# RADIAL GLIA IN THE DEVELOPING SUPERIOR COLLICULUS: EVIDENCE FOR A MIDLINE BARRIER

by

**DA-YU WU**

B.S., Beijing Teachers College, 1982
M.S., Fordham University, 1985

Submitted to the Department of Brain and Cognitive Sciences
in Partial Fulfillment of the Requirements for the
Degree of
DOCTOR OF PHILOSOPHY

at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
May 1991



Signature of Author _____
Department of Brain and Cognitive Sciences, May 1991

Certified by _____
Gerald E. Schneider
Professor, Brain and Cognitive Sciences
Thesis Supervisor

Accepted by _____
Emilio Bizzi
Chairman, Department of Brain and Cognitive Sciences

To my wife, Shwu
and to my grandparents, Wu Liang-shu and Xie Yue-xian

Their love, encouragement, and support made this possible.

## TABLE OF CONTENTS


| | | |
|---|---|---|
| ABSTRACT | | 4 |
| INTRODUCTION | | 6 |
| CHAPTER I | Retinal Axons Cross the Tectal Midline After Unilateral Tectal Ablation Only When the Midline Glia Are Damaged | 19 |
| CHAPTER II | The Glial Environment and Its Changes in the Developing Superior Colliculus | 51 |
| CHAPTER III | Recrossing of Retinal Axons in the Optic Tectum After Selective Damage to the Midline Glial Processes | 84 |
| CONCLUSION | | 111 |
| BIBLIOGRAPHY | | 119 |
| ACKNOWLEGEMENTS | | 133 |

# RADIAL GLIA IN THE DEVELOPING SUPERIOR COLLICULUS: EVIDENCE FOR A MIDLINE BARRIER

by

DA-YU WU

Submitted to the Department of Brain and Cognitive Sciences at the Massachusetts Institute of Technology, May 1991, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

## ABSTRACT

In a developed nervous system, information is conducted between sensory organs and neurons, between various neurons, and between neurons and effectors such as muscles and glands through an intricate but precise network. To form such a network, axons, namely nerve fibers, have to grow out of somata of neurons to reach their innervating target, sometimes over long distances. Although much information has been accumulated in recent years, the mechanisms by which axons grow towards and locate their terminals within the destined target area during development are still largely unclear.

In the previously reported findings of abnormal crossing of the tectal midline by retinal axons after unilateral tectal lesions (Schneider, 1973; Schneider et al., 1985), damage to tissue medial to the lesioned superficial gray layer of the superior colliculus (SC) was shown to be necessary to permit the crossing (So and Schneider, 1978). This prompted the hypothesis that during retinal axon development in normal animals there exists a midline barrier which prevents these axons from growing into the wrong side of the tectum. The work reported here specifically tests this "midline barrier" hypothesis using anatomical, histological and immunohistochemical procedures: (1) Unilateral tectal surgery was performed on neonatal hamsters in which one of the SCs, along with the midline tissue was damaged with heat. This procedure had been shown to induce the abnormal crossing of the midline by retinal axons. The eye on the lesioned side was enucleated to eliminate competition in the intact SC after the anomalous axon crossing. The changes in glial structures were followed by immunostaining with two of the conventional glial markers, antibodies raised against glial fibrillary acidic protein (GFAP) and vimentin. Retinal axon growth into the intact SC in the same brain was

observed by injecting horseradish peroxidase (HRP) into the remaining eye before sacrifice of the animal. The abnormal distribution of retinal axons and the changes in glial structures were described to elucidate the relationship between the midline glia and retinal axon growth in the SC. The results showed that retinal axons cross the midline only where the midline glial processes had been disrupted. (2) Anti-GFAP and anti-vimentin antibodies were used to immunostain glial cells at various embryonic and postnatal stages. This approach made it possible to see the correlation between the reported timing of retinotectal axons arriving at and terminating within the SCs and the differentiation, morphology, and distribution of glial cells in the midline area and in SC parenchyma in the normal brain. The results from this work were further confirmed and substantiated with labeling of radial glia with a fluorescent dye (DiI) during perinatal ages. (3) To more directly test the barrier function of midline glia, a hooked tungsten wire was used as a surgical blade to undercut the glial processes in the tectal midline at birth. The surgery was designed to exclusively disrupt the midline glial structure while leaving the midline pia intact. The initial damage caused by the surgery, the glial reaction to the surgery, and the resulting influence on the growth of retinal axons in the SCs were evaluated with a double labeling procedure: DiA labeling of midline radial processes combined with DiI labeling of retinal axons promptly after surgery. Glial-axonal relationships were also detected by immunostaining with the two antibodies for glia and by HRP tracing of retinotectal axons.

The results from these studies provide strong evidence that during the period of retinotectal axon ingrowth, a group of radial glia in the tectal midline differentiate differently from the rest of the radial cells and organize themselves into a special glial barrier which helps establish the laterality of retinal axon distribution in each SC. Since similar midline structures have been reported in rats (Raedler et al., 1982) and in opossum (Barradas et al., 1989), this kind of compartmentalization of the developing brain during axonal ingrowth might provide a general mechanism of axon guidance to ensure correct laterality in information input and neuronal wiring.

Thesis Supervisor: Dr. Gerald E. Schneider
Title: Professor of Brain and Cognitive Sciences

# INTRODUCTION

During development of the nervous system, neurons extend axons to reach their targets, sometimes over long distances. How axons are guided to their destined target areas is largely unclear. However, significant progress has been made in understanding some important aspects. For example, in vertebrate nervous systems, there is strong evidence that chemotropic factors (Tessier-Lavigne et al., 1988; Dodd and Jessell, 1988), substrate pathways with specific adherence to growing axons (Hammarback et al., 1985; Gundersen, 1987; Lander, 1987), and recognition through cell-cell contact and recognition molecules (Rutishauser, 1985) are important mechanisms in axonal guidance. Besides these inductive, promotive mechanisms, inhibitory factors which provide "negative guidance" have been proposed recently as another important type of mechanism to ensure correct axonal extension and termination (Patterson, 1988; Schwab, 1990). The present research has been carried out to address the distribution, differentiation, and probable axon growth inhibitory functions of a special group of radial glia in the optic tectum during the development of retinotectal projections in Syrian hamsters.

The vertebrate nervous system is composed of mainly two types of cells, the neuronal cells and the glial cells. Both cells originate from the

neuroepithelial wall of the neural tube during embryogenesis. Later there is also secondary gliogenesis in so-called nongerminal sites, as in the developing rodent forebrain and cerebellum (Hajós and Bascó, 1984) or adult optic nerve (Wolswijk et al., 1990). Neuronal cells generate and conduct electrical signals. Glial cells, depending on their type and location, provide nutritional and structural support to the neurons, form myelin sheaths, remove debris after injury, and maintain the ionic balance and chemical concentration of the extraneuronal environment (Kandel and Schwartz, 1985). Importantly, glial cells also guide neuronal migration, and they may direct axonal growth during brain development (Rakic, 1971,1981; Sotelo, 1978; Noble et al., 1984; Silver and Rutishauser, 1984; Mason et al., 1990).

Glial cells are subdivided into different types according to their birth date, distribution, immunocytochemistry, morphology and function (Choi and Lapham, 1978; Choi, 1981; Levitt and Rakic, 1980; Ghandour et al., 1983; Bovolenta et al., 1984; Misson et al., 1988; Raff, 1989; Scoff, 1990). Two intermediate filaments, the glial fibrillary acidic protein (GFAP) and vimentin, are commonly detected in glial cells in both developing and adult central nervous systems (CNS) in mammals.

