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Attraction of specific thalamic input by cerebral grafts depends on the molecular identity of the implant

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ABSTRACT **The cerebral cortex of mammals differentiates into functionally distinct areas that exhibit unique cytoarchitecture, connectivity, and molecular characteristics. Molecular specification of cells fated for limbic cortical areas, based on the expression of the limbic system-associated membrane protein (LAMP), occurs during an early period of brain development. The correlation between this early molecular commitment and formation of specific thalamocortical connections was tested by using a transplantation paradigm. We manipulated the phenotype of donor limbic and sensorimotor neurons by placing them in different cortical areas of host animals. Labeling of transplanted tissue with the lipophilic dye 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine was used to assay host thalamic neurons projecting to the donor tissue. We found that limbic thalamic axons successfully projected into cortical transplants (*i*) when LAMP was expressed by early committed limbic cortical neurons, irrespective of their host location, and (*ii*) when LAMP was expressed by uncommitted sensorimotor progenitor cells whose fate was altered by their new host locale. Thus, the response of cortical neurons to both intrinsic and environmental cues that influence their molecular phenotype has an important anatomical correlate, the development of specific patterns of thalamocortical connectivity.**

The emergence of specific phenotypes of developing neurons contributes to the assembly of distinct areal domains in the cerebral cortex. The regulation of phenotypic expression appears to be complex, influenced by an as yet ill-defined array of intrinsic and environmental factors that operate as the cortex forms during ontogeny. Some of the controversies surrounding the issue of cortical specification in the mammalian brain relate to the different effects that environmental manipulations may have on specific phenotypes. For example, the host environment appears to define the projection patterns of layer V neurons of transplanted visual or parietal cortex (1). Afferent interactions with cortical targets modulate some features of cellular organization, such as the barrels in somatosensory cortex (2). The ontogenetic plasticity of these phenotypes has been suggested by some to indicate an absence of early cortical specification, resulting in interchangeable regions that rely on environmental interactions for histotypic and functional differentiation (3, 4).

Examination of other phenotypic traits indicates that there may be some fundamental differences between areas of developing cortex. Thalamic afferent patterning in cortex seems to occur in an ordered fashion during development (5, 6), suggesting that growing fiber systems are somehow able to recognize differences in the developing cortical mantle. Previous studies showed that the limbic system-associated membrane protein (LAMP) (7, 8) is expressed early in the formation of the cerebral cortex (9), at a time [after embryonic day (E)14] when postmitotic neurons destined for limbic cortical regions migrate from the proliferative zone along the lateral ventricle to limbic regions (9–12). We found that the host milieu can regulate the molecular fate of cortical stem cells, so that E12 cells placed into host perirhinal cortex express LAMP regardless of their cortical origin in the donor (13). Although these studies demonstrate an early commitment of neurons to the limbic phenotype, they have not addressed whether the molecular specification of cortical neurons has other phenotype correlates, such as patterns of connectivity.

In the present study, we have used this feature of manipulating molecular phenotype in transplantation studies to test the potential of cortical tissue to attract appropriate thalamic projections, based on whether neurons in the transplants express LAMP.

MATERIALS AND METHODS

Transplantation of Fetal Tissue to Neonatal Host. Fetal Sprague–Dawley rats were obtained from timed pregnant dams. Plug date is considered to be E0. Dams were anesthetized with Nembutal (50 mg/kg of body weight), and animals at E12 and E14 were removed. Areas of the cerebral wall containing presumptive perirhinal and sensorimotor cortex were excised and prepared as described (13). E12 and E14 correspond to times before and after neurons initiate their commitment to the limbic molecular phenotype, respectively. Slabs of tissue were labeled with fast blue, rinsed, and transplanted into newborn [postnatal day (P) 0] host rat pups; thalamocortical projections are still developing in newborn rats. Transplants were placed heterotopically or homotopically into either sensorimotor or perirhinal cortex. Two weeks after grafting, rat pups were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed from the skull and postfixed by immersion for 24 hr. Control animals receiving no transplants were also perfused.

