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11. The major source of luminance contrast artifacts are the axial chromatic aberrations, since lateral chromatic aberrations appear to be corrected by the nervous system [R. Held, in *Visual Coding and Adaptability*, S. Harris, Ed. (Erlbaum, Hillsdale, NJ, 1980), p. 69]. For the display monitor used in these experiments (R = 627 nm, G = 525 nm) when the green color was at zero diopters from the fixation, the red was estimated to be at −0.4 diopters [P. A. Howarth and A. Bradley, *Vision Res.* **26**, 361 (1986)]. For the sinusoidal gratings of the highest used spatial frequency (1 cycle per degree) or for the filtered random dot stereograms, and for a maximum pupil size of 4 mm, the artificial contrast induced by the axial chromatic aberrations at isoluminance was computed to be 1.02%. When we used achromatic stimuli of a contrast less than 2.00%, performance was never improved in the psychophysical experiments and the typical frequency doubling of the magnocellular cells described in this paper was never elicited. We, therefore, believe that the contrast artifacts produced by chromatic aberrations cannot underlie either the effects of color contribution to the perception of motion and stereo or the residual activity of the broad-band system at isoluminance.
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Target Control of Collateral Extension and Directional Axon Growth in the Mammalian Brain

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Individual neurons in the brain send their axons over considerable distances to multiple targets, but the mechanisms governing this process are unresolved. An amenable system for studying axon outgrowth, branching, and target selection is the mammalian corticopontine projection. This major connection develops from parent corticospinal axons that have already grown past the pons, by a delayed interstitial budding of collateral branches that then grow directly into their target, the basilar pons. When cocultured with explants of developing cortex in three-dimensional collagen matrices, the basilar pons elicits the formation and directional growth of cortical axon collaterals across the intervening matrix. This effect appears to be target-specific and selectively influences neurons in the appropriate cortical layer. These in vitro findings provide evidence that the basilar pons becomes innervated by controlling at a distance the budding and directed ingrowth of cortical axon collaterals through the release of a diffusible, chemotropic molecule.

MOST REGIONS OF THE MAMMALIAN neocortex send axons to the basilar pons, establishing a major connection that is essential for the control of motor behavior (*1*). The corticopontine projection, which arises from neurons in layer 5 of the neocortex (*2*), develops by a delayed interstitial budding of axon collaterals, rather than through the direct ingrowth of primary axons or of branches formed by bifurcation of their growth cones (*3*). Cortical axons initially grow past the basilar pons and only later do they extend branches into it. This mechanism is used regardless of whether the parent axon's post-pontine segment, which forms the corticospinal projection, is retained (motor cortex) or later eliminated (visual cortex) (*3, 4*). Thus, in this system, unlike others characterized to date (*5*), the target is not recognized by the growth cones of the primary axons. However, the subsequent budding of collaterals at stereotypic positions directly over the basilar pons suggests that cues do identify it as a target of cortical axons.

What mechanism controls the budding and ingrowth of collaterals? One possibility is that collaterals are produced at predetermined points along the axon, or at some predetermined time in its extension according to a program intrinsic to the cortical neuron. Alternatively, the process could be in response to local cues that develop selectively in the region of the corticospinal tract overlying the basilar pons. Another possibility is that the maturing basilar pons induces its own innervation by producing a long-range, chemotropic signal that acts on the overlying cortical axons.

One means of distinguishing among these possibilities is to coculture at a distance explants of cortex and basilar pons within three-dimensional collagen matrices and examine the extent to which cortical axon outgrowth may be stimulated and directed across the intervening collagen matrix. This procedure enables the detection of diffusible, chemotropic activities emanating from target tissue and excludes the action of possible local guidance cues (*6*). Accordingly, explants of postnatal day 0 to 1 rat motor or visual cortex were cultured alone (n = 73) or cocultured (n = 397) in collagen gels with age-matched explants of their normal target tissue, the basilar pons, or a nontarget, control tissue, or both (*7*). Control tissues used were olfactory bulb, hypothalamus, mammillary bodies (tissues at a similar developmental stage as basilar pons, but which do not receive neocortical input),

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and neocortex. The pontine and control explants were positioned on the flanks of the cortical explants at a spacing of 150 to 300 μm, a distance roughly equivalent to that separating corticospinal axons from the basilar pons in vivo (*3*). The cultures were incubated for 24 to 48 hours and examined with phase-contrast optics and by fluorescence microscopy after dye injections into the explants.

