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Early ingrowth of thalamocortical afferents to the neocortex of the prenatal rat

(development/somatosensory/initial connectivity/cortical differentiation)

SUSAN M. CATALANO*, RICHARD T. ROBERTSON*, AND HERBERT P. KILLACKEY*†‡

Departments of †Psychobiology and *Anatomy and Neurobiology, University of California, Irvine, Irvine, CA 92717

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ABSTRACT **The initial ingrowth of thalamocortical afferents into the presumptive somatosensory cortex was examined in the fetal rat. Thalamic fibers were labeled in fixed brains with the carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). On embryonic day 16, thalamocortical afferents arrive in the neocortex and course tangentially within the intermediate zone immediately underneath the cortical plate. By embryonic day 17, thalamocortical fibers have begun their radial growth into cortex and their arbors span the cell-sparse zone between layer VIb and the bottom of the cortical plate. By the day of birth (embryonic day 21), thalamocortical fibers form a dense plexus within layers VI and V below the dense cortical plate. Our observations indicate that in the rat thalamic afferents arrive in the cortex at a very early age and arborize within the forming cortical layers without an apparent "waiting" period.**

The mechanisms that control the development and differentiation of the neocortex remain the subject of intense interest. Recent suggestions (1, 2) that thalamocortical projections play some role in these processes imply that thalamocortical projections make contact with the neocortex at a relatively early stage. However, the available evidence on this point is equivocal. On the one hand, Lund and Mustari (3) have reported that thalamocortical fibers reach the occipital cortex of the rat on embryonic day (E) 18 and then progressively invade the visual cortex on the following days. On the other hand, it has been reported that in the rat somatosensory cortex the thalamocortical fibers accumulate in the white matter below the cortical layers for several days and commence their innervation of the neocortical layers at about the time of birth (4, 5). This observation, as well as those of several other investigators (6, 7), has led to the generally held belief that the development of thalamocortical projections is characterized by a "waiting" period. Further, it has been suggested that during this waiting period thalamocortical projections make contact with a transient population of cortical neurons, the subplate, and that these transient interactions play a particularly important role in the development and differentiation of the neocortex (8).

We chose to investigate this question further because until recently the methods available to delineate thalamocortical projections were not particularly well-suited for use in the fetal brain. The introduction of carbocyanine dyes, such as 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), as neuronal tracers in previously fixed tissue (9) overcomes many of the shortcomings of previous methods. Our study focuses on the earliest development of thalamocortical projections to the presumptive somatosensory cortex of the rat (E16 to the day of birth). The later development of these afferents and their adult morphology has been studied (10–12). The available evidence suggests that the somatosensory cortex is one of the first portions of neocortex to differentiate (13), making this region ideal for the study of the initial invasion of the neocortex by thalamic afferents.

MATERIALS AND METHODS

Timed-pregnant Sprague–Dawley rats were obtained by overnight mating. A sperm-positive vaginal smear the following morning was considered an indication of probable conception and the 24-hr period beginning at the time of confinement for mating was designated E0. By this criterion, birth usually occurred late on E21. Rat fetuses between E15 and E21 were employed in this study. Thalamocortical projections were examined in at least four animals at each embryonic age. Pregnant dams were deeply anesthetized with ketamine/xylazine. Fetuses were individually removed from the uterus and further anesthetized with sodium pentobarbital if E19 or older. They were then perfused transcardially with 4% (wt/vol) paraformaldehyde. Heads were removed and postfixed in 4% paraformaldehyde overnight, and then the brains were removed. Brains were cut in the mid-sagittal plane and crystals of DiI (Molecular Probes), approximately 100 μm in diameter, were inserted into the thalamus with a glass micropipette. Other crystals were placed in the vicinity of the central tegmental tract at the level of the mesencephalic flexure to delineate the trajectory of brainstem afferents to the cortex. Brains were stored in fixative at room temperature for periods of up to 5 weeks. Brains were then embedded in agar and 75-μm sections were cut on a Vibratome into phosphate buffer (0.02 M monobasic sodium phosphate and 0.08 M dibasic sodium phosphate). Wet-mounted sections were examined under epifluorescence illumination (excitation wavelength, 547 nm) and sections were selected for photoconversion. The remaining sections were counterstained with bisbenzimide (Sigma) to visualize cortical cytoarchitecture, placed on a slide with phosphate buffer, coverslipped, and sealed with DePeX mounting medium (Gurr, Santa Monica, CA).