Fluorescent Labeling. To examine the afferent thalamocortical projections of the transplant, a lipophilic fluorescent tracer, DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate; Molecular Probes), was inserted into the transplants (Fig. 1) to retrogradely label host thalamic neurons. While many laboratories have used the DiI for anterograde labeling of axon projections and analysis of growth patterns, it has been shown also to be an excellent means of retrograde labeling of projection neurons in fixed tissue (14). For the present analysis, DiI was dissolved in 100% ethanol, precipitated by evaporation onto small pieces of pulled glass micropipettes, and inserted into the transplantation sites of the fixed brains. Care was taken to keep the micropipette within

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Abbreviations: DiI, 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate; E, embryonic day; LAMP, limbic system-associated membrane protein; P, postnatal day.

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FIG. 1. Color fluorescence micrographs showing both transplantation site of E14 perirhinal cortical tissue located heterotopically in host sensorimotor cortex and neurons within the thalamus of the same animal that were retrogradely labeled with DiI inserted into the graft. *A* and *C* were counterstained with *m*-phenylenediamine (a fluorescent Nissl stain). (*A*) Coronal section of P13 brain showing transplant site 2 weeks after surgery, visualized under fluorescein optics. Yellow-orange labeling indicates bleed-through of the signal from DiI. (Bar = 1 mm.) (*B*) Higher power of area outlined in *A* showing transplant indicated by the fast-blue-labeled cells (blue-white spheres). Graft is well integrated. Bright pink-white labeling is bleed-through of the signal from DiI. Note that the DiI insertion site does not reach the white matter (wm). (Bar = 0.5 mm.) (*C*) Section through mid-thalamus from the same host as in *A* and *B*, illustrating densely labeled fibers and scattered neurons retrogradely labeled with DiI (bright orange) in the lateral dorsal nucleus. (Bar = 0.25 mm.)

the cortical wall itself without penetrating the underlying white matter. Brains were incubated at 37°C for 8–9 weeks and were then sectioned on a Vibratome into 200-μm coronal slices. The sections were wet mounted onto slides and coverslipped with 70% glycerol in 0.1 M phosphate buffer. Fast blue and DiI at the transplant site were photographed, the coverslip was removed, and the sections were re-coverslipped with *m*-phenylenediamine in glycerol/phosphate buffer and reexamined for integrity of the transplant, insertion site and depth of the DiI label in transplant, and retrogradely labeled cells in thalamus. Homotopic and heterotopic grafts used for analysis were well integrated, and the DiI insertion site was limited to the boundaries of the transplant.

Quantification of Retrogradely Labeled Thalamic Neurons. Only brains with both well-integrated transplants and DiI labeling in the correct position (micropipette placed directly into the transplant site and glass not penetrating white matter) were used for analysis. Approximately 25% of the integrated transplants (>90% of total surgeries) had proper placement of the DiI and were used for this study. Four to eight brains were analyzed for each category. All thalamic nuclei were analyzed, but due to the absence or small number of retrogradely labeled neurons in some nuclei, these were not included in the data shown. For each brain, retrogradely labeled neurons in all sections through the thalamus were counted and a percentage of the total population of retrogradely labeled neurons was calculated. Histograms display the mean percentage ± SEM of neurons in each thalamic nucleus for each control or transplant category. Labeling of normal somatosensory cortex resulted in an average of ≈250 retrogradely labeled thalamic neurons per animal; normal perirhinal cortex labeling resulted in ≈80 retrogradely labeled neurons per animal. In the transplants situated in the sensorimotor region of the host, an average of ≈180 retrogradely labeled thalamic neurons per animal were obtained; labeling of transplants placed in the perirhinal area of the neonatal host (except E14 sensorimotor tissue) resulted in an average of ≈50 retrogradely labeled thalamic neurons per brain.

RESULTS

Normal Projections of Cortex. In order to establish the normal pattern of thalamic afferent connectivity to perirhinal and sensorimotor cortical regions at P14, each area in control brains was labeled with DiI. We found that ≈30% of all labeled thalamic neurons following DiI insertion into normal perirhinal cortex reside in limbic-associated nuclei: the lateral dorsal, parafascicular, and ventral medial thalamic nuclei (Fig. 2*A*). Labeled neurons (30%) are also present in the posterior thalamic and centrolateral/centromedial group, which project to all areas of cerebral cortex. The labeling seen in the medial geniculate was due to spread of the DiI out of the limbic cortical area and into neighboring auditory cortex. This spread was less evident in the transplant cases (see below). Placement of DiI in normal somatosensory cortex resulted in labeling in primarily two thalamic groups, the ventral basal nuclei (57%) and posterior group (43%) (Fig. 2*B*). These projection patterns correspond to basic thalamocortical organization (reviewed in ref. 15). We have based our general categorization of limbic and nonlimbic thalamic nuclei on the nontransplant projection patterns that we obtained and on previous work from other laboratories showing that certain areas, such as the lateral dorsal and ventral medial nuclei, clearly have a major projection to association limbic cortices, with little if any input to primary sensory or motor regions (16, 17). Some nuclei have been shown to clearly project widely and relatively evenly across the cerebral cortical mantle (16, 18).