GFAP, first described for human tissue undergoing gliosis (Eng et al., 1971), has been widely used as a marker for astrocytes (Uyeda et al.,

1972; Bignami et al., 1972; Raff et al., 1979, 1984; Dixon and Eng, 1981). Vimentin, although first described in mesenchyme-derived cells (Franke et al., 1978), has been detected in many immature glial cells (Schnitzer et al., 1981; Sommer et al., 1981; Dahl et al., 1981, 1982), including radial glia in the embryonic and neonatal CNS (Bignami et al., 1982; Drager et al., 1984; Hirano and Goldman, 1988). Sometimes, either GFAP or vimentin is localized in a particular group of glial cells whereas at other times both are found in the same cell (Sharp et al., 1982; Wang et al., 1984). Since it has been shown that in some cases, a decrease of vimentin expression in the developing brain is coupled with an increase in GFAP expression (Dahl, 1981), and that there are "transitional" cells with cytological and immunohistochemical features intermediate between those of radial glia and astrocytes (Pixley and De Vellis, 1984; Hirano and Goldman, 1988), and between those of astrocytes and oligodendrocytes (Choi et al., 1983a,b), it is conceivable that immature vimentin-expressing glial cells differentiate into mature, GFAP-expressing astrocytes and/or myelin-forming oligodendrocytes at late stages during development. Such transitions have been clearly demonstrated in *in vitro* experiments (Fedoroff et al., 1983; Ogawa et al., 1985).

Neuroglia play important roles in neuronal cell migration and in axonal growth and guidance. Rakic has observed that in the fetal monkey

cerebellum, postmitotic neurons migrate to their final destination along the processes of radial glia (Rakic, 1971). His studies with Levitt, using GFAP immunostaining, further provide evidence that glia and neurons are present concomitantly during neurogenesis and that the radial glia themselves later transform into astroglia after completion of neuronal migration (Levitt and Rakic, 1980; Levitt et al., 1981). Glial processes are also known to be involved in axon development and pattern formation. Double labeling with GFAP and neurofilament immunostaining reveals that in rodents, subpial axons are closely intermingled with astroglial processes which are well organized into a "palisading pattern" (Bitner et al., 1987). Direct evidence that astrocytes promote axon growth *in vivo* comes from Silver's study of callosal axon regeneration. When a cellulose bridge is implanted into the neocortex of young acallosal mice, astrocytes migrate onto the cellulose and cover the bridge. This glia-coated bridge can then support the *de novo* growth of commissural axons (Silver and Ogawa, 1983; Smith et al., 1986).

The involvement of glial cells in neuronal survival, differentiation, and axon growth has specifically been shown in many *in vitro* studies. For example, it is reported that 60%-80% of embryonic mouse cerebellar neurons can survive after five days when they are co-cultured with certain glial cells whereas less than 5% survive if they are co-cultured with

non-glial cells under the same circumstances (Alliot et al., 1988). Neurons in culture are found to be more adherent to glia than to other neurons but are more adherent to other neurons than to non-glia; also glia promote greater neurite outgrowth than non-glia (Noble et al., 1984). Recently, Prochiantz and his colleagues have shown that neuronal morphogenesis and polarity are regulated by astrocyte-derived factors (Rousselet et al., 1990). The capability of astrocytes in regulating the rate of axonal growth is further shown by the finding that immature astrocytes promote longer neurite outgrowth by retinal ganglion cells (RGCs) than mature astrocytes *in vitro* (Smith et al., 1990). They also find that immature astrocytes promote a higher rate of neurite outgrowth and that with maturation astrocytes have a reduced capacity and different molecular bases for supporting neurite outgrowth. Therefore, the extent of trophic support by glia varies with the age of glia.

As in other parts of the brain, vimentin and GFAP are the major cytoskeletal components of glial cells during development of the visual system (Raff et al., 1979, 1983; Raff and Miller, 1984; Schnitzer, 1988). Vimentin and GFAP expression in glial cells of the visual system have been shown to be spatiotemporal events correlated with neuronal development and axonal growth (Abney et al., 1981; Pixley and De Vellis, 1984; Bovolenta et al., 1987). Much evidence from both *in vivo*

and *in vitro* studies of visual systems indicates possible interactions between glial cells and neuronal cells and implies that glial cells also play important roles in retinal axon survival and guidance. Armson and colleagues have demonstrated that the survival of embryonic rat RGCs depends on Müller cells, a group of glia in the retina, while postnatal RGC survival depends on target tissues (Armson et al., 1987). Bonhoeffer's group reported that in the developing chick optic tectum, there also exists a palisading pattern of radial glia similar to that reported in the rodent midbrain, and that retinal axons course through the area densely packed with highly organized radial glia which are oriented perpendicular to the direction of axonal ingrowth (Vanselow et al., 1989).

In terms of the cellular and molecular bases of various glial functions, many studies suggest that glia-derived extracellular matrix and membrane molecules are responsible for mediating interactions between neurons and glia (Rutishauser, 1985; Lander, 1987; Matsunaga et al., 1988; Letourneauet al., 1988; McLoon et al., 1988). For example, NCAM is localized at the endfeet of neuroepithelial cells which line the route of the retinotectal pathway during retinal axon development (Silver and Rutishauser, 1984). Laminin is co-localized with neuroepithelial endfeet lining the optic pathway prior to and during retinal axon outgrowth from the eye in chicks (Cohen et al., 1987). In the developing

rat optic nerve, laminin is also found to be distributed only transiently during the period of retinal axon growth and is co-localized with astrocytes expressing GFAP (McLoon et al., 1988). In addition, N-cadherin and integrin are found to mediate ciliary ganglion neurite outgrowth on cultured astrocytes (Tomaselli et al., 1988). L1 on Schwann cell surfaces is shown to elicit neurite outgrowth from embryonic retinal explants (Kleitman et al., 1988). Recently, L1 on the surface of Müller cells has been found to promote RGC neurite outgrowth (Drazba and Lemmon, 1990).

Based on evidence from these studies, one can be quite certain that some glial cells are supportive (as in formation of substratum pathways, etc.) and sometimes promotive (as in longer and faster neurite growth) for neuronal differentiation and axonal growth. Nevertheless, it does not exclude the possibility that there may also exist some glia which are inhibitive (when they actively suppress axon growth) or nonpermissive (when they are not suitable for or passively block axon growth). Indeed, glia with such properties may direct axonal growth cones by so-called "negative guidance" (Schwab, 1990). Much evidence has emerged in recent years which indicates that glial cells (astrocytes and oligodendrocytes) are an important source of inhibitory factors influencing both axonal growth and regeneration in the CNS. Schwab and

Caroni have discovered that two membrane proteins associated with oligodendrocytes, NI-35 and NI-250 of CNS myelin are nonpermissive to axonal growth in the rat spinal cord (Caroni and Schwab, 1988a,b; Schwab and Caroni, 1988). Only when the inhibitory effect of these proteins is neutralized by antibodies raised against them does some regeneration of the severed corticospinal tract occur in the adult spinal cord (Schnell and Schwab, 1990; Schwab, 1990).

In the visual system, glia serving as a barrier in guiding axon elongation has been indicated in studies of retinal axon pathfinding. Silver and colleagues report that, in mice and rats, the upper wall of the distal half of the primitive eye stalk is formed by a group of neuroepitheliel cells which transiently become pigmented prior to and during the passing of pioneer optic axons. It has been observed that all the outgrowing axons avoid this melanotic tissue and prefer the pigment-free area (Silver and Sidman, 1980; Silver and Sapiro, 1981). In another study, Silver has reported that a knotlike structure of glia is found at the mouse optic chiasm which directs ventronasal axons from the retina to grow contralaterally (Silver, 1984). It is suggested that the mechanism of glial guidance is, at least to some extent, a blocking effect of the glial knot in preventing the ventronasal axons from growing rostrally. In other words, the glial cells serve as a barrier to the ventronasal axons.

Recently, evidence from Mason's laboratory indicates that along the midline of the chiasm, crossed and uncrossed retinal axons respond to midline cues differently. The uncrossed axons seem to encounter some substrates which are not permissive to their growth (Godement et al., 1990). With antibody labeling, Mason and colleagues have revealed a group of radial glia in the midline of the chiasm. They suggest that there may be a recognition mechanism between retinal axons and glial cells by which uncrossed axons are pushed to stay on the side ipsilateral to the eye of origin (Mason et al., 1990; personal communication).