Individual sections were photoconverted according to the protocol of Sandell and Masland (14). Briefly, sections were preincubated in a solution of 10 mg of diaminobenzidine in 6.7 ml of 0.1 M Trizma base (pH 8.2) at 4°C for several hours. Sections were then placed on a depression slide in a pool of the diaminobenzidine solution and illuminated using a rhodamine filter set for 1–3 hr with a 200-W halogen bulb through a ×20 objective. The diaminobenzidine solution was changed at half-hour intervals. At the end of this period, the sections were rinsed, placed on gel-coated slides, dried, counterstained with cresyl violet, and coverslipped with Permount.

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Abbreviations: DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; E, embryonic day.

‡To whom reprint requests should be addressed.

RESULTS

A typical crystal placement in which the dye is clearly confined to the dorsal thalamus is illustrated in Fig. 1. It is at this age (E16) that fibers can be first traced to the neocortex. In the section illustrated in Fig. 1, fibers can be seen to form a compact bundle traversing the striatum. On the preceding day (E15), thalamic fibers also formed a compact bundle but only reached the medial edge of the striatum, the vicinity of the arrow in Fig. 1 (unpublished observations). We also determined that fibers labeled by brainstem injections travel in a more ventrally situated bundle and do not intermingle with thalamic fibers (unpublished observations).

The initial growth of thalamic afferents into the neocortex is illustrated in Fig. 2 *A* and *A'*. On E16, thalamocortical fibers have passed the region of the ventricular angle and course tangentially within the upper part of the intermediate zone. Many of the axons are tipped by elongate growth cones with a few filopodia, a morphology typical of growth cones located within fiber tracts (15). At this age the neocortex can be divided into an outer marginal zone, a cortical plate, an intermediate zone, and a ventricular zone. Laterally, but not medially, the lowermost layer of cortex, layer VIb, can be seen as separate from the overlying cortical plate (16). This observation is consistent with the general lateral to medial wave of maturation in cortex (13). On subsequent days, we retained this same terminology but extended it by designating the cortical layers as they differentiated from the dense cortical plate (see Fig. 2). On E16 and the following days, a few retrogradely labeled cells were found in the cortical plate, lamina VIb, and the intermediate zone (see Fig. 2*A'*).

On E17 (results not illustrated), some fibers had grown further medially within the intermediate zone coincident with the further medial differentiation of layer VIb from the overlying cortical plate, and others had reached the vicinity of the occipital pole. Laterally, in the region of cortex first encountered by the thalamocortical fibers, a few individual fibers can be seen to turn from their tangential course in the intermediate zone and course obliquely through the widening cell-sparse zone separating layer VIb from the cortical plate (i.e., the bottom of layer VIa). Thus, E17 is the day on which thalamic afferents commence their invasion of the cortical layers.

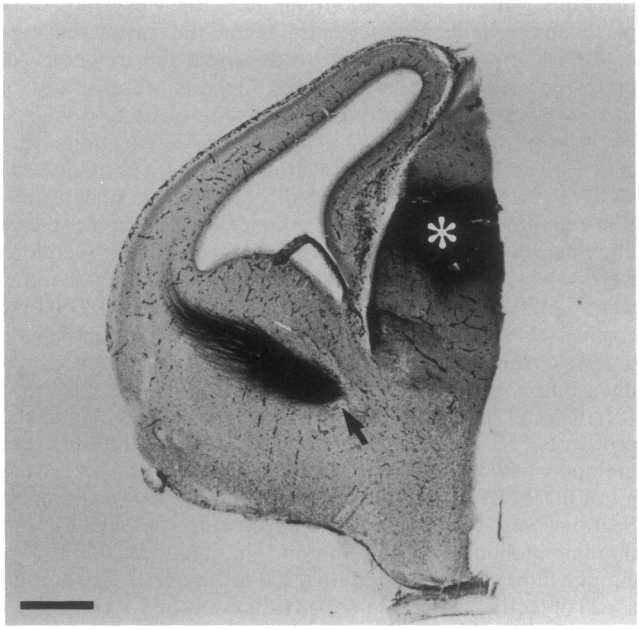

FIG. 1. Photomicrograph of a coronal section through the thalamus of an E16 rat illustrating the site of DiI crystal placement (*) and the resultant label (photoconverted). The course of thalamic fibers through the striatum and their initial entry into neocortex are evident. The arrow indicates the place at which thalamic fibers enter the striatum. (Bar = 500 μm.)

