torials in Motor Behavior II, G. E. Stelmach and J. Requin, Eds. (Elsevier North-Holland, Amsterdam, 1992), pp. 195–209.
11. I. Israël, M. Fetter, E. Koenig, *Exp. Brain Res.* **96**, 335 (1993).
12. I. Israël, S. Rivaud, A. Berthoz, C. Pierrot-Deseilligny, *Ann. N.Y. Acad. Sci.* **656**, 472 (1992); C. Pierrot-Deseilligny, I. Israël, A. Berthoz, S. Rivaud, B. Gaymard, *Exp. Brain Res.* **95**, 166 (1993); I. Israël, A. Berthoz, S. Rivaud, C. Pierrot-Deseilligny, *Brain*, in press.
13. A. S. Etienne, R. Maurer, F. Saucy, *Behaviour* **106**, 81 (1988).
14. M. L. Mittelstaedt and S. Glasauer, *Zool. Jahrb. Physiol.* **95**, 427 (1991).
15. The two front wheels of the robot are driven by two independent 300-W motors that ensure propulsion of a 120-kg maximal mass at a maximal linear velocity of 1.2 m/s, with a maximal acceleration of 1 m/s^2. Steering is achieved by controlling the relative speed of the two drive wheels. The robot can be controlled either remotely (by a personal computer through a wireless modem) or directly (by a joystick on the robot itself). The joystick controls the robot's linear velocity at steps of 0.05 m/s (robot velocity directly proportional to joystick angle), with a 0.2-s delay. Positioning accuracy and linearity of the trajectory are ensured by proportional integral derivative control loops operating at 100 Hz (using optical encoding of position with a resolution of 1 mm) and a trajectory generation and control algorithm operating at 250 Hz. Participants were secured to the seat by means of three safety belts. Their heads were restrained by a cushioned support (to impede displacements and yaw rotations) and a bite bar (to prevent pitch rotations). They also wore headphones that relayed a wide-band noise ("pink" noise) to prevent perception of external acoustic cues, as well as a pair of goggles with blacked-out lenses to suppress visual information. Optically encoded digital odometry (50 Hz) was transferred from the robot through the modem to the computer after each trial.
16. E. C. Poulton, in *Handbook of Physiology: The Nervous System*, vol. II, *Motor Control*, V. B. Brooks, Ed. (American Physiological Society, Bethesda, MD, 1981), part 2, pp. 1337–1389; S. S. Stevens, *Am. Sci.* **48**, 226 (1960).
17. L. R. Young and J. L. Meiry, *Aviat. Space Environ. Med.* **39**, 606 (1968); B. K. Lichtenberg, L. R. Young, A. P. Arrott, *Exp. Brain Res.* **48**, 127 (1982); M. Shelhamer and L. R. Young, *J. Vestib. Res.* **4**, 1 (1994).
18. L. R. Young, in *Handbook of Physiology: The Nervous System*, vol. III, *Sensory Processes*, I. Darian-Smith, Ed. (American Physiological Society, Bethesda, MD, 1984), part 2, pp. 978–1023.
19. S. M. O'Mara, E. T. Rolls, A. Berthoz, R. P. Kesner, *J. Neurosci.* **14**, 6511 (1994).
20. B. L. McNaughton, C. A. Barnes, J. O'Keefe, *Exp. Brain Res.* **52**, 41 (1983); S. I. Wiener, C. A. Paul, H. Eichenbaum, *J. Neurosci.* **9**, 2737 (1989); B. L. McNaughton, L. L. Chen, E. J. Markus, *J. Cogn. Neurosci.* **3**, 190 (1991); S. I. Wiener and A. Berthoz, in *Multisensory Control of Movement*, A. Berthoz, Ed. (Oxford Univ. Press, Oxford, 1993), pp. 427–455; P. E. Sharp, H. T. Blair, D. Etkin, D. B. Tzanetos, *J. Neurosci.* **15**, 173 (1995).
21. We thank S. Glasauer for computer software design, A. Treffel for adapting the Robuter, and the Association des Paralysés de France and the Cercle Sportif des Invalides (Paris, France) for their kind cooperation. Supported by the European Economic Community (EEC) Esprit Basic Research program, project 6615 (Multisensory Control of Movement); the Cogniscience program (Axe Thématique: Représentation de l'Espace, CNRS, France); an EEC Human Capital and Mobility grant (R.G.); and a grant from the Fyssen Foundation (T.T.).