In Syrian hamsters, retinal axons emerge from the eyes around embryonic day 11 (E11) and grow towards their target zones rapidly. The first axons reach the optic chiasm at about E11.5 and the SCs at around E13.5 (Jhaveri et al., 1983, 1990, 1991; Schneider et al., 1985). Along the route of extension, retinal axons segregate at the optic chiasm to grow either contralaterally or ipsilaterally. After the optic chiasm, retinal axons normally do not cross the midline again, but remain on the side either contralateral or ipsilateral to the eye of origin. This can be changed if the midline tissue separating the two SCs was damaged when one of the SCs was ablated. Then the retinal axons can recross the midline, often in fascicles, and terminate at the opposite SC (Schneider, 1973; So, 1979). If, in addition to the unilateral SC ablation and the

midline tissue damage, the eye on the lesioned side is enucleated at the same time to eliminate competition from axons normally terminating in the opposite SC, the recrossed retinal axons from the remaining eye span the entire opposite SC (Schneider, 1973; So and Schneider, 1978). It became obvious from these experiments that both SCs can accommodate retinal axons from either side after the chiasm. Somehow, retinal axons in a normal situation are prevented from growing into the "wrong" side during development, and they remain so during maturity.

What blocks retinal axons from growing across the midline of the optic tectum in normal development? Or, what was damaged in the cases with lesions so that the abnormal crossing was permitted?

During study of the unilateral SC ablation cases, there were cases which had gone through the same surgical procedure but failed to produce crossing at the SC midline. When these cases were examined, it was noticed that the medial SC tissue was somehow spared during surgery (So and Schneider, 1978). In addition, experiments carried out in this laboratory (Hsiao and Schneider, 1978) and in Lund's laboratory (Jen and Lund, 1979) showed that when a surgical blade was used to make a cut through the skull above the tectal midline into the tissue between the two SCs, the two tecta fused into one. If one of the eyes was enucleated during the surgery retinal axons from the remaining eye grew into both sides. It

was therefore speculated that a certain structure existed in the tectal midline which was normally inhibitory or nonpermissive to the growth of retinal axons. Only when this nonpermissive structure was damaged at the time of the SC ablation was the crossing permitted.

Recently, Snow and Silver reported that a group of glial cells in the spinal cord roof plate express keratan sulfate proteoglycan (KSPG), which has been shown to inhibit neurite outgrowth both *in vivo* and *in vitro* (Snow et al., 1990a,b). The roofplate in the spinal cord forms the midline of the dorsal column. It was observed that when axons of the dorsal column and the ventral commissural pathway extend dorsomedially, they never invade the glial roof plate. The glial cells express KSPG transiently, primarily during the elongation period of the dorsal column and of the commissural axons. It was therefore proposed that a glial barrier existed at the roof plate during development to confine axon growth through an 0inhibitory influence.

The midbrain and hindbrain midline have been shown to possess some distinctive cells during CNS development. Raedler and his colleagues reported that a cell group at the seam at the midline of the mesencephalic roof of the rat brain developed between E12 and E15 and differentiated into a distinctive "median ventricular formation" (MVF) (Raedler et al., 1982). These cells had a short ventral process attached to

the lining of the ventricle and a long dorsal process that could be followed up to the pial basement membrane. With immunostaining, the existence of such midline glial cells was subsequently reported in the postnatal optic tectum of opossum (Barradas et al., 1989) and in the postnatal brainstem of cats and rabbits (Mori et al., 1990). Authors of these studies speculated that these midline cells might have some role in structural support, communication, and/or axon-astroglial interactions during development. Recently, it was observed through electron microscopic studies that when retinal axons crossed the midline after a unilateral tectal lesion, the processes of the midline cells were damaged (Poston et al., 1988). It was therefore suggested that a group of midline cells might form a boundary for developing retinal axons.

The research presented here addresses three questions: 1) Is there a glial structure involved in forming a barrier for retinal axons between the two SCs during development and, if there is, what is its spatiotemporal relationship with the developing visual pathway in normal and abnormal growth? 2) How and when do the glial populations differentiate during the normal development of SCs and is there a distinctive group of glia forming any specific midline glial structure? 3) What happens if the midline glial processes are selectively compromised during development of the retinotectal axons?

Answers to these questions could provide insights into the possible additional roles of glia in CNS development and on how during development axons are guided to and confined within their destined target areas. The new knowledge could also point towards better understanding of how and why recovery and regeneration might or might not occur in certain neurological diseases and after injuries.

# CHAPTER I

## Retinal Axons Cross the Tectal Midline After Unilateral Tectal Ablation Only When the Midline Glia Are Damaged

### INTRODUCTION

During development of vertebrate nervous systems, axons sometimes extend long distances to reach their target. Although the mechanisms through which axons are guided to their destined terminal areas are still largely unclear, significant progress has been made in recent years. Among the important aspects of axonal growth and guidance in the vertebrate nervous system it has been demonstrated that chemotropic factors (Tessier-Lavigne et al., 1988; Dodd and Jessell, 1988), substrate pathway (Hammarback et al, 1985; Gundersen, 1987; Lander, 1987), and recognition through cell-cell contact and recognition molecules (Rutishauser, 1985; 1989) are important mechanisms in axonal guidance. Besides these promotive mechanisms, inhibitory factors which provided "negative guidance" has been recently proposed as another important mechanism to ensure the correct axonal extension and termination (Patterson, 1988; Schwab, 1990).

In rodent visual system development it had been shown that retinal axons were possibly "guided" by axon-repulsive neuroepithelial or glial

structures at some decision-making points along their pathway. Silver and colleagues reported that, in mice and rats, all the outgrowing retinal axons at the optic stalk avoided a melanotic tissue area formed by pigmented neuroglial cells and preferred the pigment-free intercellular tunnel (Silver and Sidman, 1980; Silver and Sapiro, 1981). In another study, Silver reports that a knotlike structure of glia was found at the mouse optic chiasm which directs ventronasal axons from the retina to grow contralaterally by preventing these axons from growing rostrally (Silver, 1984). Recently, evidence from Mason's laboratory indicated that along the midline of the chiasm, crossed and uncrossed retinal axons responded to midline cues differently. The uncrossed axons seemed to encounter some substrates at the midline area which were not permissive to their growth towards the contralateral side (Godement et al., 1990). With antibody labeling, Mason and colleagues revealed a group of radial glia in the midline of the chiasm. They suggested that there might be a recognition mechanism between retinal axons and glial cells by which uncrossed axons were pushed to stay on the side ipsilateral to the eye of origin (1990 SN abstract, and personal communication).

Previous studies of the development of the hamster visual system by Schneider and his colleagues had shown that a unilateral tectal lesion at birth could result in a vast termination of optic tract axons in the opposite

SC. The retinal axons which already grew to the SC contralateral to the eye regenerated and grew across the tectal midline and terminated in the ipsilateral SC (Schneider, 1973; So, 1979). However, the crossing did not occur if the medial tissue of the SC was not damaged (So and Schneider, 1978). This had prompted the hypothesis that certain structures along the midline prevented the crossing in these cases and in normal brains so that the laterality of the retinotectal topology was established in development.

Recently, two peices of evidence were reported by Jerry Silver and colleagues. First, a group of glial cells in the roofplate of the developing spinal cord in rats expressed keratan sulfate proteoglycan (KSPG), which was suggested to be an axon-repelling molecule to confer the axon barrier function of the roofplate (Snow et al., 1990a). In collaboration with our laboratory, KSPG was also located along the hamster tectal midline at the neonatal period (Snow et al., 1990a). Second, in the unilateral tectal lesion case, certain radial cells in the midline area were found to be damaged and degenerated away from the lesion site (Poston et al., 1988). However, how was the damaged glia related to the crossing retinal axons and whether an early reported tissue bridge was necesary for the crossing were unanswered.

To test the possibility that 1) glial structures in the tectal midline area were indeed involved as part of the barrier to prevent axons from

growing into the wrong side of the tectum during development of normal retinotectal projection, and 2) damage to glial structures after unilateral tectal lesion was responsible for the recrossing of retinal axons at the midline, two conventional glial markers, antibodies raised against glial fibrillary acidic protein (GFAP) and vimentin, were used to immunostain the normal and lesioned neonatal tectum. The results from immunostaining were compared with retinal axon projections in the same brain to examine the relationship between the changes of glial structure and growth of retinal axons after the lesion.