Transplants. Does the specificity of thalamocortical projections correlate with the molecular phenotype of the developing target? We tested this by performing both homotopic and heterotopic transplants of either presumptive perirhinal or sensorimotor cerebral wall into either perirhinal or sensorimotor cortical areas of the P0 host. In general, homotopically located transplants, which we have shown previously (13) maintain their appropriate molecular phenotype, also receive their correct class of thalamic afferents, although the contributions from different nuclei varied. Homotopically located E14 perirhinal cortex received 92% of its

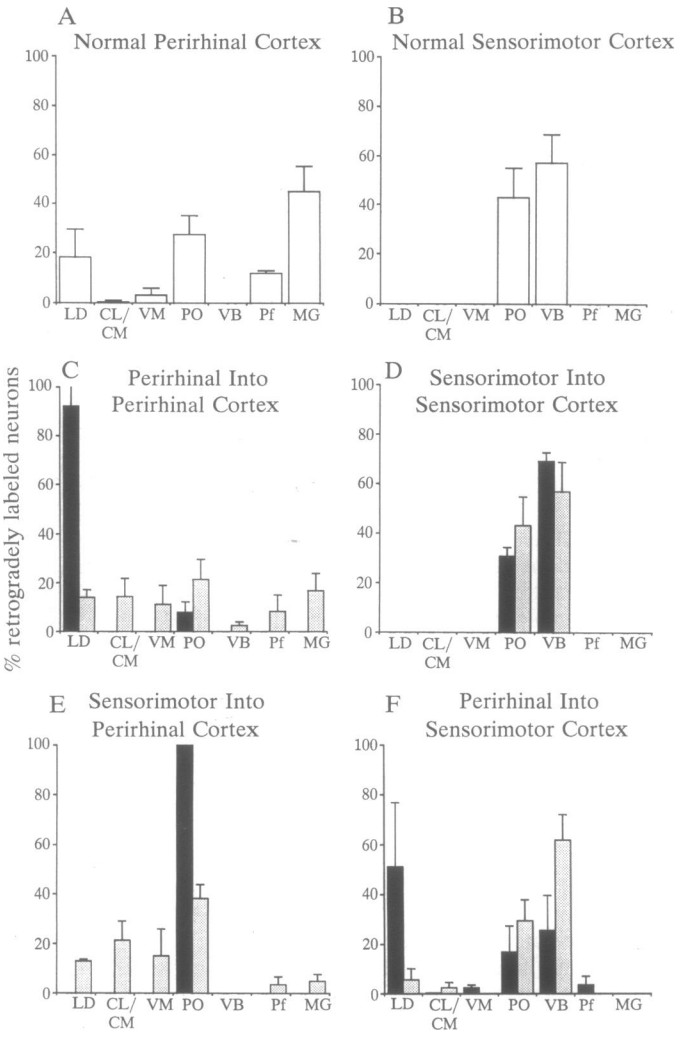

FIG. 2. Histograms showing the mean percentage of the total number of retrogradely labeled neurons in each thalamic nucleus from brains of different control and transplantation experiments. LD, lateral dorsal; CL/CM, central lateral and central medial intralaminar nuclei; VM, ventral medial; PO, posterior group; VB, ventral basal group; Pf, parafascicular; MG, medial geniculate. (*A*) DiI inserted into perirhinal cortex of control (no transplant) P13 rat pups. Largest percentages of retrogradely labeled neurons are located in LD, PO, and MG nuclei ($n = 8$). MG labeling reflects diffusion of DiI into neighboring auditory cortex. (*B*) DiI inserted into sensorimotor cortex of control (no transplant) P13 rat pups. All labeled thalamic neurons are located in either VB (somatosensory) or PO (general) groups ($n = 4$). (*C*) Perirhinal cortex from E14 (■) and E12 (▨) fetuses transplanted homotopically into newborn perirhinal cortex. DiI labeling of E14 grafts ($n = 4$) results in retrogradely filled neurons primarily in limbic LD. Transplants of E12 ($n = 5$) show labeling of neurons in LD, CL/CM, PO, and MG. Note the reduced labeling in MG in these cases compared with nontransplanted cases, reflecting the smaller amount of DiI spread. (*D*) Sensorimotor cortex from E14 (■) and E12 (▨) transplanted homotopically into sensorimotor cortex of newborns. Transplants of E14 ($n = 4$) and E12 ($n = 4$) show retrograde labeling only in PO and VB groups. (*E*) Sensorimotor cortex of E14 (■) and E12 (▨) transplanted heterotopically into perirhinal cortex. Only one of the four brains with E14 transplants had retrogradely labeled neurons, located exclusively in the widely projecting PO nucleus. In contrast, DiI labeling of heterotopically located E12 sensorimotor cortex ($n = 4$) results in retrogradely labeled neurons primarily in limbic thalamic nuclei (LD, VM, Pf). Note the absence of labeling in VB. Also note the paucity of neurons labeled in MG (compared with normal controls in *A*), reflecting a lack of diffusion of the DiI in these cases. (*F*) Perirhinal cortex of E14 (■) and E12 (▨) transplanted heterotopically into sensorimotor cortex. Transplants of E14 perirhinal ($n = 4$) have approximately equal percentages of retrogradely labeled neurons located in both limbic (LD) and somatosensory (VB) thalamic nuclei. In contrast, brains receiving E12 heterotopic perirhinal grafts ($n = 5$) have the largest percentage of retrogradely labeled neurons in somatosensory thalamic nuclei (VB), with only a small contribution from LD. Three of the five brains with E12 heterotopic perirhinal transplants did not have any retrogradely labeled neurons in LD.