The pontine target tissue elicits an increase in the number of axons emerging from the proximal face of both motor and visual cortical explants when compared with controls (Figs. 1 and 2). This greater outgrowth could be due either to a trophic effect of the pons, which would increase the survival or enhance the axonal growth of cortical neurons that lie on the proximal side of the explant, or to the directed growth of cortical axons or axon collaterals toward the pons, that is, a tropic effect. That this enhanced neurite outgrowth is due to directional growth (a tropic effect) is, however, indicated by further observations of axons in the collagen matrix. First, substantially more cortical axons grow directly toward the pontine explants than toward control explants (2564 axons scored; pontine intercept coefficient, 0.79; see Fig. 1D for determination), and second, axons that would have missed the pontine explant if they had maintained their initial trajectory turn preferentially toward it (593 axons scored; pontine turning coefficient, 0.96; see Fig. 1D for determination).

The behavior of axons within the cortical explants was visualized by injecting pontine (n = 97) and control (n = 53) explants in 24- to 48-hour cocultures with the fluorescent axon tracer, 1,1-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI, Molecular Probes) (*8*). Retrograde filling of cortical axons contacting the pontine explant reveals that they grow by one of two means. Some primary axons turn from their initial ventricular-directed course to emerge on the lateral, pons-facing surface of the explant (Fig. 3A). Many primary axons, though, maintain their original trajectory toward the ventricular surface but give off collaterals, which grow laterally toward and into the pontine explant (Fig. 3, B to E). The cell bodies of axons and collaterals retrogradely labeled from the pontine explant are distributed across the width of the cortical explant (Fig. 3, D and E) giving further indication that the observed effect is not due to an enhanced viability of cortical neurons that lie on the side facing the pontine explant, but rather is due to a tropic influence. Most of these cell bodies are found at a position within the cortical ex-