## MATERIALS AND METHODS

Syrian hamster pups were bred in our colony or purchased from the Charles River Laboratory (Wilmington, Massachusetts). For early-lesion cases, animals that had undergone unilateral tectal ablation surgery (see below) and had their left eye injected with horseradish peroxidase (HRP) were sacrificed by an overdose of Nembutal and perfused transcardially with an initial rinse of 0.9% saline containing 0.25% sodium nitrite followed by 4% paraformaldehyde made up in 0.1 M phosphate buffer (pH 7.4). The brains were not post-fixed so that the HRP activity could be better preserved. Fixed brains were dissected out and cryoprotected in phosphate buffered 30% sucrose. Transverse

sections were cut at 40 micrometers on a cryomicrotome. Alternate sections were collected in 0.1 M phosphate buffer (pH 7.4) for immunohistochemistry and for HRP processing (described below).

Surgical Procedures:

All pups were operated at the age of P1. The ablation of one side of the tectum was made according to the protocol established in this laboratory. All the tectal ablations were done on the right side so that results could be compared consistently. Briefly, hypothermia as anesthetic was quickly induced by removal of P1 pups from the nest and put onto crushed ice for 5-7 minutes. The skull above the right SC was exposed by opening the overlaying skin and removal of connective tissue between the skin and skull with a cotton swab. The superficial layers of the right SC and the midline were then destroyed by heat from an alcohol flame-heated pin-head applied to the area of exposed skull above the right SC. The right eye was subsequently removed to facilitate the crossed retinal axons to terminate in the left SC. Animals were returned to the nest and sacrificed at later ages.

HRP Eye Injection and Retinotectal Axon Tracing:

HRP was injected into the remaining eye (always the left eye) 18 to

24 hours before the animals were sacrificed. Lesioned animals at the ages of P12 and P14 were used for labeling of regenerating retinal axons. In each case, one to three microliters of 40-50% HRP (Sigma) made in 2% dimethyl sulfoxide (DMSO, Fisher Scientific) were injected into the left eye through a micropipette after the animals were fully anesthetized with Chloropent. Animals were perfused as described above. Sections from these brains were cut on a cryomicrotome and collected alternately into three groups. One group was used for HRP-filled axon labelling. The other two groups were used for vimentin and GFAP labelling. Sections with HRP-filled axons were incubated with tetramethyl benzidine (TMB, Sigma) as described in other publications (Jhaveri et al., 1988). They were then mounted onto microscope slides, counter-stained with neutral red, and coverslipped after alcohol dehydration and xylene clearing.

Immunohistochemistry:

Sections adjacent to those reacted for HRP were immunostained to observe their glial environment in the midline and were then analyzed together with the HRP-labeled regenerating retinal axons. Monoclonal antibodies against GFAP and against vimentin were purchased from Boehringer. Each was used at a dilution of 1:30 in phosphate buffered saline (PBS) with 0.1% normal horse serum (Sigma), 0.3% Triton X-100

and 0.2% sodium azide.

Sections were rinsed in PBS and the non-specific binding blocked in PBS containing 20% normal horse serum. They were next incubated in primary antibody for 18 to 24 hours on a rocker at room temperature (or for 96 hours at 4°C). They were then rinsed in PBS and incubated in biotinylated horse anti-mouse IgG solution (1:200 dilution) for one hour at room temperature. The tissue was rinsed in PBS again and placed in an avidin-biotin-HRP complex solution (Vector Labs, ABC kit) for an hour. Finally, the HRP was visualized using DAB as the chromagen. Control sections were processed according to the same protocol except that the primary antibody was omitted in the first incubation. Sections were mounted on subbed slides, dried overnight, cleared in xylene and coverslipped.

DiI Tracing of Radial Glia in the Lesioned Brain:

Animals were fixed by transcardial perfusion with the same paraformaldehyde and followed by at least two days of postfix. Brains were dissected out of the skull before postfix. To label radial glia in the tectal area, a small crystal of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) was placed onto the lining of the dorsal side of the ventricle at the cerebral aqueduct of Sylvius level. Brains were

then returned to the fixative and kept at room temperature for four to ten days. 100μm thick transverse sections were cut on a vibratome. Sections were immediately collected in 0.1 M phosphate buffer, mounted onto microscope slides and coverslipped. DiI labeling of the tissue was observed and photoed with a Nikon epifluorescence microscope using a rhodamine filter.

## RESULTS

### 1. Changes of Glial Environment After Lesion

In control animals the majority of retinal axons from the left eye crossed to the contralateral side at the optic chiasm. Some of them terminated in the right lateral geniculate body (LGB) while others continued growing caudally and terminated in the right SC. Retinal axons start to arrive at SCs on E13.5. Animals are born on E16, which is also counted as P0. At P1 almost the entire superior colliculi are occupied by retinal axons. The adult-like terminal arbors appear in the SCs between P10 and P12 (Frost et al., 1979). After heat-lesion to the superficial gray of the right SC and damage to the midline at P1, the severed retinal axons from the left eye regenerated and regrew acrossed the midline at the tectum to terminate in the left SC. To understand the neuronal-glial interactions involved during this regeneration and the abnormal growth

of retinal axons, the changes of the glial envionment after lesion are studied through immunohistochemistry.

Since pups were lesioned on P1 and sacrificed on P12 or P14, normal glial distribution in the tectum was examined at these ages as control. On P1 anti-vimentin antibody was found to stain radial cells both in SC parenchyma and between the two SCs, at the midline. These radial cells all had ventral attachment lining the ventricular zone and dorsal attachment buried in the pia (Fig.1A). There were morphological differences between the radial glia in the SC parenchyma and radial glia in the midline. The radial glia in the SC parenchyma were orderly spaced between each other (Fig.1B) while those in the midline were tightly packed together, forming a special "septum" structure dividing the two SCs (Fig.1C). Further, radial glia in the midline were stained by antibody against GFAP but those in the SC parenchyma were not (Fig.1D). On P12 and P14, rarely any radial glia in the SCs were stained by either antibody against vimentin or antibody against GFAP (Fig.2). Nonetheless, the radial glia in the midline were still positive for both vimentin and GFAP staining and maintained structural integrity. This staining thus demonstrates that a glial partition exists in the early age at the tectal midline and remains at least till the adult-like terminal arbors form in the SCs.

When the brains were fixed 18 hours after unilateral tectal ablation and transversely sectioned at the SC level for immunostaining with either anti-vimentin or anti-GFAP antibodies, two changes were observed compared to the normals. First, the vimentin- and GFAP-positive midline cell processes were found to have changed their shape and deviated from their normal locations (Figs.3A,3B). As early as 18 hours after surgery, these processes were observed to loosen up below the lesion and started to withdraw from the damaged pial surface of the SC (Fig.3C). Second, a new type of glia appeared in the area around the lesion 18 hours after surgery, seen with GFAP staining (Fig.3D). These glial cells and their processes were not arranged in an orderly way but appeared to be randomly aggregated to surround the lesion site. Their morphology matched those of reactive astrocytes (Hajós and Bascó, 1984).

In animals surviving beyond a week after the lesion, some of the normally straight dorsal-ventrally oriented midline glial processes were seen to curve along the edge of necrotic tissue towards the left, intact SC (Figs. 4, 5). As a result, a blank area, devoid of immunostaining, appeared to exist between the pial surface and the remaining portion of the glial processes at the midline. This area was termed an immunostaining "gap". The gap only appeared when the midline area was

sufficiently lesioned. In the very rostral and very caudal tectum where the heat sometimes did not reach during the surgery, the midline glial processes usually maintained their pial attachment.