On the subsequent days the ingrowth of thalamic afferents continues and appears to closely parallel the cytoarchitectonic differentiation of the cortical layers from the dense cortical plate. By E18, these afferents are located throughout the width of the intermediate zone underneath lateral cortex. Tangentially oriented fibers are confined to the intermediate zone and the immediately adjacent lamina VIb (Fig. 2*B*). The deepest part of layer VIa has further differentiated, increasing the distance between lamina VIb and the bottom of the cortical plate (bars in Fig. 2*B'*). At this age, more fibers have separated from the tangentially coursing population at the VIb border and arborize in an oblique radial direction within layer VIa deep to the border with the dense cortical plate (Fig. 2*B'*). Lamina VIa further differentiates from the dense cortical plate over the ensuing day. By E20, individual afferents tipped with small growth cones can be seen throughout layer VIa up to the border with the dense cortical plate (Fig. 2 *C* and *C'*). By E21 (the day of birth), lamina Vb has differentiated, and labeled fibers can be seen arborizing throughout this layer. The dense cortical plate is a relatively small fraction of the thickness of cortex by this time and a few labeled fibers have penetrated it in the lateral portions of cortex (Fig. 3*A'*). Coincident with the cytoarchitectonic differentiation of these layers, a few retrogradely labeled cells can be identified within them.

The close correspondence between the cytoarchitectonic differentiation of the cortical layers and the ingrowth of thalamic afferents along a medial to lateral gradient is demonstrated in Fig. 3. Fig. 3 *A* and *A'* illustrates the pattern of cortical lamination and thalamic afferent innervation in lateral presumptive somatosensory cortex on E21. Fig. 3 *B* and *B'* illustrates these same features more medially in the same section. Clearly, both cytoarchitectonic differentiation and thalamic ingrowth were further advanced laterally than medially.

DISCUSSION

The present study provides evidence that in the rat thalamocortical axons reach the neocortex by E16. These fibers continue to grow medially in a tangential direction and turn to grow radially into the cortical layers by E17. The radial growth of thalamic afferents within cortex proceeds steadily, invading each cortical layer as it differentiates cytoarchitectonically from the dense cortical plate. Only a few labeled fibers can be seen in the deeper portions of the cell-dense cortical plate. We found no evidence that the ingrowth of thalamic afferents in this species is characterized by a waiting period.

A few technical points should be noted. The most important point is whether our crystal placements did, indeed, label thalamic afferents. Brainstem monoaminergic afferents are known to reach cerebral cortex during late fetal development (17) and serotonergic fibers are distributed in a pattern analogous to the thalamocortical afferents in the neonatal rat (18). However, we have determined that these brainstem fibers travel in a pathway distinct from the thalamic afferents and are not labeled by our thalamic placements (unpublished observations). Further, brainstem afferents enter the cortex at its rostral pole rather than laterally beneath the ventricular angle as the thalamic fibers do. A second point is the possibility that the labeled fibers in cortex are processes of neurons in the intermediate zone and layer VIb labeled either retrogradely or transneuronally. It is known that DiI can diffuse transcellularly (9). We have noted faint labeling of radial glia in some of our material that must be regarded as transneuronal. However, we regard it as highly unlikely that

