are guiding neurons not only during the early postmitotic migratory phase of development but also during the later formation of functional divisions such as layers and nuclei.

For some time now, we have been interested in the mechanisms that result in the presence of distinctive cell layers in the lateral geniculate nucleus (LGN), the major thalamic target of retinal fibers in mammals (1–4). One aspect of these studies has been to examine correlations between the various events involved in lamination in the LGN of the tree shrew (Tupaia belangeri), one of the few species in which LGN cell layers develop postnatally (1–4).

Lamination is generally defined as areas packed with neuronal somata that alternate with relatively cell-free interlaminar zones. However, this describes only one of at least three types of lamination in the LGN. Each type has a distinct time course and may involve a distinct mechanism (1–4). Retinal terminals innervating the LGN are found in layers at birth in tree shrews (1–4) and probably begin and end their segregation process during the last 2 weeks of embryonic development (V.A.C. and G. J. Condo, unpublished data). (Gestation in the tree shrew is 43 days. We have designated the day of birth as postnatal day zero (P0); therefore, embryonic day 43 is equivalent to P0.) This afferent lamination is contrasted with cytoarchitectonic lamination, the differential packing density of cells (i.e., into cellular and cell-free zones). The former process occurs during postnatal week 1 in tree shrews (1–4). Finally, the LGN exhibits cytoarchitectonic lamination — differences in the size and shape of cell bodies. In the tree shrew LGN, this process occurs during postnatal weeks 2 and 3 (1–4).

At present, it is unclear what mechanisms are involved in the development of cell layers. A role for glial cell involvement in the development of more elaborate cytoarchitectonic arrangements of neurons is suggested by recent studies (5, 6) that show arrangements of glia during early development of the barrel fields of rodent somatosensory cortex and of the neostriatum that are strikingly similar to neuronal patterns present in the adult. A specific interaction of glia and neurons in vitro, involving ultrastructural specializations, has been demonstrated (7, 8). Similar specific neuronal-glial interactions may operate in vivo as well.

The present paper reports evidence that glial cells are distributed in layers just before neurons show a cytoarchitectonically defined laminar pattern. Thus, it is possible that glial cells may play a part in forming the final arrangement of neuronal cells in the LGN.

MATERIALS AND METHODS

Perfusion-fixed tree shrew brains were sectioned and processed for immunohistochemical localization of glial cell markers, glial fibrillary acidic protein (GFAP) and vimentin. Both antibodies recognize intermediate filament proteins thought to reside only in glial and ependymal cells in adult brain tissue (9). Two types of controls were performed to ensure staining specificity: adjacent sections were processed as nonimmune controls, and immunoblotted NaDodSO4/PAGE gels of homogenized tissue at corresponding ages were used to characterize the proteins bound by the primary antibodies used for immunohistochemical staining.

Immunohistochemistry. Tree shrews were obtained from our breeding colony within 24 hr after birth (on P0) or at the ages indicated. A lethal overdose of anesthetic was administered, and the animals were perfused with lactated Ringer's solution followed by a mixed aldehyde fixative (4% freshly depolymerized paraformaldehyde/0.1% glutaraldehyde/0.1 M cacodylate buffer, pH 7.4). Brains were removed and postfixed in the same mixture for 1 hr. A second postfixation (2% paraformaldehyde in cacodylate buffer at 4°C) followed. For correlated light and electron microscopic studies, tissue was sectioned in the horizontal plane on a Vibratome (Pelco) at 50–100 μm; the sections were taken through an ascending glycerol/sucrose series, flash-frozen, and then thawed and processed for immunohistochemistry as described below. This procedure allows penetration of immunoreagents with minimal disruption of tissue ultrastructure. For studies using light microscopy only, the tissue was equilibrated in cacodylate-buffered 30% sucrose and sectioned frozen at 40 μm on a sliding microtome. Sections were rinsed in phosphate-buffered saline (PBS: 0.8% NaCl/50 mM phosphate buffer, pH 7.4) and then preincubated in PBS containing 10% (vol/vol) normal horse serum and 0.1% Triton X-100 (Triton was omitted from all incubations with tissue destined for EM). Incubation in primary antibody followed. Monoclonal antibodies (mouse origin) to GFAP and vimentin were obtained from Boehringer Mannheim. Sections were incubated free-floating on a shaker platform in primary antibody at 5 μg/ml in PBS/0.1% Triton X-100 for 3 days at 4°C. The primary antibody incubation was terminated by three rinses in PBS, followed by incubation (2–8 hr at room temperature)
with biotinylated horse anti-mouse IgG (1:100, Vector Laboratories, Burlingame, CA). Another series of rinses terminated the second antibody incubation, which was followed by incubation in "ABC" reagent (a biotinylated horseradish peroxidase/avidin mixture, obtained from Vector Laboratories). The tissue was rinsed and assayed for bound horseradish peroxidase by using either 3,3′-diaminobenzidine (at 2 mg/ml in 0.1 M phosphate buffer) with 0.02% H2O2 or the same mixture with 0.02% CoCl2 added to enhance the immunostaining. Sections were rinsed, mounted on slides coated with gelatin/chrome alum, dehydrated, and covered with a coverslip. Alternate sections were usually processed for Nissl staining with cresyl violet to compare cytoarchitectonic features to glial cell staining.