2. Where Do Retinal Axons Cross The Lesioned Tectal Midline Abnormally?

HRP injected into the left eye was picked up by retinal ganglion cells. It was then transported anterogradely to the terminals of the axons within 24 hours. After reacting with tetramethyl benzidine (TMB), the trajectory and terminations of these axons could be traced in the tectum when the brain was transversely sectioned. In the normal brain, no HRP filled axons were found to cross the midline at SC (Fig.4A). Labelled axons in the SC formed a dense pack in the superficial gray up to the midline.

When HRP was injected into the left eye of lesioned animals at P11, it was transported to the terminals of the re-crossed retinal axons in the left SC. Eighteen to 24 hours after injection, the animals were perfused and the brains were transversely sectioned. Serial sections were collected alternately into three groups and were either reacted for HRP visualization or immunostained with antibodies against GFAP or vimentin. It was observed that after unilateral tectal lesion numerous

HRP-filled axons had regenerated and re-crossed the midline and grew into the intact left SC (Fig.4B). Retinal axons crossed the midline via two routes: one was above the damaged tissue along a tissue bridge. Retinal axons grew along this route were usually in fascicles. The other route was below the damaged tissue. Axons in this route grew across the midline in a more diffuse pattern. Both routes allowed the re-crossing axons to grow into the left SC. When sections showing the re-crossing axons were compared with adjacent sections immunostained with the two antibodies, it was seen that re-crossing retinal axons crossed the midline through the newly created immunostaining "gap" only (Figs. 4, 5). If, at certain areas along the rostral-caudal axis some midline radial glial processes escaped the heat damage and the immunostained processes could be followed up to the pia, retinal axons stayed within the small remnant of the right SC without crossing. On the other hand, axons were not always observed to pass the gap where the midline processes were damaged and withdrew. Instead, whenever the re-crossing axons were observed crossing the midline, the midline vimentin- and GFAP-positive glial processes were found to be absent from the pia.

3. DiI Labeling of Midline Radial Glia in the Lesioned Brain

Both vimentin and GFAP are intermediate filaments which take

part in forming cytoskeletons of radial glia. To confirm the observation from immunostaining that the midline radial glia indeed withdrew the processes from pia after the heat-lesion, instead of merely a lack of staining in the "gap" area, midline radial glia in animals with unilateral tectal lesion were labeled with DiI from the ventricle.

A crystal was placed onto the lining of the ventricle after the lesioned animals were sacrificed at P9 and P12. When brain sections from these animals were examined, the midline radial glia labeled by DiI were found to be similar to those stained by anti-vimentin antibody or anti-GFAP antibody (Fig. 6). A "gap" was also seen between the damaged pia and the withdrawn midline processes. Since the fine cellular structures were seen with DiI labeling, the end of the midline glial processes below the necrotic tissue was clear, as they were marked by a congregation of endfeet-like enlargements. The curving of the processes next to the lesion towards the intact SC were also observed (Fig.6).

4. How Retinal Axons Cross the Midline

The upper route: As was reported earlier, retinal axons crossed the midline in the tectum after unilateral tectal lesion along a tissue bridge in the regenerated pia above the necrotic tissue. When the lesioned brain was immunostained for GFAP, it appeared that the tissue bridge was filled

by GFAP-positive glial cells. These glial cells were spaced in an orderly fashion from one another and were oriented perpendicular to the long axis of the tissue bridge (Fig.7). Therefore axons might have grown along the tissue bridge at a right angle to the glial processes, just as what happened during the normal elongation period of retinotectal axons in the optic tectum -- the direction of incoming axons were perpendicular to the radial glial processes distributed in the SC parenchyma.

The lower route: A previous study (So, 1979) reported that there was rarely, if any, successful crossing of the tectal midline by regenerating retinal axons ventral to the necrotic tissue. This was not so in the present study. With HRP labeling of the regenerating axons, substantial successful crossing was observed through the area below the lesion through the immunostaining "gap" (Figs. 4, 7). The discrepancy might have arisen from the fact that in the early report, some different tracing methods were used. In contrast to the upper route, the crossing axons were rarely fasciculated into a single narrow tract, but in a more or less diffuse pattern. It appeared likely that no tissue bridge formed as specific substrate pathway on which retinal axons grew across the midline. If this is true, then a tissue bridge is not necessary for the crossing of retinal axons after unilateral tectal ablation.

## DISCUSSION

1. Radial glia have been demonstrated to guide postmitotic neurons in the developing monkey brain (Rakic, 1971; Levitt and Rakic, 1980). However, this seems not to be the role performed by radial glia described in the hamster SC after birth. For example, in the hamster optic tectum, neurogenesis peaks at around E12 and ends at E13 (Crossland and Uchwat, 1982). If the newly generated neurons complete their migration to their final laminae in the SC in two to four days, as is seen in mice (Edwards et al., 1986a), the neurons finish their migration towards the superficial layer of the tectum around E16, which is the date of birth. Therefore, the existence of well developed radial glial processes and midline structure after birth may not only serve the purpose of guiding neuronal migration. On the other hand, retinal axons grow into the SC with a preformed retinotopic order (Frost et al., 1979; Thanos et al., 1984). Since this ordered distribution of axonal terminals is established before the eye opening, cues provided by the target environment must have played an important role in guiding axons to topographically appropriate sites.

2. The glial environment has been shown to be closely related to axonal growth. For instance, Rutishauser and Silver have reported that retinal axons grow along a preformed adhesive pathway on

neuroepithelial endfeet (Silver and Rutishauser, 1984). Vanselow and colleagues also find that retinal axon growth cones growing into chick optic tectum have very close relationships with radial glia endfeet (Vanselow et al., 1989). In Syrian hamsters, retinofugal axons emerge from the eyes at around E11. The first retinotectal axons reach SC at E13.5 (So et al., 1977; Jhaveri et al., 1983). After a waiting period, the majority of the retinal axons start their arborization in the contralateral SC at around P3 and form adult-like terminal arbors by P14 (Frost et al., 1979; Schneider et al., 1985). Although we do not know exactly when synaptogenesis in SC starts and ends, work on rats suggests that the majority of retinal terminals form their synapses in rat SCs before P12 (Lund and Lund, 1972). Naturally, considering the distribution and differentiation of radial glia in hamster optic tectum at the neonatal period, the glial environment is more likely to have a role in influencing or dictating axonal growth than in neuronal migration.

3. The immunohistochemistry of midline cells and their processes is intriguing because of their unique and constant expression of both GFAP and vimentin during the early postnatal period. This dual expression indicates that the midline glial cells and their processes comprise a unique glial population. What are the possible functions of these cells and processes? Earlier studies, by Raedler and colleagues

(Raedler et al., 1982), find that in the rat SC area, a group of ventricular midline cells, mostly generated during E12 through E15, has a unique morphology. They have short and broad ventral processes and long and thin dorsal processes which can be followed up to the pial basal lamina. Lately, the Cavalcante group also find that there is a glial midline structure that is distinctive in its immunohistochemistry and morphology in the developing opossum SC (Barradas et al., 1989). In these studies it is suggested that the midline glial structure may have a role of mechanical support for the young tectum or provide guidance for migrating neurons in the SC. In our experiment, a unilateral tectal lesion with the destruction of this midline tissue in newborn hamsters which also have one eye removed results in the recrossing of the midline by axons from the remaining retina (as expected from previous work). Identical procedures without midline damage does not result in retinal axon crossing. Therefore, the midline glia has likely participated in some sort of barrier for the growing retinal axons in the normal SC. If this is true, there must have been a form of compartmentalization which prevented the axons from growing into the wrong side of the brain during development. Through this mechanism the laterality of the sensory input is established and maintained to ensure the appropriate functions in animals' survival.

4. If one side of the tectum is heat-ablated in the hamster brain between P0 and P3, the retinal axons grow across the tectal midline and terminate in the intact SC. Meanwhile, if the left eyes of P12 and P14 animals are injected with HRP 18 to 24 hours before sacrifice, HRP is transported anterogradely to the fibers and terminals of the crossed and uncrossed retinal axons in the tectum. These axons can then be traced along their entire trajectories. In such cases, numerous HRP-filled axons are observed to cross the midline via two routes. One is above the damaged tissue along a newly formed tissue bridge, as is described in detail in papers published earlier by this laboratory. The other is below the damaged tissue through the immunostaining "gap" between the necrotic tissue and the withdrawn midline glial processes. Both routes allow the crossing axons to grow into the left SC.