4 January 1995; accepted 17 April 1995

Disruption of Retinal Axon Ingrowth by Ablation of Embryonic Mouse Optic Chiasm Neurons

D. W. Sretavan*, E. Puré, M. W. Siegel, L. F. Reichardt

Mouse retinal ganglion cell axons growing from the eye encounter embryonic neurons at the future site of the optic chiasm. After in vivo ablation of these chiasm neurons with a monoclonal antibody and complement, retinal axons did not cross the midline and stalled at approximately the entry site into the chiasm region. Thus, in the mouse, the presence of early-generated neurons that reside at the site of the future chiasm is required for formation of the optic chiasm by retinal ganglion cell axons.

During embryonic mammalian development, retinal ganglion cell axons exit the optic nerves to grow into the site of the future ventral hypothalamus (*1*). There, axons from the two eyes, upon leaving the optic nerves, turn within the neuroepithelium in a medio-posterior direction to meet each other and lay down an X-shaped pattern of intersecting retinal axon pathways known as the optic chiasm. Subsequently, retinal axons arriving later undertake a second task in which axons originating from the nasal retina project across the midline to the opposite side of the brain, whereas a group of axons from the temporal retina turn away from the chiasm midline to project toward ipsilateral targets. Ipsilateral and contralateral axon routing appear to involve interactions of retinal growth cones with guidance cues present in the neuroepithelial environment of the optic chiasm (*2*).

Previous work has shown that the first retinal axons entering the ventral hypothalamus encounter a population of early generated neurons arranged as an inverted V–shaped array pointing anteriorly at the future site of the chiasm (*3*). These are among the first neurons generated in the embryonic mouse brain (*4*) and are already present in the ventral hypothalamus at embryonic day 11 (E11), 1.5 days before the arrival of the first retinal axons (*3*). Axons leaving the optic nerves make a medio-posterior turn toward these embryonic chiasm neurons and their processes. Upon reaching these neurons, retinal axons intermix with and run within the most anterior elements of this array to cross the midline to the other side. In this manner, retinal axons from the two eyes cross over each other to form the X-shaped optic chiasm.

Chiasm neurons express cell surface molecules capable of influencing retinal axon growth. These include L1, a member of the immunoglobulin (Ig) superfamily, which has been shown to be a potent promoter of retinal axon outgrowth in vitro (*5*) and CD44, a transmembrane glycoprotein known to bind components of the extracellular matrix (*6*), which exerts a negative influence on embryonic retinal axon outgrowth in vitro (*3*). These findings suggest that these early-generated neurons may play a role in the initial formation of the X-shaped optic chiasm.

To determine whether these neurons are involved in the establishment of the optic chiasm in vivo, we used a monoclonal antibody (mAb) to CD44 to direct complement-mediated ablation of these cells in E11 mouse embryos in utero before the arrival of retinal axons at the ventral hypothalamus. Embryos were then allowed to develop in utero until E16, an age when retinal axons have formed a well-defined X-shaped optic chiasm in normal animals.

For neuronal ablation, a rat mAb that recognizes mouse CD44 (*7*) was injected with guinea pig complement into the lateral and third ventricles of E11 mouse embryos (n = 22) (*8*). Antibodies labeled the CD44$^+$ chiasm neurons within 4 hours (Fig. 1, B and D) (*9*). As described previously (*3*), labeled neurons were organized in a layerlike fashion below the pial surface of the ventral hypothalamus (arrowheads, Fig. 1D), forming a subset of the cells present (Fig. 1E). Twenty-four hours after ablation, very few CD44$^+$ chiasm neurons could be visualized with antibody to CD44 (Fig. 1F), although the overall cell density in this region was not detectably reduced (Fig. 1G). DiI(1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate deposited at the ventral hypothalamic midline in normal embryos labels the axons of the CD44$^+$ chiasm

D. W. Sretavan, Neuroscience Program, Department of Ophthalmology, and Department of Physiology, University of California, San Francisco, CA 94143, USA.
E. Puré, Wistar Institute, Philadelphia, PA 19104, USA.
M. W. Siegel, Department of Pharmacokinetics and Metabolism, Genentech, South San Francisco, CA 94080, USA.
L. F. Reichardt, Neuroscience Program, Department of Physiology, and Howard Hughes Medical Institute, University of California, San Francisco, CA 94143, USA.
*To whom correspondence should be addressed at Department of Ophthalmology K107, University of California, 10 Kirkham Street, San Francisco, CA 94143, USA.

neurons that extend dorsally along the lateral wall of the diencephalon (3). DiI labeling at this site in treated embryos (n = 3) (10) failed to result in similar axon labeling, which is consistent with successful ablation.

