Postnatally Induced Formation of the Corpus Callosum in Acallosal Mice on Glia-Coated Cellulose Bridges

Abstract. *Developing axons of the corpus callosum of mice are guided across the cerebral midline by a slinglike glial structure that forms transiently between the hemispheres. If the "sling" is cut at precallosal stages, the would-be callosal fibers whirl into paired neuromas adjacent to the longitudinal cerebral fissure. In experiments on such surgically acallosal animals, the aberrant commissural axons maintained a potential to regrow across the hemispheres at prenatal and early postnatal stages if they were presented with a properly aligned, glia-covered scaffold spanning the hemispheres.*

What are the guiding forces within the normally developing brain that control the growth routes of axons? This question, unanswered since Ramón y Cajal first discovered the axonal growth cone (*1*), becomes of considerable importance when, in pathological situations, embryonic axons fail to reach their appropriate synaptic targets. Perhaps the most dramatic of all such axonal disorders is the inherited acallosal malformation, in which all or most fibers of the largest axonal pathway in the mammalian brain, the corpus callosum, fail to cross into opposite cerebral hemispheres. These misplaced axons do not die. Instead, as the fibers arrive on schedule at the hemispheric midline, they gather into massive, paired neuromas (Probst's bundles) adjacent to the longitudinal cerebral fissure (*2*). The fibers persist in this location and configuration throughout subsequent ontogeny.

Recently a guidance pathway has been identified midsagittally in the normally developing brain that provides directionality cues to the callosal axons. During embryogenesis of the mouse forebrain and 2 days before the appearance of the pioneer fibers of the callosum at the cerebral midline, on embryonic (E) day 17, a population of primitive glial cells migrates medially through the septum from the ependymal zones bordering the lateral ventricles of each hemisphere. At the midline, the glial cells coalesce to form an interwoven bridgelike structure or "sling," suspended below the longitudinal cerebral fissure and attached at the two ventricles (*3*). The earliest forming callosal axons grow upon, and are guided by, the upper surface of this cellular bridge as they make their way toward the contralateral side of the brain. The glial sling fails to form in genetically acallosal BALB/cCF mice, and is absent in normally acallosal opossums (*3*). The formation of the corpus callosum can be completely blocked by surgically disrupting the sling in normal precallosal, C57BL/6J mice. This intrauterine lesion, however, has no effect on other crossed telencephalic projections, such as the anterior and hippocampal commissures (*3*). In general, the callosal pathology in animals with surgical lesions is identical to that described for the genetically and chemically induced callosal defects present in a variety of mammalian species including humans (*2–4*).

Among the aberrant callosal axons residing in Probst's bundles, do some still maintain a growth potential during prenatal and early postnatal stages? If they do, would the advancing axons move in the proper direction contralaterally provided they are reassociated with a copy of the slinglike glial tissue that guides them across the cerebral midline during normal development?

Mouse embryos of the C57BL/6J strain (N = 175) were made acallosal by sectioning the sling through the uterine wall on E16 (*3*).

In one group of 70 embryos a bridgelike sling prosthesis [a 1-mm^2 ⌂-shaped piece of cellulose membrane filter (Millipore), 0.45-μm pore size] was placed into the cerebrum (*5*). After the first lesion to cut the sling, a second and slightly larger stab wound was made in the cranium with the use of a No. 11 scalpel blade. The blade was inserted horizontally into the longitudinal cerebral fissure. The cellulose structure was then inserted, pointed side first, into the stab wound. Our goal was to introduce the implant in the same orientation and position as that of the original sling. The fetus was then reinserted into the body