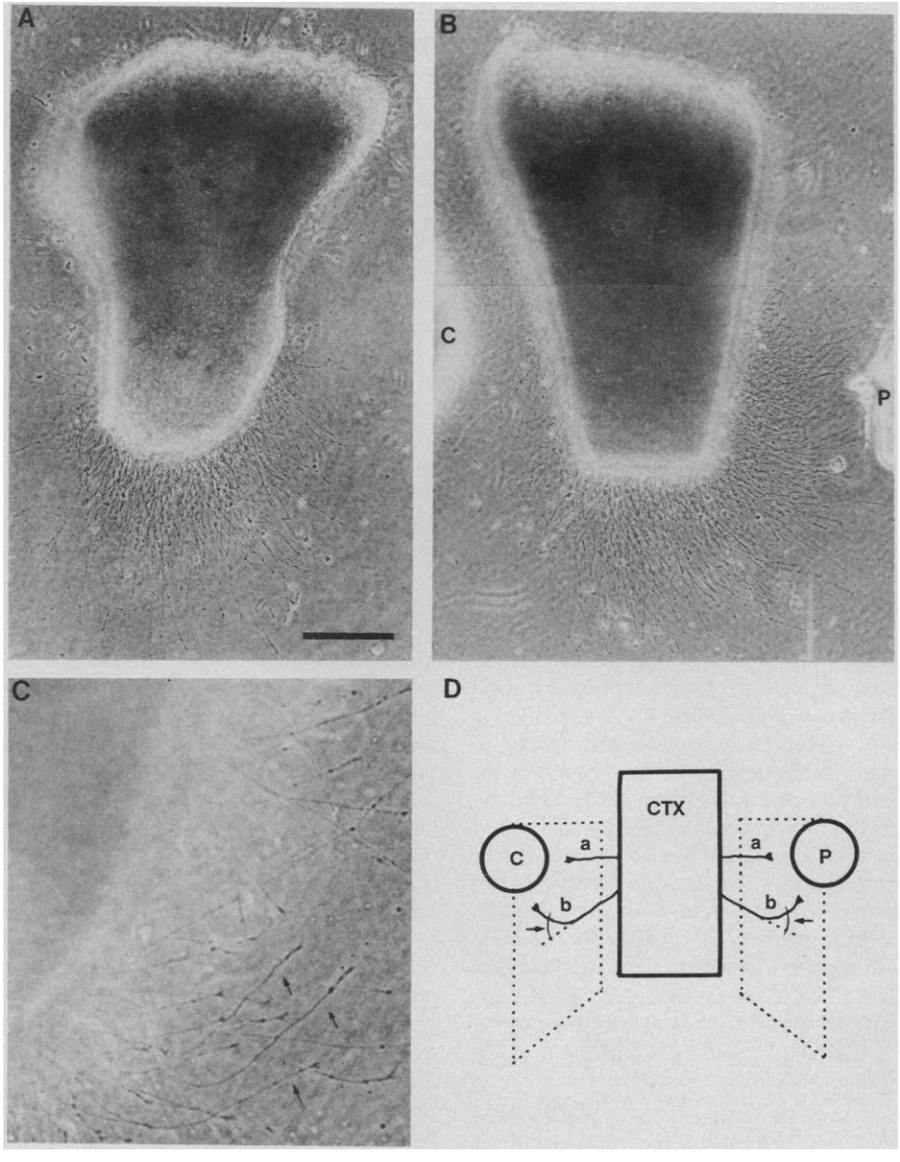

Fig. 1. Phase-contrast images of cortical axon growth in three-dimensional collagen matrices. (**A**) Motor cortex cultured alone for 24 hours. Most axon growth is directed inferiorly from the ventricular surface of the explant (pial surface is to the top). (**B**) Motor cortex cultured for 24 hours with control tissue (c) to the left and basilar pons (p) to the right. Cortical axon growth is predominant on the side facing the pons. Further, most cortical axons on this side are directed laterally toward the pons, whereas those on the control side maintain their inferior direction of growth. (**C**) Higher magnification of cortical axons turning within the collagen matrix to grow toward the pontine explant (located out of the field to the upper right). Examples are indicated with arrows. Scale bar, 200 μm in (A) and (B); 400 μm in (C). (**D**) Method for quantifying directional growth of cortical axons. Data are from a consecutive series of motor cortex cocultures (n = 122) of the type shown in (B). Cortical axons were scored as intercepting if their trajectory directs them toward the pons or control tissue explants (axons a). Turning axons are those that have an initial trajectory when exiting the cortical explant that would miss the pontine or control tissue explants, but is subsequently turned >30 degrees toward either target explant (axons b). Cortical axons were counted only if their growth cones were visible in the areas defined by the dotted lines. These areas are displaced 50 μm from the sides of the cortical explant. CTX, cortical explant; C, control tissue explant; P, basilar pons explant. The pontine intercept coefficient was calculated by dividing the number of axons intercepting the pontine explant by the total number of axons intercepting the pontine and control tissue explants. Thus, if all "intercepting" axons are directed toward the pontine explant, the coefficient would be 1; if all would intercept the control tissue explant, the coefficient would be 0. The pontine turning coefficient was calculated in a similar manner. Thus, if all "turning" axons turn toward the pontine explant, the coefficient would be 1; if all turn toward the control tissue explant, the coefficient would be 0.

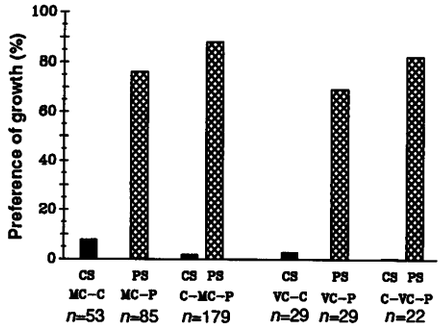

Fig. 2. Preference of cortical axon growth. For each type of coculture (indicated beneath the histogram), the bars indicate the percentage of cortical explants (MC, motor cortex; VC, visual cortex) with axon growth predominantly from the side facing the pontine explant (PS, cross-hatched bars) or the control tissue explant (CS, filled bars). No differences were observed between the control tissues used, and they are displayed as a single group. Example of abbreviation of coculture type: C-MC-P = an explant of motor cortex with a control tissue explant (C) on one side and a pontine explant (P) on the other.