In nonimmune controls, primary antibody was omitted from the first incubation, and the tissue was processed in parallel with immunostained tissue in all subsequent steps. Nonimmune controls showed only a faint background staining with either cobalt-enhanced or unenhanced diaminobenzidine processing.

EM. After immunocytochemical processing, tissue destined for electron microscopic studies was postfixed in 1% OsO4 and dehydrated in an ethanol series. Tissue was cleared in propylene oxide and embedded in Epon/Araldite resin. For high-voltage EM, sections were cut at a thickness of 1 μm, dried onto filmed slot grids, and examined at 750 kV in the high-voltage EM facility at the University of Colorado, Boulder.

RESULTS

The monoclonal antibodies used in this study did not stain cells in the adult tree shrew LGN to any significant degree; the only labeling seen after immunohistochemical processing of adult tissue occurred in the end-feet of presumed glial cell processes near blood vessels traversing the nucleus. This pattern was in sharp contrast to the situation early in postnatal development. At the day of birth (P0) GFAP-like immunoreactive (GFAP+) processes were clustered in the inchoate layers of the LGN (Fig. 1A) before these layers were apparent with Nissl staining techniques (Fig. 1B). A number of astrocytes were seen at this age in the developing LGN (Fig. 1C). However, the fine processes of these cells that make up the laminar staining pattern at early ages could not be resolved well by light microscopy. The overall pattern of vimentin immunoreactivity in the tree shrew LGN at this age was less distinctive (Fig. 1D). Some tendency toward labeling in discrete layers was seen but was not as evident as with the tissue immunostained for GFAP. The nucleus was laced with bundles of vimentin+ processes, presumably in association with axon bundles entering the nucleus. This layering of glial cell processes was apparent at least 1–2 days before the first signs of cytoarchitectonic lamination in the tree shrew LGN; at later developmental ages, it was always more pronounced than the corresponding Nissl staining pattern.

After the lamination of neurons in the LGN was complete (in tree shrews about P8 and older), the vimentin+ and GFAP+ bands were found preferentially in these layers. Several examples are shown in Fig. 2. Compare the bands of vimentin+ processes at P9 (Fig. 2A) to the Nissl staining pattern in an adjacent section (Fig. 2B). By P14, the bands of vimentin+ processes had dissolved and almost no vimentin+ glial processes were apparent in low-power micrographs (Fig. 2C). There was a great deal of variability in the staining pattern at this age, and other cases showed remaining bands of vimentin+ processes. At the same age in adjacent sections, banding of GFAP+ processes was still consistently obvious (Fig. 2D). Thus, the characteristic six cell layers were apparent in the developing nucleus at 2 weeks after birth (P14) when either GFAP immunoreactivity or Nissl staining was used as a marker, but not always with vimentin. A week later, at P21, the GFAP immunostaining pattern was adult-like (that is, almost no immunoreactivity was apparent in the nucleus at low power), and no banding of immunoreactive material was seen with this technique (Fig. 2E). By this time, however, an adult-like pattern of Nissl staining was established in the LGN, and the characteristic cytoarchitecture and cytoarchitectonics of the six cell layers was apparent (Fig. 2F).

The difficulty in resolving fine GFAP+ and vimentin+ processes using the light microscope led us to pursue preliminary work on the localization by EM of GFAP and vimentin immunoreactivity. An example of a GFAP+ cell (presumably a glial cell) from a P0 tree shrew is shown as two stereo-pair micrographs (Fig. 3A and B). In Fig. 3A, a relatively small GFAP+ perikaryon gives rise to a fine GFAP+ process extending into an incipient LGN lamina. Filamentous GFAP+ bundles extend through the fine processes of this cell. The nucleus is only 4 μm in its largest dimension, and the perikaryon rapidly gives rise to a set of fine, filament-carrying processes less than 0.5 μm in diameter. The soma size, general morphology, and immunostaining of this cell are all consistent with its preliminary identification as a glial cell. Based on correlations between the light and
DISCUSSION

There is considerable evidence to support the idea that glia can act as guides for migrating neural cells during development (12–21). However, in many areas of the nervous system, neurons that migrate to a target zone later become further reorganized into distinct nuclei and layers. For example, cells destined to become the LGN, the major thalamic target of retinal fibers, are born at the wall of the third ventricle at the midline and migrate on radial glial guides to their final positions at the lateral margin of the thalamus (20). In many species, these LGN cells are subsequently arranged into layers.