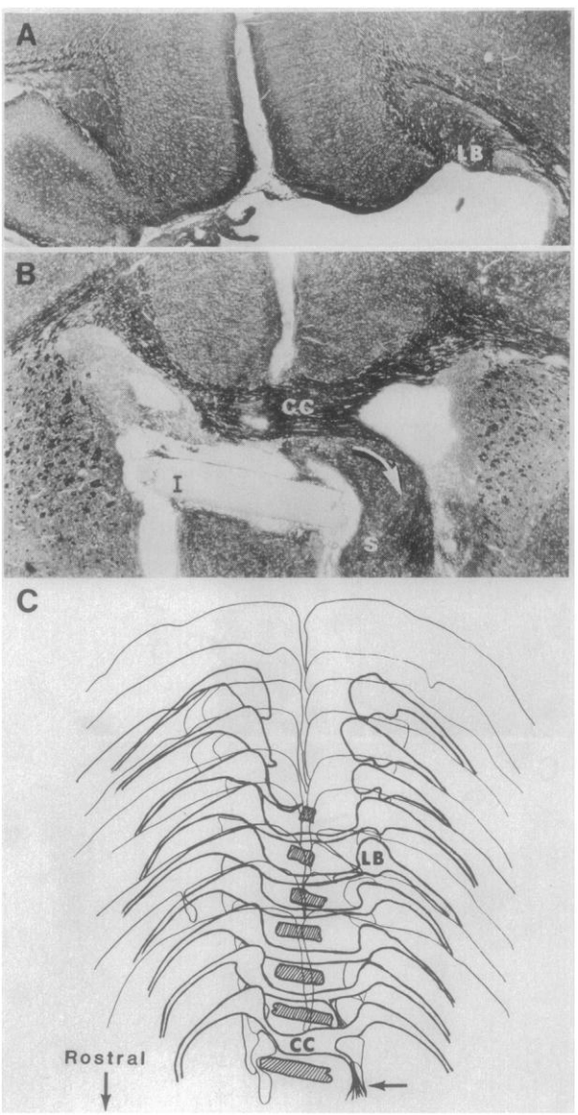

Fig. 1. (A and B) Coronal silver-stained sections (×75) through the forebrain of an acallosal animal that had been given an implant (*I*) on day P4. The animal was killed on P9. Arrows indicate where some commissural fibers have grown into the contralateral septum (*S*). (C) Camera lucida serial reconstruction of the brain shown in (A) and (B). The newly formed callosum appears only above the implant (hatched structure). Abbreviations: *CC*, corpus callosum; *LB*, longitudinal bundle of Probst.

cavity, an additional length of uterus was exposed, and the two operations were repeated until all embryos were treated. Of these animals, 20 survived.

In a second group of 60 surviving (of 105) surgically acallosal animals, the cellulose bridge was introduced into the brain after birth [on postnatal (P) day 2 or 4], through the use of a procedure identical to that described above. Our goal was to bridge the gap between the two Probst's bundles. After the second operation, 50 animals recovered.

All animals were allowed to survive until P4 or P9. In some, the brains were sectioned and stained with silver (*6*); in others, the brains were embedded in plastic and processed for serial 1-μm section analysis and electron microscopy (*3*). Among the 70 animals that endured both operations, 45 were acallosal. Since our procedures were accomplished without the use of a stereotaxic device, however, we have been able to direct bridges that span the midline and come to lie near one (N = 7; 3 prenatal, 4 postnatal implants) or both Probst's bundles (N = 5; all postnatal implants) in only 12 animals. It is in this group that we have had consistent success in redirecting commissural axons, and we report here on several of our most instructive cases.

The patterning of "callosal" axons in surgically acallosal animals given sling replacements was contrasted with that of acallosal animals not given implants. In acallosal animals with implants, many fibers that would have remained within Probst's bundles grew out of the bundles, crossed the cerebral midline, and—in the five cases in which the bridge came to rest near both neuromas—continued into the opposite cortex. One animal (prosthesis implanted P4, killed P9) with a massive amount of new callosal development is shown in Fig. 1. This originally acallosal animal (identified as such by the presence of Probst's bundles) developed a well-formed callosum (about 600 μm long) in the rostral half of the forebrain, but foward of the lamina terminalis (Fig. 1, A and B). A camera lucida serial reconstruction of the brain of this animal revealed that the novel callosum appeared only in those regions that contained an implant (Fig. 1C). Immediately caudal to the bridge, only well-formed Probst's bundles, and no crossing fibers, were present (Fig. 1, A and C).