projections from a single limbic nucleus, lateral dorsal, while homotopically located E12 perirhinal cortex received projections in approximately equal proportions from limbic (lateral dorsal and ventral medial; 24%) and widely projecting (posterior, central lateral/central medial; 35%) thalamic nuclei. In only one case was any labeling (2% of total) seen in the nonlimbic ventral basal group (Fig. 2*C*). The differences in specific labeling patterns between the E12 and E14 homotopic perirhinal transplants may be due to the more difficult task of isolating, at E12, cells giving rise exclusively to lateral limbic cortex. As expected, both E14 and E12 homotopically transplanted sensorimotor cerebral wall received projections from the ventral basal nuclei (31% and 43%, respectively) and posterior group (Fig. 2*D*).

Heterotopic transplants afforded us the opportunity of manipulating molecular phenotype, allowing direct comparison of how developing thalamic projections organize under such circumstances. We examined thalamic afferents of grafts of both presumptive perirhinal and sensorimotor cortex from E12 donors [at E12, only progenitor cells are present in the cerebral wall (10–12)] and found that they received thalamic afferents that reflected the grafts' new position. More than half of the thalamic neurons labeled retrogradely from E12 sensorimotor tissue transplanted heterotopically to perirhinal cortex were situated in limbic nuclei (Figs. 2*E* and 3*C*), including the lateral dorsal, central intralaminar, and ventral medial nuclei. Moreover, the neurons in the ventral basal group, which usually project to sensorimotor cortex, were never labeled in these cases. The broadly projecting posterior group contained most of the other labeled neurons. Neurons in the E12 perirhinal tissue, placed in sensorimotor cortex of the host, received the greatest proportion of their thalamic input from the ventral basal group (62%). Only 8% of the retrogradely labeled neurons were situated in limbic thalamic nuclei (Fig. 2*F*). In both instances, the grafting of stem cells into a different region of the host cortex results in the development of specific thalamic inputs that reflect both their new location and a new pattern of LAMP expression.

In contrast to the cerebral wall at E12, these same regions at E14 contain both progenitor cells and postmitotic neurons (10–12). We have shown previously that more than half of the surviving perirhinal neurons transplanted heterotopically maintain LAMP expression, but the remaining cells fail to express this protein (13). When we analyzed thalamic input to this graft, we found that there was a mixture of input types that reflected both the original donor location (57% of the projections were from the limbic lateral dorsal, parafascicular, and ventral medial thalamic nuclei) and the new host location (26% from the somatosensory ventral basal thalamic nuclei) (Figs. 2*F* and 3*A*). These data suggest that growing limbic thalamic axons recognize their correct cortical targets, even when placed in an inappropriate location. In contrast, the E14 sensorimotor transplants to perirhinal host regions, in which <10% of the neurons express LAMP, fail to induce DiI-detectable afferents from limbic-associated or ventral basal thalamic nuclei. In only one case were neurons in the thalamus labeled, and these were all in the posterior group (Fig. 2*E*). The general failure of the E14 sensorimotor transplants to attract somatosensory thalamic fibers, when placed in the lateral perirhinal cortex, may reflect early mechanical constraints of these axons within the internal capsule, thus preventing them from reaching areas through which they normally do not pass (5, 10). The successful innervation of all