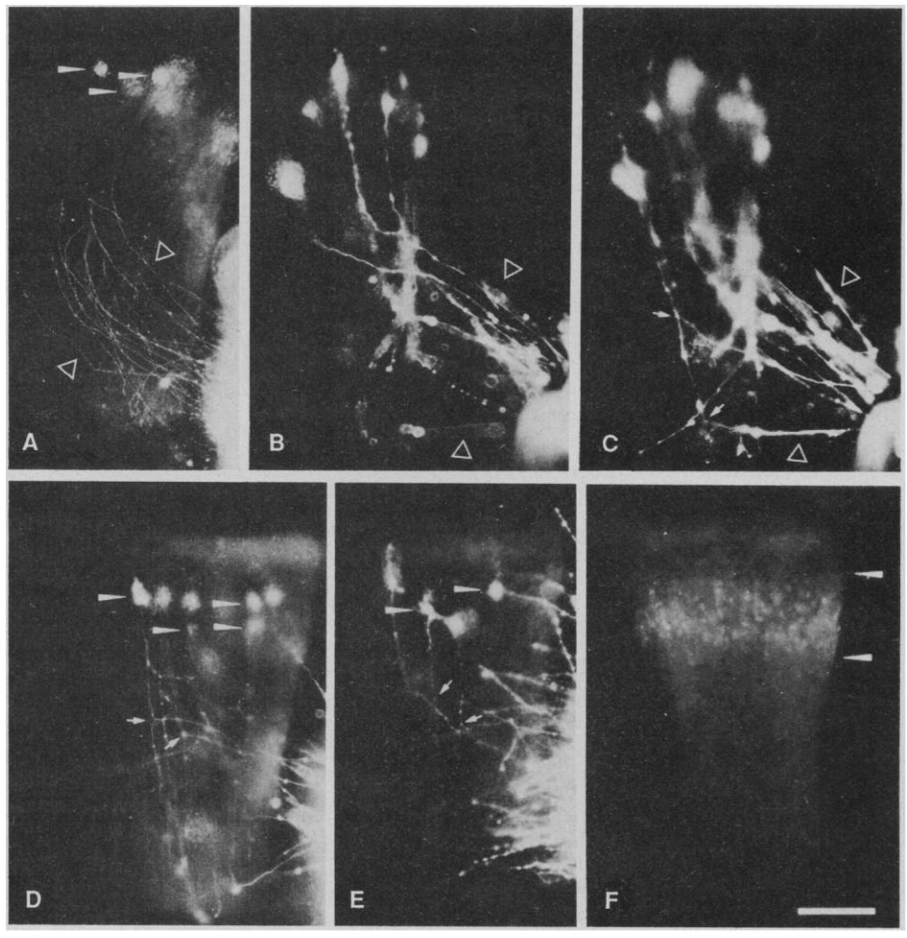

Fig. 3. Fluorescence images of axon turning and branching in cortical explants. (A through E) Motor cortex cocultured with control tissue to the left and basilar pons to the right as in Fig. 1B; only the cortical explant is in the field of view. After aldehyde fixation, DiI was injected into the pontine explant. In the examples illustrated, the pontine explants were only partially filled with DiI to facilitate observations. Because of explant thickness, only some labeling is in focus at any focal plane; segments of labeled axons are not visible when well out of the focal plane. (**A**) Fixed at 48 hours. Many cortical axons retrogradely labeled with DiI turn within the cortical explant (trajectory marked with open arrowheads) to exit the side facing the pontine explant. Some retrogradely labeled parent cells are marked with arrowheads. (**B** and **C**) Different focal planes of another cortical explant fixed at 24 hours. Many retrogradely labeled cortical axons contacting the pontine explant are collateral branches; their path within the collagen matrix is delineated with open arrowheads. The branch points of two collaterals visible in the cortical explant are marked with arrows in (C). (**D** and **E**) Two different cortical explants fixed at 24 hours. Retrogradely labeled cell bodies in the cortical explants (some marked with arrowheads) appear to be in layer 5 [compare to (F)]. Again, many retrogradely labeled cortical neurons contact the pontine explant by collateral branches—some branch points visible in the cortical explant are marked with arrows. (**F**) An explant of motor cortex taken 24 hours after rhodamine isothiocyanate was injected into the spinomedullary junction of a newborn rat to label retrogradely the band of corticospinal-corticopontine neurons in layer 5 (marked with arrowheads). Scale bar, 200 μm in (A) through (F).

plant that corresponds to the layer 5 distribution of corticospinal-corticopontine neurons (*9*) (Fig. 3, D to F). This observation suggests that the chemotropic effect may be specific for the class of cortical neuron that innervates the basilar pons in vivo. DiI injections into control explants reveal that few cortical axons make contact and provide little indication of collateral branching.

Thus, axon labeling substantiates and extends the inferences drawn from phase-contrast observations. Not only does the pontine explant affect the directional growth of cortical axons, but it can affect at a distance the extension and directed growth of axon collaterals. These results suggest that at the appropriate time in development the basilar pons releases a diffusible molecule that elicits budding and directed growth of corticopontine collaterals, either by direct action on the parent axons or by conditioning the intervening matrices, neural or collagenous.

We suggest that in vivo the corticopontine projection forms by a two-stage process. First, the primary axons of a subset of layer 5 neurons grow down a defined pathway that leads them past the pons and into the spinal cord, likely as a response of their growth cones to local cues associated with the axon tract. Second, the basilar pons, coincident with its maturation (*10*), induces and attracts axon collateral branches through the release of a diffusible molecule. Our in vitro findings support such a chemotropic mechanism and suggest that the location of collateral budding is governed by the basilar pons rather than by a length or timing program intrinsic to the corticospinal axons or by the development of local cues within the corticospinal pathway. Collaterals directed to the pontine explant in our cocultures bud from axons at positions much more proximal to the cell body than those in vivo. Furthermore, our finding that primary axons can turn toward the pontine explant suggests that their growth cones are able to respond to the pontine attractant. The failure of the primary growth cones to respond in vivo may be due to the late maturation of the basilar pons (*10*), to a preference for the axon tract, or possibly because they pass by the pons too rapidly to be adequately influenced.