Injected embryos were allowed to continue development in utero until E16. In normal E16 embryos, DiI crystals placed into the optic disk regions of the retinas heavily labeled the retinal projections, showing clearly the optic nerves, the X-shaped chiasm, and the two optic tracts (Fig. 2A). Control embryos, injected on E11 with either mAb alone (n = 5) (Fig. 2B) (11) or with complement alone (n = 4) (10), revealed retinal projections indistinguishable from those in normal embryos (12). In contrast, embryos in which chiasm neurons had been ablated exhibited highly abnormal projections at the ventral region of the developing hypothalamus (Fig. 2, C through F). Optic nerves appeared to be similar in size to those present in normal embryos. However, retinal axons in these animals failed to extend beyond the approximate junction of the optic nerves with the future site of the optic chiasm (Fig. 2, C through F). This position is approximately where the earliest retinal axons would normally encounter embryonic chiasm neurons (3). Figure 2 shows some variability in the distance at which retinal axons stopped from the midline (50 to 100 μm). Also note a few axons crossing the midline in Fig. 2E. Nevertheless, in all cases, retinal axons entering the region at E16 failed to form an X-shaped optic chiasm and optic tracts.

Light microscopic analysis of mAb complement–treated embryos at E16 showed that cells remaining in the region were normal in morphological appearance (Fig. 3B). Electron microscopic analysis (13) showed that the region was free of debris or cellular infiltrate (Fig. 3D), and there was no apparent tissue scarring that may have impeded axonal growth. Consistent with this observation, developing neocortical tissue has been transplanted (14), and embryonic cortical subplate neurons ablated chemically (15), without scarring or damage to remaining cells. Ultrastructural examination also confirmed the lack of a significant projection crossing the ventral midline (Fig. 3D).

To provide further evidence for specificity, experiments were carried out in living E12 retina-chiasm preparations in which the retinas were isolated together with the optic nerves and the part of the developing brain giving rise to the optic chiasm (16). To assess whether general damage to the neuroepithelium can lead to the arrest of retinal axon ingrowth, glass micropipettes were used to puncture the neuroepithelium at the junction of the optic stalks and the