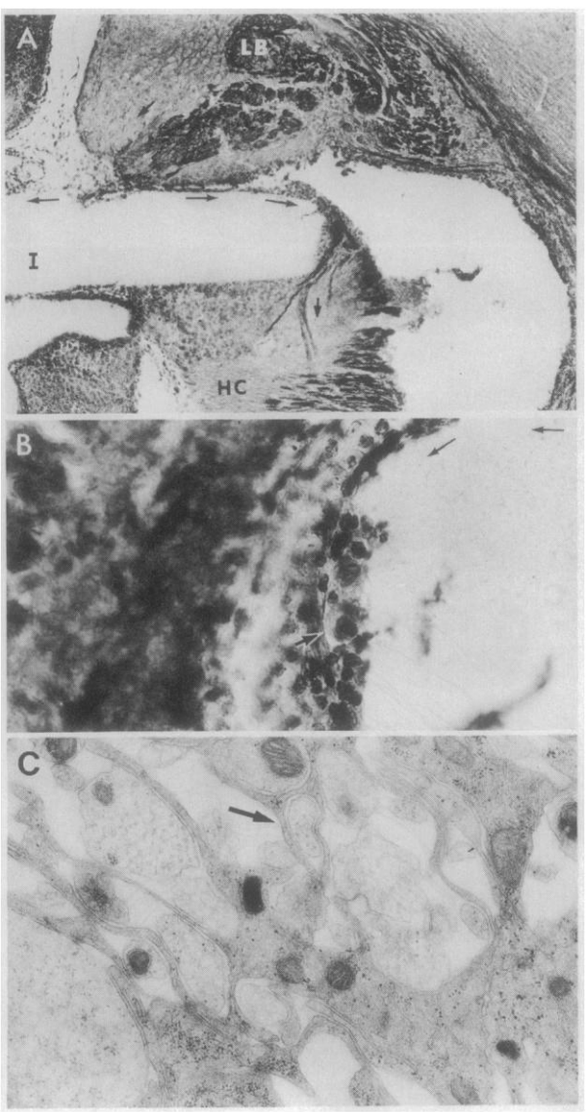

Fig. 2. (A) Coronal silver-stained section (×75) of the brain of an acallosal animal that had received a cellulose implant (*I*) on E16 and been killed on P4. Fibers leave the confines of one Probst's bundle (small arrows) to grow medially and laterally among the cells that encase the implant. (B) Left-sided continuation of (A) but at higher magnification (×400). Axons can be seen among the coating cells (large arrow). (C) Electron microscopic view (×24,000) of the implant surface of an animal that had received an implant embryonically and been killed on P9. Note the presence of axons ensheathed by glial (astrocytic) processes (arrow). Abbreviations: *HC*, hippocampal commissure; *LB*, longitudinal bundle of Probst.

For several reasons, we believe that this previously acallosal animal generated a partial callosum postnatally.

1) Although we have surgically created about 75 acallosal individuals in the course of all our past studies (*3*), neither we nor those who have analyzed the congenital anomaly (*2, 4*) have ever seen an affected animal with a smaller than normal callosum located far rostrally. Although partially acallosal animals do infrequently occur, in them the crossing fibers consistently appear much farther caudally, above the hippocampal commissure (*3, 4*).

2) In animals with partial callosa, Probst's bundles and great numbers of crossing fibers are never seen in the same region. The two formations are, in a sense, antitheses of each other. The animal shown in Fig. 1 is unique in that massive Probst's bundles and a well-formed callosum are present in the same territory. We do not know whether the apparent additional growth has resulted from a sprouting of damaged fibers or of healthy fibers (*7, 8*), or whether the original growing tips of the callosal axons found their way out of the whorls.

3) An unusual, asymmetric, contralateral corticoseptal projection formed in this brain (arrows in Fig. 1, B and C). Thus, not all commissural fibers grew into the cortex. Since, so far as we know, a similar tract has not been described in the brain of any mammal (normal or acallosal), this projection, as well as the callosum, must have arisen as a result of the implanted sling prosthesis. To our knowledge, this rebuilding of a major axon tract is the first demonstration that epigenetic "axonal engineering" can be used to restore a malformed fiber pathway in the mammalian brain.

The acallosal animals shown in Fig. 2 are representative of those that received implants embryonically. In them, fibers have left the boundaries of one Probst's bundle to grow upon the surface of the bridge. The replacement sling was embedded in only one of the ectopic bundles, and had neither united the two hemispheres nor successfully redirected the callosal axons contralaterally. However, one can appreciate, in these animals, the cellular nature of the surface of

the implant and the close positional relationship between this cell coating and the outgrowing axons (Fig. 2, B and C). Although, in the animal shown in Fig. 2, A and B, a few fibers grew medially for relatively long distances and seemed to terminate within the cellular coat, others grew laterally but eventually abandoned this surface to grow ventrally toward the hippocampal commissure (Fig. 2A) (*9*).

Our ultrastructural analyses suggest that the cells that encase the cellulose bridges are a form of glia, probably astrocytes. They have the ability, characteristic of young astrocytes, to actively enwrap outgrowing fibers (*10*). In addition, they are rich in glycogen granules and intermediate filaments (*11*) (Fig. 2C). We do not yet know, however, if these glia are remnants of the original sling glia population that normally occupies the subcallosal region.