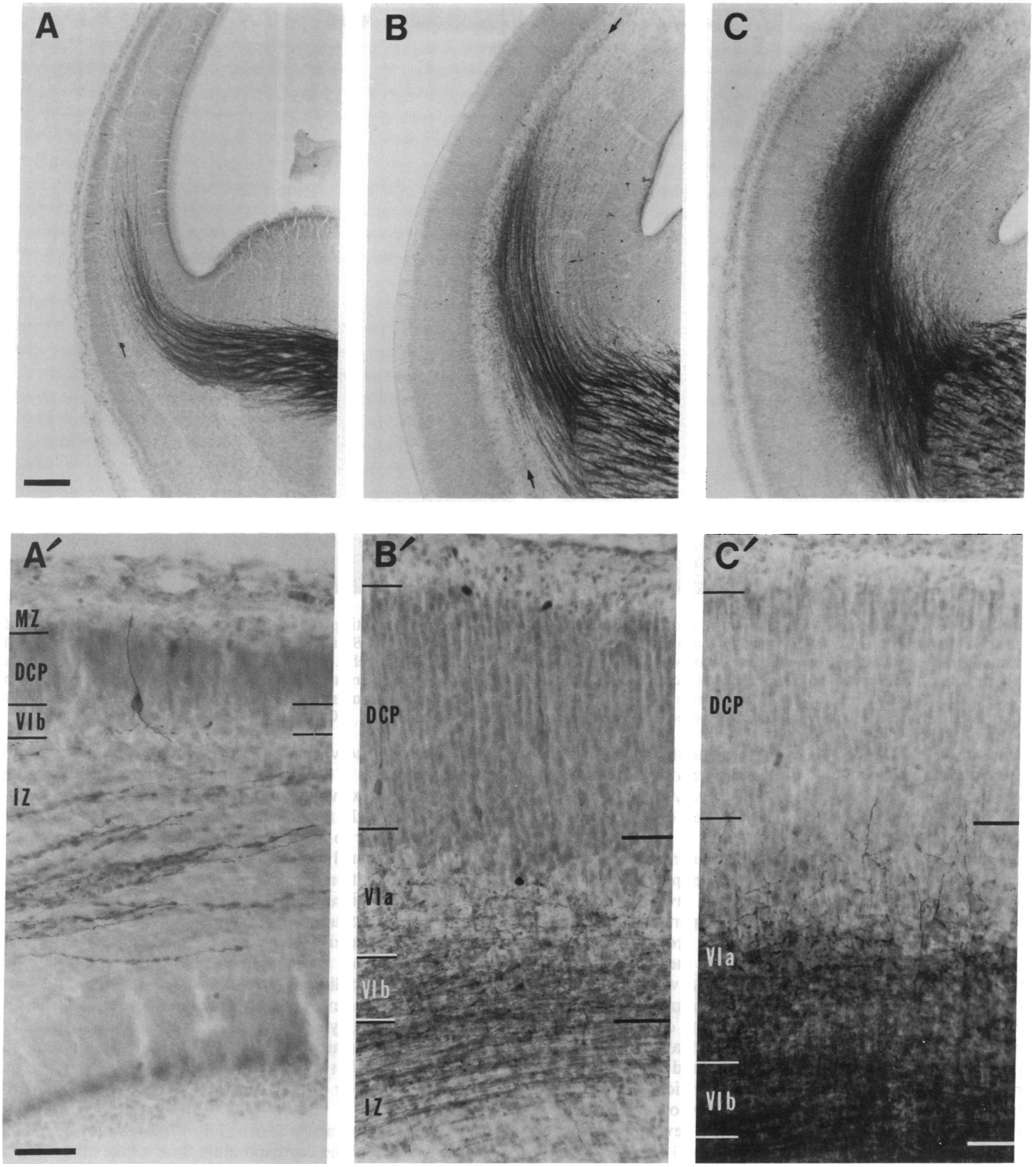

FIG. 2. Photomicrographs of photoconverted and Nissl-counterstained coronal sections illustrating the distribution of thalamic afferents and their relation to the developing neocortex on E16 (*A* and *A′*), E18 (*B* and *B′*), and E20 (*C* and *C′*). The arrows in the upper photomicrographs indicate layer VIb. The cortical layers are indicated on the left of the lower photomicrographs. The upper bar on the right in each of the lower photomicrographs indicates the lower border of the dense cortical plate (DCP). We define the dense cortical plate as the undifferentiated zone of closely packed cells just under the marginal zone (MZ). As development proceeds and the neocortex expands, the lower border of the dense cortical plate moves upward relative to the differentiating cortical layers and the intermediate zone (IZ). The lower bar on the right indicates the bottom layer of VIb. Note that on successive days the space between the upper and lower bars increases reflecting the differentiation of the cortical layers. On E18 (*B′*) and E20 (*C′*), the space between the bars is also filled with arborizing thalamic fibers. (Scale bars: *A–C*, 200 μm; *A′–C′*, 50 μm.)

collaterals or processes of labeled elements form a significant fraction of what we interpret as thalamic label as one can clearly trace labeled thalamic fibers from the internal capsule into the cortical layers. Further, we are struck by the paucity of labeled cortical cells in our material. The most likely explanation of the sparse retrograde label is that few cortical