Our results suggest that a diffusible, chemotropic signal allows cortical axons to recognize the basilar pons as an appropriate target. Although chemotropism was first proposed by Cajal in the last century (*11*), the concept lost favor for want of supporting evidence. However, there is now evidence that an exogenous source of nerve growth factor can act as a chemoattractant for sympathetic and sensory neurites (*12*), and, recently, natural chemoattractant activity has been detected in developing periph-

eral and central neural systems (*6, 13*). The latter observations indicate a chemotropic effect of the maxillary process on trigeminal ganglion neurons in the mouse (*6*) and of the floor plate of the spinal cord on dorsolateral commissural neurons in the rat (*13*). In these studies, the crucial demonstration was that a peripheral target (*6*), or an intermediary structure in a central axonal pathway (*13*), isolated in vitro could elicit oriented axonal outgrowth from specific types of neurons. Despite this long-awaited support for what is arguably a simple and effective mechanism for selective axon guidance (*14*), the prevailing view holds that a growth cone is guided by "signals encoded in the structures with which it is in direct contact" (*15*). Here we provide further evidence for a target-derived influence acting at a distance on axon directionality, extend this mechanism to the developing brain, and report that the target can induce the remote formation of axon branches.

The corticopontine projection is a major efferent connection of the mammalian neocortex, yet it is established in an indirect fashion (*3*). Interestingly, this projection is formed and retained by most regions of neocortex (*1*), whereas the post-pontine segment of the primary axon, which develops in a direct fashion, is subsequently lost by large regions of neocortex, including the primary visual and auditory areas (*3, 4, 16*). Our evidence that a signal derived from the pontine target can operate over a distance and affect the elaboration of this projection suggests a crucial role for diffusible molecules in the establishment of connections in the mammalian brain.

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7. These ages were chosen because in vivo corticospinal axons develop pontine collaterals between birth and postnatal day 2 (*3*). Postnatal day 0 is the first 24 hours after birth. Cortical explants spanned the full radial thickness of the cortex; this orientation was maintained in the cultures, but the sidedness was random. The side of the cortical explant on which the control and pontine explants were placed was varied. Explants were positioned above the floor of the culture dish within a hemisphere-shaped matrix of rat tail collagen (*6*). Culture medium was a 50:50 mixture of Ham's F12 and Dulbecco's modified essential medium (MEM) (Gibco) with 5% fetal bovine serum (Gibco).
8. Living cultures and cultures fixed with 15% paraformaldehyde in MEM were injected with 2% DiI in dimethylformamide through a glass micropipette. DiI is a fluorescent, lipophilic dye useful as an axon tracer in living tissue [M. G. Honig and R. I. Hume, *J. Cell Biol.* **103**, 171 (1986)] and fixed tissue [P. Godement, J. Vaselow, S. Thanos, F. Bonhoeffer, *Development* **101**, 697 (1987)]. DiI diffuses through aldehyde-fixed cell membranes and labels the entire axon tree and cell body from uptake by the primary axon or a collateral. After 5 to 8 hours (living) or 1 to 2 days (fixed and stored at room temperature), cultures were examined and photographed with a fluorescence microscope with green light excitation.
9. The layer 5 position of corticospinal-corticopontine neurons in the explants was determined by retrograde labeling with rhodamine isothiocyanate [S. Thanos and F. Bonhoeffer, *J. Comp. Neurol.* **219**, 420 (1983)] injected at birth into the spinomedullary junction (*16*) 24 hours before explanting.
10. The timing of collateral budding correlates with maturation of the basilar pons (*3*): The constituent neurons are generated between embryonic (E) days E16 and E19 in the dorsal medulla and migrate ventrorostrally and arrive at their definitive positions between E18 and birth (E22) [J. Altman and S. A. Bayer, *J. Comp. Neurol.* **179**, 49 (1978)]. By birth, when the first collaterals begin to form, pontine neurons are immature, but have extended a number of short primary dendrites, some of which give rise to secondary and tertiary branches [C. E. Adams, J. G. Parnavelas, G. A. Mihailoff, D. J. Woodward, *Brain Res. Bull.* **5**, 277 (1980)].
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"While your scientific integrity is commendable, Hartley, I might remind you that Brand X never tests out ahead of our product."