Because retinal axons are seen to prefer growing into the left SC instead of non-target areas for the visual system either on the lesioned side or on the intact side, there must be some kind of recognition mechanism or chemotropic mechanism involved during the abnormal crossing. Also, retinotectal axons are not always observed to grow through the gap at all levels where the midline processes are disrupted or withdraw. Along the rostral-caudal axis, the beginning of the gap and the end of the gap usually does not have crossing axons. However, whenever retinal axons are

observed to cross the midline, the midline vimentin- and GFAP-positive glial processes are always found to have withdrawn and a gap exists. This observation is confirmed through DiI labeling of midline radial glia. Evidence like these indicated that the damage to the midline is permissive but not inductive for the retinal axon crossing.

5. Recently, Snow and Silver reported that, in the rat, the spinal cord roof plate is formed by a special group of glial cells which acts as a barrier to axonal growth. With antibody labelling, they find these cells contained KSPG on their membrane. Since KSPG is a glycosaminoglycan that repels growing axons *in vitro* (Snow et al., 1990b), Snow and Silver speculated that KSPG sequestered by the midline cells results in a molecular barrier to repel or inhibit the growth cone of axons in the spinal cord. During collaboration with this laboratory, KSPG was also found to be expressed in the neonatal hamster SC midline area. Furthermore, preliminary data shows that KSPG disappears from the area where the midline glia are damaged in the unilateral tectal lesion. Regrowing retinal axons crossed the midline after the lesion from where KSPG is missing (personal communication).

6. If there is a barrier at the midline participated by the midline glial cell processes, what kind of barrier is it? Anatomically, there are intertectal axons between the two colliculi. It is therefore possible that the

midline barrier is selective. To some of the axons, the crossing is permitted. To others, it is not. It is reported that in the developing ferret auditory system, a similar barrier in the midline is observed, but the growing axons from the midline trapezoid body use very fine glial processes perpendicular to the midline as a guide or permissive mechanism in crossing the midline (Brunso-Bechtold et al., 1988). How such permissive and non-permissive contacts are regulated, and the nature of the interactions between the midline glial cells and retinal axons in the development of retinotectal projection, remain important questions to be addressed through future research.

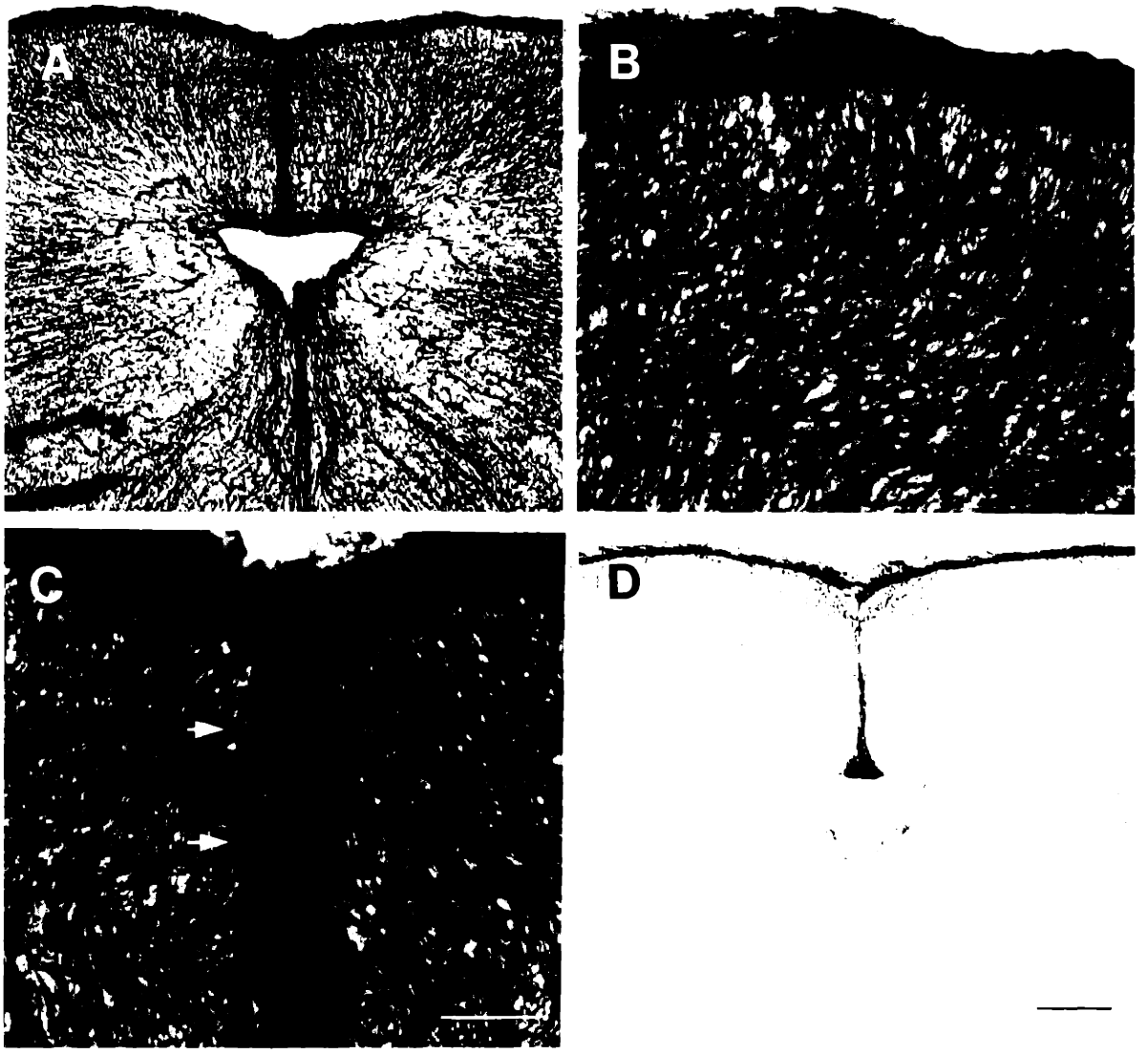

A
B
C
D

Figure 1: Immunostaining of P1 brain with antibodies against vimentin (A, B, C) and GFAP (D). Two vimentin-positive populations are seen in the SC. The first population is made up of lateral radial glial cells evenly distributed in the entire SC (B). The second population consists of a group of midline radial glia tightly packed together (C). They are also GFAP-positive (D). Bar: (A, D) 200μm; (B, C) 50μm.


B

Figure 2: Immunostaining of P12 brain with antibodies against vimentin (A) and GFAP (B). Lateral radial glial cells are no longer observed with anti-vimentin staining (A) but midline radial glia are still vimentin- and GFAP-positive and maintain their integrity (A,B).
Bar: 200μm.