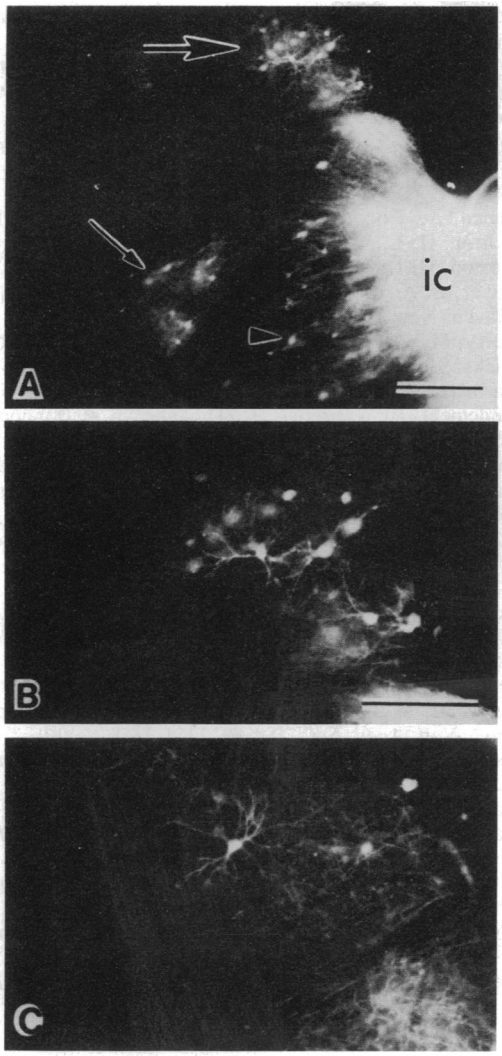

FIG. 3. Photomicrographs showing examples of thalamic neurons that were retrogradely labeled with DiI. (*A*) Coronal section from a P13 brain that received a transplant of E14 presumptive perirhinal cortex grafted heterotopically into P0 sensorimotor cortex. Hosts were killed 2 weeks after surgery, and DiI was inserted into graft. Limbic, somatosensory, and "nonspecific" thalamic neurons were labeled in this case, as seen in the lateral dorsal nucleus (large arrow), ventral basal group (arrowhead), and posterior group (small arrow), respectively. DiI-labeled axons also are present in the internal capsule (ic). (Bar = 0.5 mm.) (*B*) Higher power of same section as *A*, illustrating retrogradely labeled neurons in lateral dorsal nucleus of thalamus. (Bar = 0.25 mm.) (*C*) Coronal section from a DiI-labeled P13 brain that received a transplant of E12 presumptive sensorimotor cortex grafted heterotopically into the P0 perirhinal cortex. Neurons in the limbic lateral dorsal nucleus are retrogradely labeled.

LAMP-positive grafts located in the host perirhinal cortex (E12 sensorimotor and the E12 and E14 perirhinal transplants), however, demonstrates that placement itself into the lateral hemisphere is not a limiting factor. In addition, these same transplants exhibited extensive corticocortical projections (data not shown), suggesting that the minimal amount of thalamic labeling reflects a true organizational feature and not a technical artifact.

DISCUSSION

Our previous and current transplantation data reveal two important features about cerebral cortical development. First, molecular phenotype is an early-determined feature that defines distinct areas of the mammalian cerebral cortex (13). Second, the pattern of thalamic afferent organization correlates with the molecular phenotype of the target (Fig. 4). The specification of mammalian cerebral cortex may rely on a combination of intrinsic and environmental factors; their interactions at specific developmental stages probably modulate different phenotypic traits. Therefore, the interchangeability of cortical areas is likely to depend on the developmental stage and the expression of specific phenotypic traits of the region at the time of transplantation. For example, molecular specification of cortical neurons can occur early after final mitosis (9, 19), in the absence of normal environmental interactions (19), and is not solely a characteristic of associational cortex (Y. Atimatsu, M. Miyamoto, I. Nihonma, K. Hirata, and Y. Uratani, unpublished data). Determination of laminar position is a phenotypic trait that has been linked to the cell cycle (20, 21). Even early migrating (22) or malpositioned (23) postmitotic cortical neurons exhibit projections that are appropriate for their cell class. Some efferent projection patterns (1) and cytoarchitectonic distinctions (3), however, may be later-developing features of cortex. Since neurons within a cortical region are generated