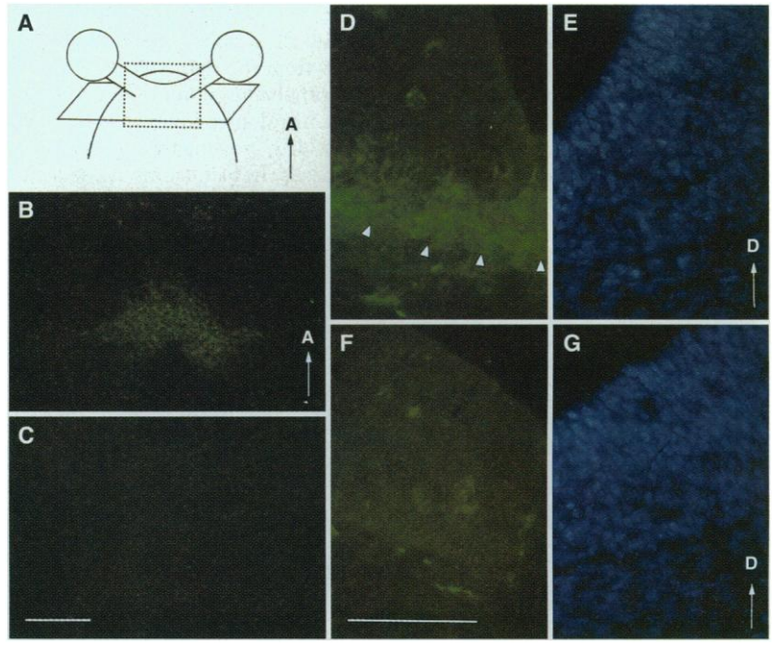

Fig. 1. Embryonic tissue sections showing anti-CD44 and 4′,6′-diamidino-2-phenylindole (DAPI) cell staining at the ventral hypothalamus. (**A**) Schematic diagram of the retinas, optic nerves, and future region of the optic chiasm in an E12 mouse embryo. A, anterior. The dotted rectangle depicts the horizontal plane of section and delineates the approximate areas shown in Fig. 2, B and C. The solid rectangle represents the coronal plane of section shown in (D) through (G). (**B**) Horizontal section through an E11 embryo showing the inverted V array of $CD44^+$ neurons after intraventricular injection of antibody to CD44, visualized with a fluorescein isothiocyanate (FITC) secondary antibody. (**C**) Horizontal section through the ventral hypothalamus of an E11 embryo that did not receive antibody to CD44 but was stained with FITC secondary antibody. (B) and (C) are at the same magnification. Scale bar in (C), 100 μm. (**D** and **E**) A coronal section through the ventral hypothalamic region of an E12 embryo after injection of antibody to CD44 at E11 and double-labeled with antibody to CD44 (D) and DAPI (E). CD44 immunoreactive chiasm neurons (arrowheads) were found immediately adjacent to the ventral pial surface and represented a subset of the cells in this region. D, dorsal. (**F** and **G**) A coronal section through the ventral hypothalamic region of an E12 embryo after injection of antibody to CD44 and complement at E11. This section was double-labeled with antibody to CD44 (F) and DAPI for cell nuclei staining (G). Anti-CD44 immunoreactivity was virtually absent. DAPI staining showed that this region appeared normal in cell size and cell density. D, dorsal. (D) through (G) are at the same magnification. Scale bar in (F), 100 μm.

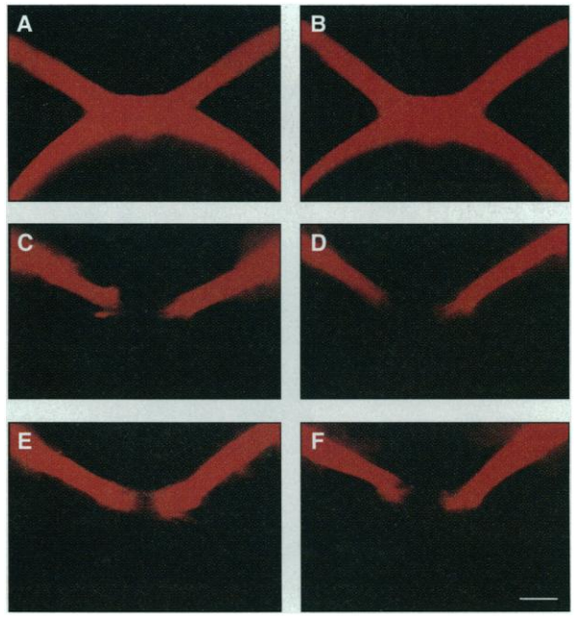

Fig. 2. DiI-labeled retinal axon projections in E16 embryos. (**A**) Retinal axon projections in a normal mouse embryo. (**B**) Retinal projections in a control embryo that was injected in utero at E11 with mAb to CD44 alone. (**C** through **F**) Retinal projections in embryos that received mAb to CD44 and complement injections at E11. The optic nerves appeared normal but the retinal axons failed to form an optic chiasm. Scale bar, 200 μm.

ventral hypothalamus before the ingrowth of retinal axons (Fig. 4, A and B), and rhodamine-labeled microspheres were used to mark the sites. After culture for 48 to 72 hours, tissue preparations (n = 8) were fixed and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) was applied at the optic disks to label the retinal projections (Fig. 4, D and E). Results showed that retinal axons grew from the optic nerves into the ventral hypothalamus along routes similar to those in control preparations (Fig. 4C) and in vivo (*17*), growing through the sites marked by beads. In fact, axons crossed the midline and began to form an optic chiasm–like structure. Thus, simple tissue damage is apparently rapidly healed, and nonspecific disruption of neuroepithelial cell populations in the ventral diencephalon does not lead to the stalling of retinal axon growth.

Previously, we found that CD44 in vitro can exert an inhibitory influence on embryonic retinal axon outgrowth (*3*). If CD44 in vivo functions in a similar manner, the inverted V array of $CD44^+$ neurons may serve two developmental roles. First, they facilitate ingrowth of the earliest retinal axons into the developing optic chiasm. Second, they may also deflect retinal axon growth laterally into the optic tracts. Guidepost neurons during grasshopper limb bud development (*18*) and subplate neurons in the developing mammalian neocortex (*15*) are specialized early generated neurons that provide guidance cues for formation of axon pathways. Our results demonstrate that $CD44^+$ neurons are critical for retinal axon ingrowth into the mammalian ventral hypothalamus and suggest that they may guide retinal axons as they leave the optic nerves to enter the brain. By analogy with grasshopper guidepost neurons and mammalian subplate neurons, this guidance may result from direct interactions between retinal axons and $CD44^+$ chiasm neurons. However, we currently cannot rule out the possibility of indirect effects.