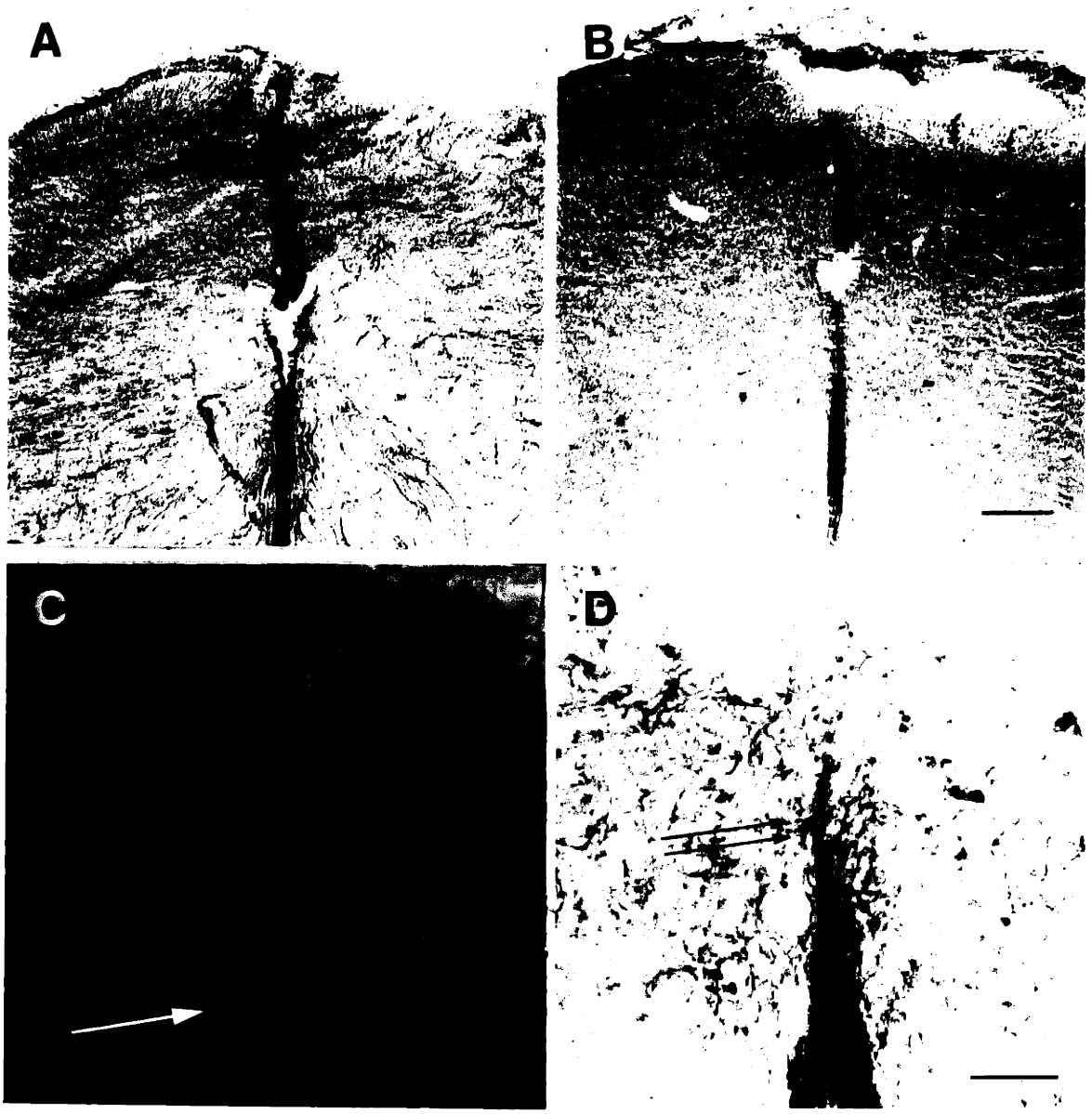

A
B
C
D

Figure 3: Withdrawal of midline glial processes 18 hours after heat lesion to the right tectum: vimentin staining (A, C) and GFAP staining (B,D). The end of withdrawn midline processes are shown (arrow). Bar: (A, B) 200μm; (C, D) 50μm.

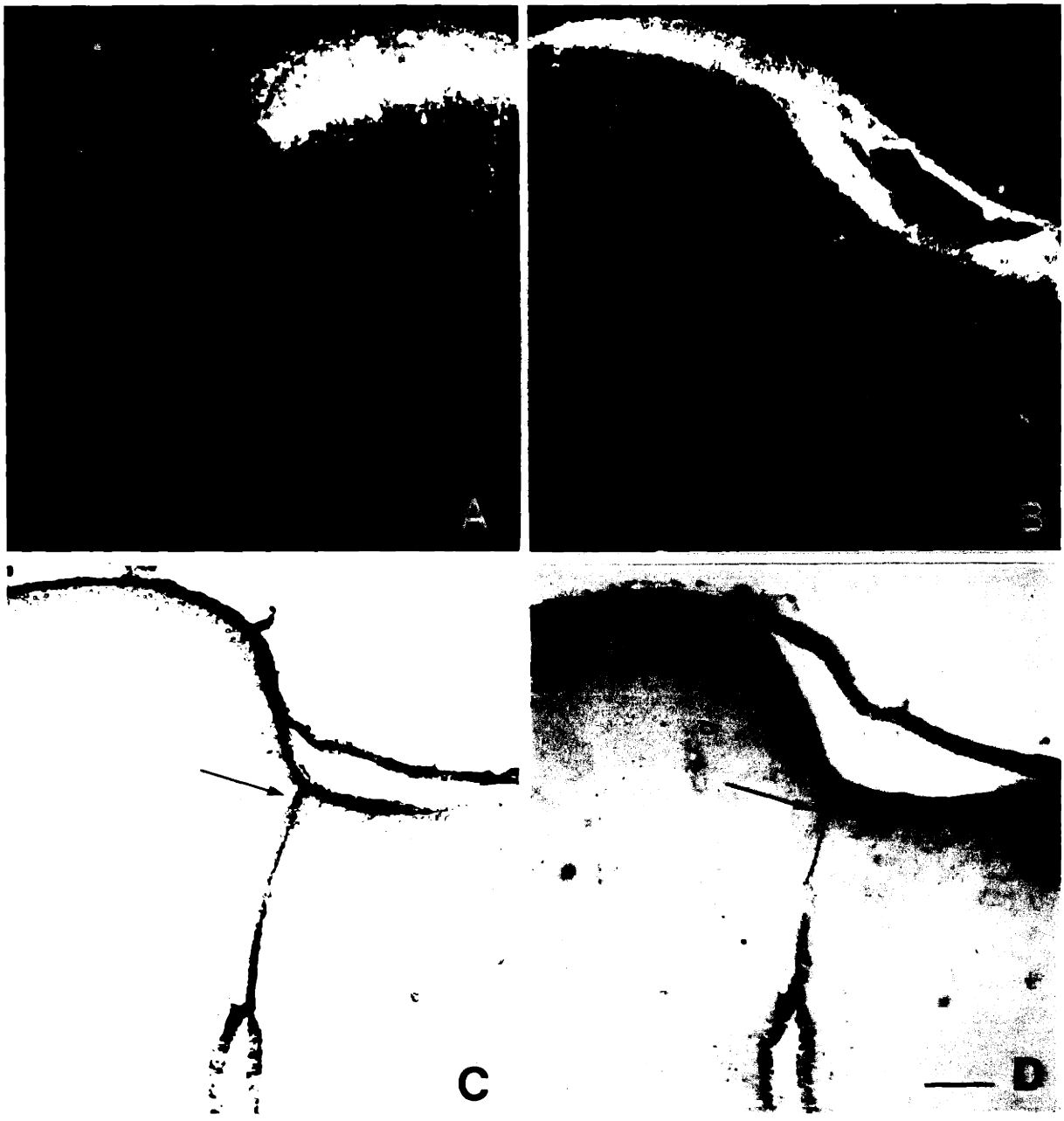

A
B
C
D

Figure 4: HRP-labeled retinal axons in P8 normal brain (A) and P12 unilateral tectal ablated brain (B). Adjacent sections of lesioned brain are immunostained for vimentin (C) and GFAP (D). The ends of withdrawn midline glial processes are shown (arrow). The stain-free area is shown in detail in the next figure. Bar: 200μm.