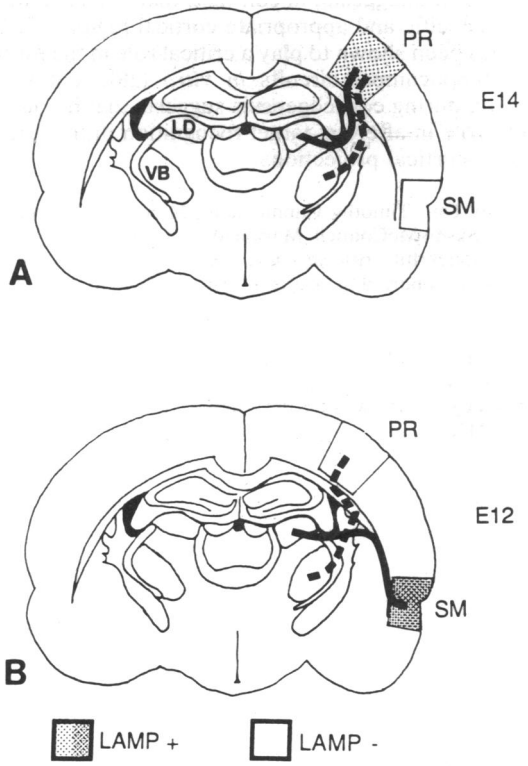

FIG. 4. Summary diagram illustrating the results of the heterotopic transplants from E14 and E12 donors. Attraction of appropriate thalamic input is linked to the molecular phenotype of the cortical grafts. (*A*) The light stippling of E14 grafts of presumptive perirhinal cortex into neonatal sensorimotor cortex represents the presence of both LAMP-positive and LAMP-negative neurons. This type of graft attracts projections from both limbic (solid line; LD) and somatosensory (broken line; VB) thalamic nuclei. E14 sensorimotor transplants maintain their original molecular phenotype (LAMP-negative) but fail to attract projections from either group of thalamic nuclei. Sensorimotor afferents may be prevented by mechanical constraints in the internal capsule from innervating the laterally placed sensorimotor tissue. (*B*) E12 transplants of presumptive perirhinal cortex into neonatal sensorimotor cortex fail to express LAMP as they would have in their normal location. This change in molecular phenotype is coincident with the development of projections from primarily somatosensory thalamic nuclei (VB). Dense stippling of E12 presumptive sensorimotor cortex indicates that, when transplanted into host perirhinal cortex, most neurons are LAMP-positive. They receive thalamic projections from primarily limbic-associated thalamic nuclei (LD).

over an extended period of time, neuronal differentiation within a transplanted tissue, placed in an altered environment, may reflect a mixture of phenotypes. This would, in effect, produce a hybrid tissue exhibiting phenotypic features driven by factors from both host and donor regions. This appears to occur in primate occipital cortex following prenatal eye removal (24, 25). Likewise, presumptive limbic cortical tissue transplanted at E14 to a heterotopic location contains both LAMP-positive and LAMP-negative cells. This same type of transplant receives both limbic and nonlimbic thalamic afferents. The early molecular differences exhibited by cortical neurons may thus provide a mechanism for specific interactions with growing fiber systems. Preliminary examination of corticocortical projections in our transplants suggests that this particular phenotype also is regulated in a manner similar to thalamocortical projections and LAMP expression (data not shown). The apparent lack of demonstrated specificity of projections of thalamic explants to cortical targets *in vitro* (26) may reflect promiscuous growth unique to the culture system or inherent difficulties in labeling single classes of thalamic afferents in the explants. Analysis *in situ* suggests, in contrast, that thalamic afferents seek out specific and appropriate cortical regions (5, 6, 27). LAMP has been shown to play a critical role in the formation of septohippocampal circuits *in vitro* (28), and its early expression during corticogenesis suggests that it could have a similar role in afferent–target recognition in the formation of thalamocortical projections.

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