The arrest of retinal axons after ablation of embryonic chiasm neurons is reminiscent of mutations in *Drosophila* in which axons

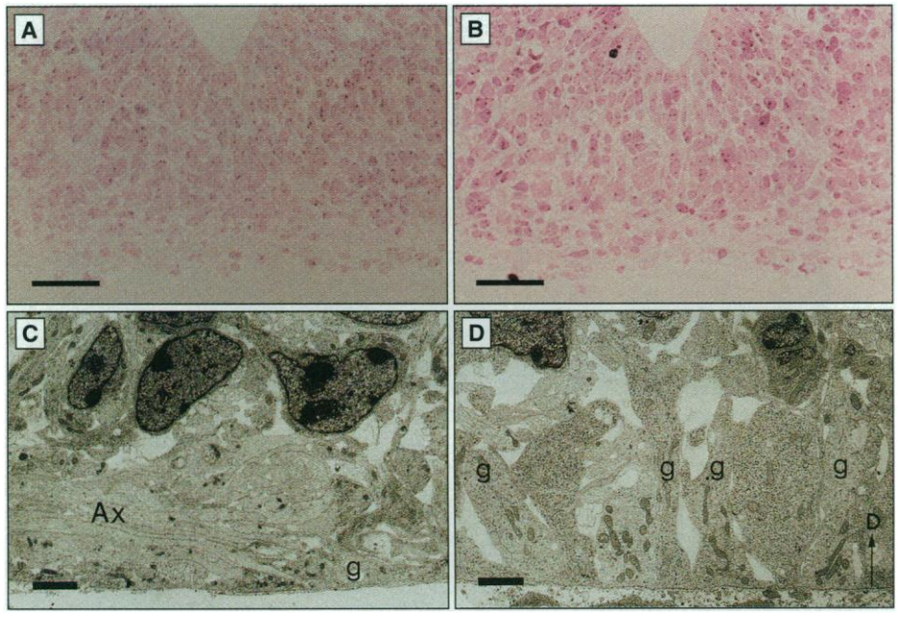

Fig. 3. Sections of the E16 ventral hypothalamus. (**A** and **B**) Plastic-embedded, hematoxylin-stained coronal sections of a normal embryo (A) and an embryo that received antibody to CD44 and complement at E11 (B). (**C** and **D**) Electron micrographs of the midline region from a normal (C) and a mAb- and complement-treated embryo (D). Ax, axon; g, radial glia-like cells. Scale bar in (A) and (B), 50 μm; in (C) and (D), 2 μm.