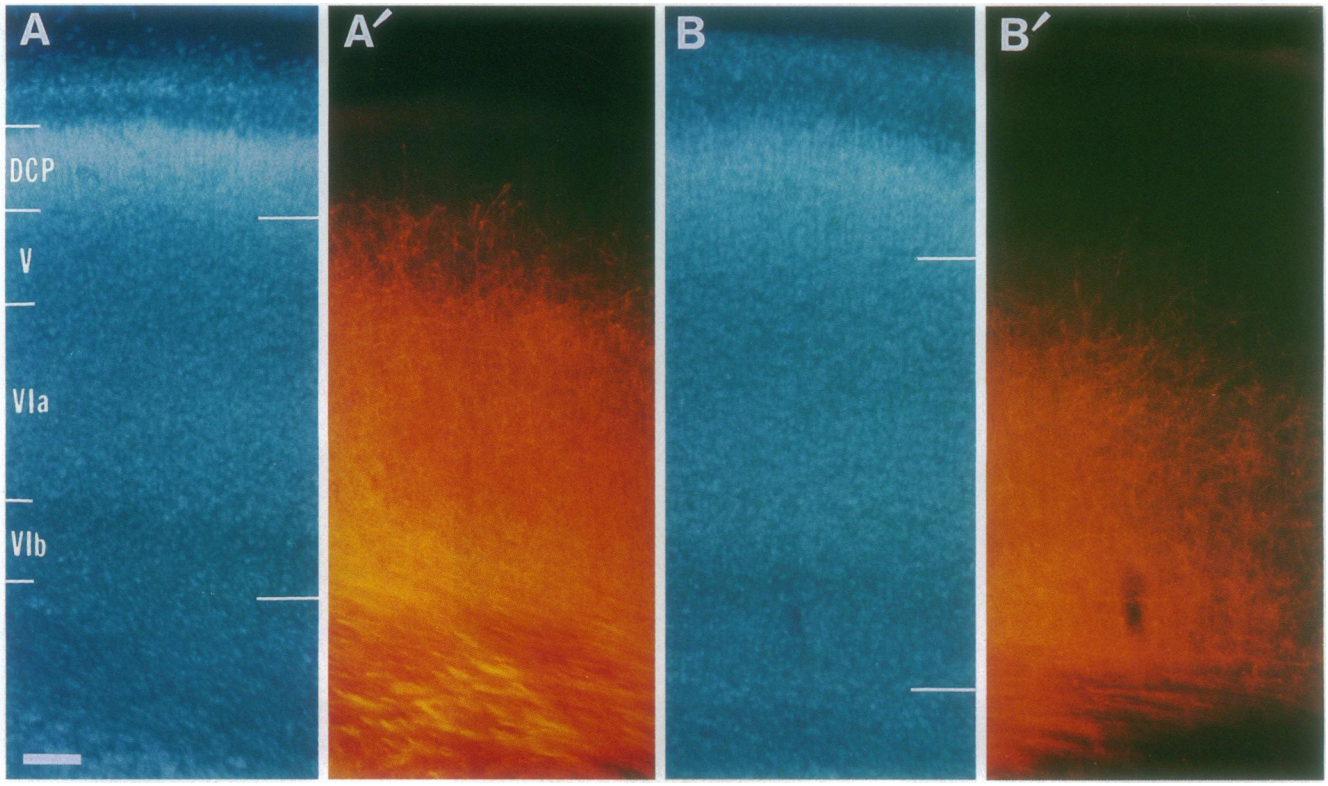

FIG. 3. High-power photomicrographs of a coronal section through the neocortex illustrating laminar differentiation and the distribution of thalamic afferents on E21, the day of birth. Each pair of photomicrographs (*A* and *A′*, *B* and *B′*) is of the same cortical field photographed with ultraviolet and rhodamine filter sets (excitation wavelengths, 340 nm and 547 nm, respectively). The pair *A* and *A′* are from lateral neocortex and the pair *B* and *B′* are from a more medial location in the same section. The cortical layers are indicated on the left side of *A*. The bars on the right in *A* and *B* indicate the lower border of the dense cortical plate and the bottom of layer VIb. Clearly, both cytoarchitectonic differentiation and afferent ingrowth have proceeded further laterally than medially. (Bar = 100 μm.)

neurons have extended an axon into the thalamus at these early ages. McConnell *et al.* (19) also reported few labeled neurons in the cat telencephalon after very early placement of DiI in the thalamus. In summary, we feel confident that we have indeed labeled thalamic afferents.

As noted above, we have found no evidence in the present study that thalamocortical fibers as a population wait and accumulate in the white matter before invading rat somatosensory cortex. We attribute this to important methodological differences between the present and previous studies (4, 5). (*i*) The previous studies assayed the distribution of thalamocortical projections postnatally with anterograde transport of tritiated amino acids as visualized by autoradiography. This technique appears to be considerably less sensitive than the DiI procedure [compare Fig. 3 of the present study with figure 7 of Wise and Jones (4)]. The relative insensitivity of the autoradiographic method has also been pointed out by Mason *et al.* (20) who have challenged the concept of a waiting period in the development of the cerebellar climbing fibers in the mouse. (*ii*) The photomicrographs of Wise and Jones (4) suggest that their analysis was focused on the more medial portions of rat somatosensory cortex. Given the developmental gradient that we report herein, the invasion of thalamocortical afferents in this region would be expected to lag behind more lateral regions.