It has been generally argued (12–21) that only elongated forms of glia (radial glia and similar cells, such as Bergmann glia) are involved in guiding neurons during development. In our material, GFAP+ and vimentin+ radial glia are found in the LGN prenatally (unpublished observations) but are absent from the LGN during postnatal cell layer formation. Although vimentin+ radial glia are absent from the LGN after birth, they are obvious in the same sections of the nervous system, particularly cortex. In contrast, the vimentin+ glia that are present in the LGN at this time have the stellate form typical of astroglia. Several recent in vitro studies of immature cerebellar astroglia have shown that the developing stellate types preferentially associate with neurons. Neurons actively attach themselves to the processes of these stellate astroglia and gradually cluster together in large numbers along the available surfaces of these glia (22, 23). If a similar attraction exists between LGN astrocytes and developing LGN neurons, then one could easily imagine how a laminated glial pattern would eventually result in a matching neuronal laminar pattern.

Further support for this view comes from work in the developing rodent somatosensory cortex and neostriatum. The barrel fields of mouse cortex, like the layers of the tree shrew LGN, consist of clusters of cell bodies preferentially distributed away from relatively cell-sparse zones. Cooper and Steindler (5) have found that subdivisions of the somatosensory barrel fields are delineated by GFAP+ processes early in development and that the pattern of GFAP immunoreactivity disperses during maturation. This process is disrupted when glia are abnormal, as in the reeler mutant mouse (21, 24). Similarly, in the neostriatum, developing "striosomes" are apparent in early postnatal tissue stained for GFAP immunoreactivity (6). All of these studies suggest that astroglia may help mature neurons reach their final, adhesive locations to form distinctive patterns such as laminae, barrel fields, and striosomes.

In the tree shrew LGN, the layering of glia occurs after the laminating of retinal afferents but at least 24 hr before the neurons have formed into layers. The retinal afferents may form a template, which the glia follow, or vice versa. Skoff et al. (25) have shown that GFAP+ astrocyte processes are arranged in bundles in the rat optic chiasm. This "astrocytic fasciculation" suggests a possible functional role of astroglia in guiding ganglion cell axons to their targets as they are sorted at the chiasm. Alternatively, formation of glial laminarization may be coincidental to formation of the cell layers and independent of the growth of retinal fibers. In our preliminary observations of the ferret LGN (another species in which neuronal cells develop layers after birth), glial cell laminarization (in interlaminar zones) is not seen during the period when retinal afferents are segregating or when the final location of neurons can be predicted from their characteristic cytolgy. Rather, the glial cell processes form layers during the period when interlaminar spaces appear between cell layers (unpublished data). The correspondence of cytoarchitectonic and glial cell laminarization in the tree shrew is shown diagrammatically in Fig. 4. Although there are differences in the details.

electron microscopic immunohistochemistry, this micrograph most likely shows a portion of a fibrous astrocyte. This is also corroborated by Fig. 3C, which is a conventional (ultrathin section) micrograph from a P0 animal. The stained glial cell process is adjacent to the pericyte making up a blood vessel in the LGN.

In pilot studies, immunoblots of gels, prepared according to a modification of the method of Towbin et al. (11), showed major bands at approximately 51 kDa for GFAP and 57 kDa for vimentin, in agreement with the published molecular masses of the proteins (9). These results will be presented in full elsewhere.
and timing of the key points in development, lamination of glial cell processes in both the tree shrew and ferret LGN follows the lamination of retinal afferents but precedes and is coincident with the lamination of neuronal cells. The correlation between glial cell lamination and neuronal lamination could be used to support several different models for the formation of cell layers in the LGN, including the possibility that glial cells play no direct role at all but merely indicate the position of other mechanisms that represent the "real" pattern-generating force. In any case, the results presented here cannot be used to prove that glial cells guide neurons into cell layers. For example, it may be that glial cell differentiation occurs in response to neuronal changes such as axonal growth, dendritic growth, or possibly synaptogenesis. However, radial glia are known to guide neurons during early development (12–21); specific interactions between glia and neurons have been shown to exist in vitro (7, 8, 22, 23); and the correlation between glial cell patterning in development and the final neuronal patterning in adults is consistent across species and at all levels of the nervous
LAMINATION OF TREE SHREW LGN

FIG. 4. Diagrammatic representation of critical events in tree shrew LGN development. Three of the key stages in the development of the tree shrew LGN are shown on this "time chart" of LGN development. Retinal afferents appear to segregate between embryonic day 29 (E29) and the day of birth (43 days of gestation, or E43 = P0). However, cytoarchitectonic lamination (the formation of clear cell-rich layers interleaved with relatively cell-sparse zones) is not complete in the tree shrew until P8. Note that the glial cell lamination reported here is contemporaneous with and extends beyond the period of cytoarchitectonic lamination. Glial cell lamination is no longer seen in the LGN after P21, when the LGN exhibits its characteristic adult Nissl staining pattern.

system (5, 6, 24, 25). All of these factors argue in favor of the idea that glial cell patterns play a role in the neuronal distribution. This, in turn, raises the question of the formation of glial cell layers.

The precise mechanism(s) underlying the glial cell lamination observed in this study are still not clear. Whatever the nature of the interaction between glial cells and neurons, future work will be necessary to elucidate whether the layering of both glia and neurons is directed by retinal afferents, whether all three processes are directed by some undiscovered factor(s), or whether glia independently direct the segregation of neurons into cell layers.

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