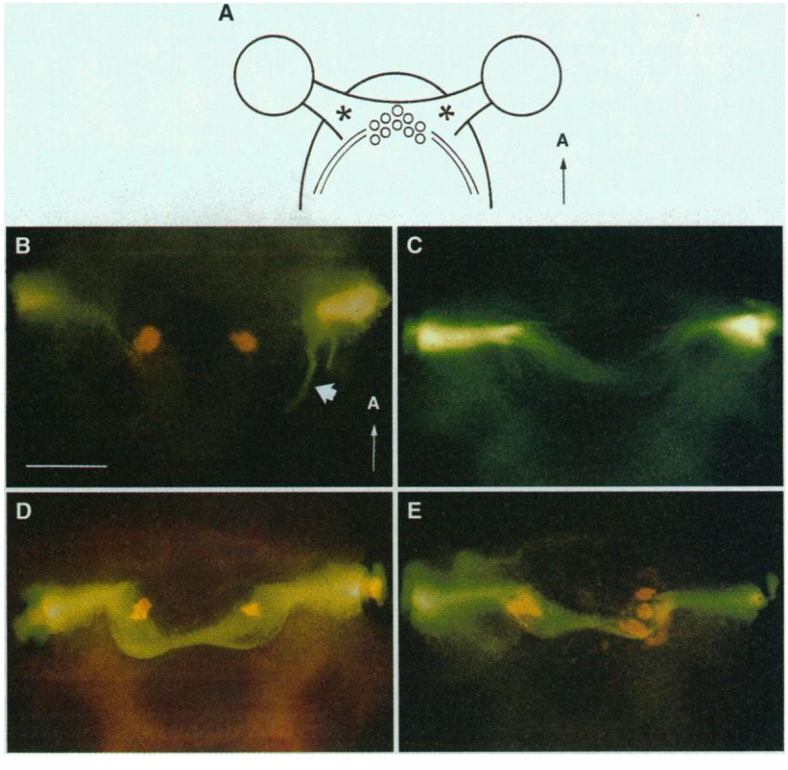

Fig. 4. Axonal growth in in vitro retina-chiasm whole mount preparations. (**A**) Schematic diagram showing the cell bodies of $CD44^+$ neurons as open circles. The location of micropipette punctures at approximately the entry site of the optic nerve into the optic chiasm region on each side is indicated by an asterisk. A, anterior. (**B**) An E12 embryo whole mount showing the retinal axon projections at the start of the in vitro culture period. DiO crystals were used to label both optic disks, and rhodamine latex beads were injected to mark micropipette puncture sites. At this age, no retinal axons have reached the ventral hypothalamus. The DiO-labeled elements marked by the arrow are most likely axons of passage near the eye region. A, anterior. (**C**) Preparation from an E12 embryo maintained in culture for 48 hours. Axons from both retinas have entered the ventral hypothalamus region and have begun to make an optic chiasm–like structure. (**D**) A whole mount from an E12 embryo subjected to micropipette-induced damage (at sites marked by rhodamine-labeled beads) and subsequently maintained for 2 days in vitro. DiO labeling revealed that embryonic retinal axons have entered the ventral hypothalamus. (**E**) A second example of a whole mount preparation from an E12 embryo maintained for 2 days in vitro. In this experiment, an extensive series of micropipette punctures (marked by three separate sites of bead labeling) were made in the right side of the preparation. DiO-labeled retinal axons have still grown into the future region of the optic chiasm. Scale bar in (B), 400 μm.

arrest at positions corresponding to pathfinding sites characterized by a change in the cellular environment (*19*). The results are also remarkably similar to the phenotype of GAP-43 null mutant mice in which embryonic retinal axons stall at the ventral hypothalamus without forming an optic chiasm (*20*).

Our experiments do not address the eventual fate of stalled retinal axons. These axons may remain at the ventral hypothalamus indefinitely, be subsequently eliminated because of a failure to reach their targets, or eventually continue into the brain by normal or alternate routes. In the achiasmatic mutation in adult dogs, retinal axons from the two eyes approach, but do not cross the midline to form an X-shaped optic chiasm. Instead, retinal axons project exclusively into the ipsilateral side (*21*). We do not know whether retinal axons in mouse embryos with ablated $CD44^+$ neurons, like retinal axons in these mutant dogs, eventually find their way to ipsilateral targets. Likewise, it is not known if embryonic $CD44^+$ chiasm neurons are affected in achiasmatic dogs. Nevertheless, the failure of retinal axons to form a normal optic chiasm in both the achiasmatic dogs and the mouse embryos with ablated $CD44^+$ neurons suggest that retinal axon ingrowth and crossing at the ventral hypothalamic midline in mammals involve pathfinding mechanisms distinct from those that mediate retinal axon growth within the optic nerves and tracts. One cellular element involved in the initial formation of the X-shaped optic chiasm appears to be early generated $CD44^+$ chiasm neurons.