The present observations confirm and extend those of Lund and Mustari (3). They reported that thalamic afferents arrive at the visual cortex of the rat at E18 and grow into layers V and VI as these layers differentiate over the next few days. Although these authors used the term wait in referring to the ingrowth of thalamic afferents, they used it to indicate that thalamic afferents do not contact target cells of layer IV until these cells have differentiated from the cortical plate. They did not observe and did not employ this term to refer to an accumulation of fibers in the white matter before invasion of the neocortex, which has come to be the generally accepted usage of the term waiting period. Thus, in the rat there does not appear to be a waiting period in either the somatosensory or visual cortex.

The accumulation of fibers in the white matter beneath the cortex and a waiting period or compartment have also been described in the course of the development of thalamocortical projections to the visual cortex of the cat and monkey (21, 6). Although both of these studies also employed relatively insensitive autoradiographic techniques, Shatz *et al.* (22) are reexamining the development of thalamocortical afferents in cat visual cortex by using DiI and have found both an earlier ingrowth of thalamic afferents than previously reported and a waiting period between the time thalamic fibers arrive at the subplate and their subsequent invasion of the neocortical layers.

The presence or absence of a waiting period may be related to brain size and the corresponding time it takes the brain to develop in a given species. For example, thymidine birth dating studies indicate that the ventral posterior neurons, which project to rat somatosensory cortex, undergo their final divisions on E14 and E15 (23). Cortical neurons of the deep layers undergo their final division during this same time span. Thus, by the time thalamic afferents reach the cortex on E16 some neurons, which are the permanent target of the thalamus in layer VI (11), are already in place and the remaining cortical neurons are generated over the next 5 days (24). In the cat, there also seems to be some overlap in the production of thalamic neurons and deep cortical neurons. The peak generation of neurons that compose the dorsal lateral geniculate appears to be around E26 or E27 (25) and

the earliest generated cortical neurons are produced between E24 and E28 (26). However, the time period over which the remaining cortical neurons are generated is much longer in the cat, approximately 1 month. Perhaps a waiting period during which thalamocortical projections make contact with a transient population of cortical neurons, the subplate, becomes necessary when cortical development is extended over a longer time period. In this regard, the issue of the presence or absence of a subplate in the rat is unresolved. Valverde *et al.* (16) have suggested that in the rat many of the neurons that they regard as analogous to the subplate (layer VIb) are not transient and survive into adulthood. However, Al-Ghoul and Miller (27) have provided evidence for a transient population of neurons in deep layer VI and the intermediate zone of the rat that may be analogous to the subplate of the cat. In either case, our own results do not suggest that the ingrowing thalamic afferents as a population wait in the vicinity of these cells. Although these cells are located in a position such that the first arriving thalamocortical afferents on E16 may make contact with them, some thalamocortical afferents have advanced beyond them into the primordial cortical layers by E17.

The close correspondence between thalamic ingrowth and the cellular differentiation of the neocortex that we have noted also deserves some discussion. At all ages, the uppermost front of arborization of the ingrowing afferent population corresponds to the lower border of the dense cortical plate (i.e., the border between cytoarchitectonically differentiated and undifferentiated cortical layers). Although this may be a simple coincidence or the reflection of a physical barrier that the fibers are unable to penetrate, perhaps the ingrowing thalamocortical afferents provide a signal for the cortical layers to differentiate in the rat. Several emerging lines of evidence suggest that this may be the case. (*i*) Windrem and Finlay (28) have reported that neonatal thalamic lesions in the hamster both increase the amount of cell death in cortex and markedly reduce layer IV, the major thalamocortical recipient layer. (*ii*) Peinado and Katz (29) have provided evidence that the morphology of layer IV stellate neurons can be altered by peripheral manipulations that presumably act through the thalamocortical projections. (*iii*) Schlaggar and O'Leary (30) have provided evidence that fetal visual cortex transplanted to the region of somatosensory cortex in the neonatal rat differentiates with characteristics unique to somatosensory cortex. In a more general context, Killackey (2) has hypothesized that thalamocortical afferents play a significant role in several aspects of neocortical specification including its subdivision into distinct cortical areas and the distribution of specific subsets of cortical connections. The present study defines the anatomical substrate within which such interactions could occur.

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