It has not yet been determined whether the newly formed callosal axons have grown to appropriate or nonappropriate targets and have formed functional synapses. In addition, we do not yet know if a critical period exists postnatally that, if allowed to pass, renders the callosal axons refractory to further growth (*12*). If so, it may be possible, in older animals, to stimulate Probst's fibers by inserting bridges impregnated with freshly "harvested" embryonic glial tissues. Such procedures are a modification of those described recently by Kromer *et al.* (*13*), who used embryonic hippocampal implants as bridges to promote the regrowth of adult septal axons.

Our data suggest that substantial numbers of potentially misplaced callosal axons in acallosal mice are capable at prenatal and early postnatal stages, not only of further growth but of correctly oriented growth as well. The callosal axons display their most vigorous, contralaterally directed growth response when they are confronted with a structure that resembles (in both cell type and orientation) the environment through which the axons would have grown in the normally developing embryo.

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Identity of HeLa Cell Determinants Acquired by Vesicular Stomatitis Virus with a Tumor Antigen

Abstract. *Growth of vesicular stomatitis virus (VSV) in HeLa cells results in progeny containing non-VSV antigens with a molecular weight around 75,000. The non-VSV antigens were detected by antiserums to HeLa cell determinants. These antiserums precipitate whole virions but do not neutralize them. Because one of the antiserums is directed to a tumor-specific surface antigen of HeLa cells, it appears that VSV specifically acquires such antigens during its passage through human tumor cells.*

When different enveloped viruses infect the same cells, they often acquire each other's surface antigens during the maturational process (*1, 2*). Vesicular stomatitis virus (VSV) is especially effective for demonstrating such phenotypic mixing because it grows in almost every cell type and acquires antigens from enveloped RNA or DNA viruses (*2, 3*). When VSV is grown in cell lines not known to harbor other enveloped viruses, the progeny will acquire a subset of the antigens normally found at the cell surface (*4–6*). With murine L cells, the "cellular" antigens acquired by VSV progeny have been identified as glycoproteins coded for by endogenous murine leukemia viruses (*7*). Therefore, with human tumor cell lines, which are not known to contain endogenous retroviruses, the "cellular" antigens acquired by VSV may, by analogy, be indicative of the expression of retrovirus-like glycoproteins.

Vesicular stomatitis virus grown in HeLa cells were precipitated, but not neutralized, by antiserums to HeLa cell determinants. Isolation of these determinants by immunoprecipitation and polyacrylamide gel electrophoresis showed two or three specific polypeptides with molecular weights between 75,000 and 100,000 (*5, 6*). Because the HeLa cells used in these experiments do not shed retrovirus-like virions (*8*), antiserum to a virus-specific glycoprotein of HeLa cells is not available. Therefore, the identity of the HeLa cell determinants acquired by VSV can be checked only with antiserum that differentiates a tumorigenic in-

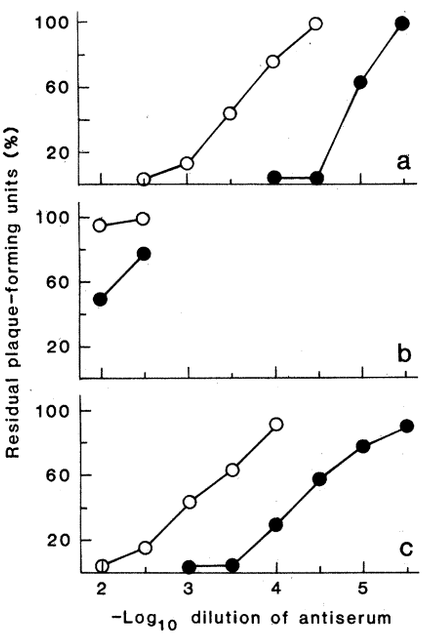

Fig. 1. Immunoprecipitation of VSV made in HeLa (●) or in HMB2 melanoma cells (○) with antibodies to HeLa cell surface antigens and *S. aureus* cells. Details concerning viruses, cells, and antiserums have been reported (*5, 6, 9*). The antiserums used were (a) sheep antiserum to VSV (tsO45) made in HeLa cells absorbed with VSV-infected Chinese hamster ovary cells, (b) rabbit antiserum to tumorigenic HeLa–human diploid cell hybrids absorbed with corresponding nontumorigenic hybrids, and (c) rabbit antiserum to HeLa cells unabsorbed. One hundred plaque-forming units of VSV in 50 μl were mixed with 50 μl of diluted antiserum, incubated for 30 minutes at room temperature, and then reacted with 1 ml of 0.2 percent *S. aureus*. The mixtures were incubated for an additional 60 minutes at room temperature, then centrifuged for 7 minutes in a microcentrifuge (Eppendorf). Supernatants were plaque-assayed on Chinese hamster ovary cells for residual VSV infectivity.