REFERENCES AND NOTES

1. In this paper, the term "hypothalamus" refers to the region where the hypothalamus will eventually develop.
2. R. J. Colello and R. W. Guillery, *Development* **108**, 515 (1990); P. Godement, J. Salaun, C. A. Mason, *Neuron* **5**, 173 (1990); D. W. Sretavan, *J. Neurosci.* **10**, 1995 (1990); _____ and L. F. Reichardt, *Neuron* **10**, 761 (1993); A. Wisenmann, S. Thanos, Y. V. Boxberg, F. Bonhoeffer, *Development* **117**, 725 (1993).
3. D. W. Sretavan, L. Feng, E. Puré, L. F. Reichardt, *Neuron* **12**, 957 (1994).
4. S. S. Easter, L. S. Ross, A. Frankfurter, *J. Neurosci.* **13**, 285 (1993).
5. V. Lemmon, K. L. Farr, C. Lagenaur, *Neuron* **2**, 1597 (1989); J. Drazba and V. Lemmon, *Dev. Biol.* **138**, 82 (1990).
6. A. Aruffo, I. Stamenkovic, M. Melnick, C. Underhill, B. Seed, *Cell* **61**, 1303 (1990).
7. Rat mAb 18C8 (IgM) mediates complement fixation in splenocyte cytotoxicity assays [C. Henry, in *Selected Methods in Cellular Immunology*, B. Mishell and S. Shiigi, Eds. (Freeman, San Francisco, CA, 1980), pp. 211–212].
8. Timed pregnant C57/B16 mice were anesthetized with sodium pentobarbital. After cesarean section, an incision was made into the uterine sac over the head region of embryos. Five microliters of mAb (1 mg/ml) was injected into each lateral ventricle and into the third ventricle with a glass micropipette attached to a 10- or 25-μl Hamilton syringe (Fisher Scientific). Guinea pig complement with low toxicity against mouse lymphocytes (cat. CL4051 Cedarlane Laboratories, Ontario, Canada) was used at a 1:10 dilution in sterile 0.9% saline.
9. To demonstrate in vivo labeling, injected embryos were returned in utero for 4 hours, after which the head was immersion fixed in 4% paraformaldehyde at 4°C for 5 min. Ten-micrometer horizontal cryostat sections were cut through the future optic chiasm region and reincubated with fluorescent secondary antibodies to rat IgM (Jackson Immunochemicals, West Grove, PA). In embryos treated with mAb and complement, cryostat tissue sections were first incubated with antibody to CD44 (KM201) followed by a fluorescent second antibody to locate remaining $CD44^+$ chiasm neurons.
10. D. W. Sretavan, E. Puré, M. W. Siegel, L. F. Reichardt, data not shown.
11. The mAb 18C8 does not block the effects of CD44 on embryonic mouse retinal axon growth in vitro (D. W. Sretavan and L. F. Reichardt, unpublished results).
12. D. Sretavan, *J. Neurosci.* **10**, 1995 (1990).
13. For electron microscopic analysis, heads were immersion fixed in 1% paraformaldehyde and 1.25% glutarylaldehyde in 0.1 M Na-cacodylate buffer (pH 7.35) for 12 to 18 hours at 4°C. Tissues were then post-fixed in OsO_4, dehydrated, and embedded in epoxy resin. Thin sections were counterstained and examined with a Phillips CM 12 electron microscope.
14. B. L. Schlaggar and D. D. M. O'Leary, *Science* **252**, 1556 (1991).
15. A. Ghosh, A. Antonini, S. McConnell, C. Shatz, *Nature* **347**, 179 (1990); A. Ghosh and C. Shatz, *Development* **117**, 1031 (1993).
16. Retina-chiasm preparations were dissected as described [D. W. Sretavan and L. F. Reichardt, *Neuron* **10**, 761 (1993)] except that preparations were obtained from E12 embryos before the arrival of retinal axons at the ventral hypothalamus. Glass micropipettes were used to puncture the neuroepithelium bilaterally in regions where retinal axons leave the optic stalks to enter the ventral hypothalamus. After 10 punctures per side, rhodamine-labeled latex microspheres were injected with a separate micropipette to mark the sites.
17. For examples of normal patterns, see figure 3 in D. Sretavan, *J. Neurosci.* **10**, 1995 (1990); and figure 2 in P. Godement, J. Salaün, C. Mason, *Neuron* **5**, 173 (1990).
18. M. Klose and D. Bentley, *Science* **245**, 982 (1989).
19. D. Van Vactor, H. Sink, D. Fambrough, R. Tsoo, C. Goodman, *Cell* **73**, 1137 (1993).
20. S. Strittmatter, C. Fankhauser, P. Huang, H. Mashimo, M. Fishman, *ibid.* **80**, 445 (1995).
21. R. W. Williams, D. Hogan, P. E. Garraghty, *Nature* **367**, 637 (1994).

22. We thank R. Camp for mAb preparation. Supported by a Muscular Dystrophy Association Postdoctoral fellowship (D.W.S.), NIH grant EY10688 (D.W.S.), the Lucille P. Markey Charitable Trust, the Retinitis Pigmentosa Foundation, and the Howard Hughes Medical Institute. D.W.S. is the recipient of a Jules and Doris Stein Professorship from Research to Prevent Blindness. L.F.R. is an investigator at the Howard Hughes Medical Institute.

9 February 1995; accepted 3 May 1995