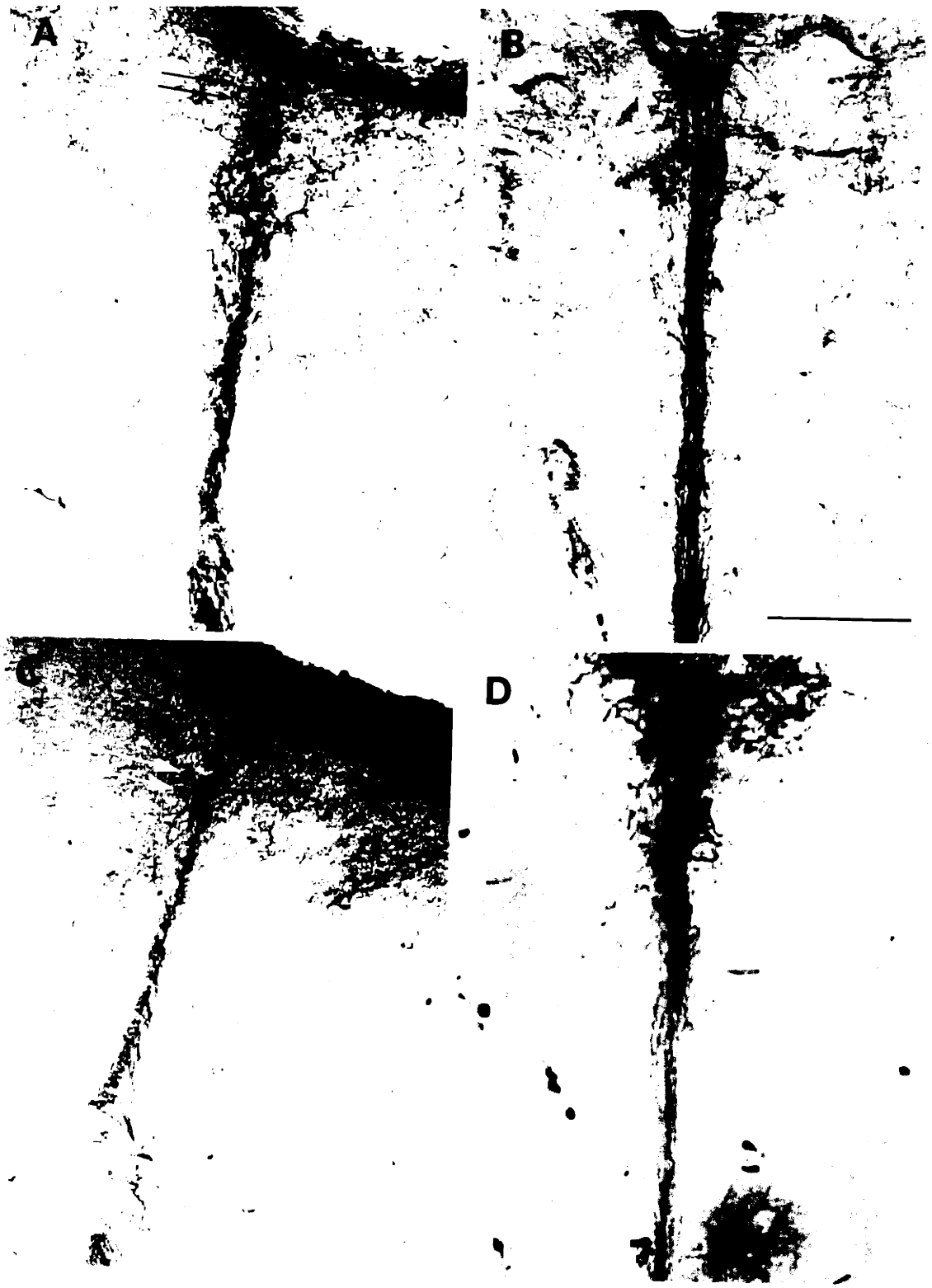

A
B
C
D

Figure 5: Higher magnification of vimentin staining (A) and GFAP staining (C) of previous figure compared with vimentin staining (B) and GFAP staining (D) in the normal brain. The enlarged end of withdrawn vimentin-positive midline processes (arrows in A) can be seen below the lesion. Similar results are seen with GFAP staining (arrows in C).
Bar: 100μm.

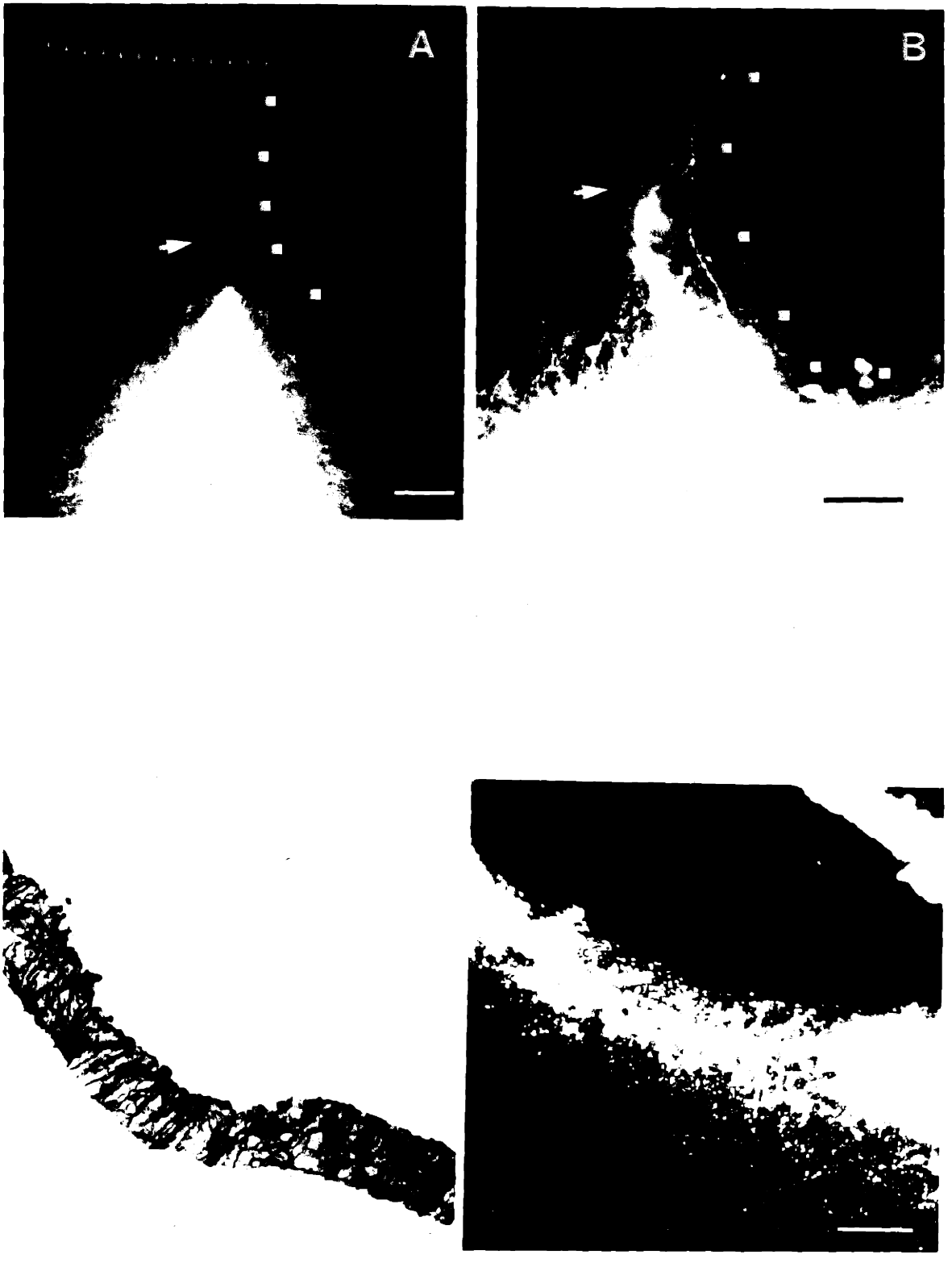

A
B

Figure 6: DiI-labeled midline processes at P9 after unilateral tectal ablation. The pial surface (small arrows) and the lesioned area is demarcated (squares). The ends of midline glial processes is shown (large arrow). Bar: (A) 200μm; (B) 50μm.

Figure 7: GFAP-positive glial processes lined the tissue bridge (left). The diffused pattern of crossing retinal axons is labeled with HRP in the lower route (right). Bar: 50μm.

# CHAPTER II

## The Glial Environment and Its Changes in the Developing Superior Colliculus

### INTRODUCTION

Vertebrate nervous systems are mainly composed of two types of cells, neuronal cells and glial cells. Both cells originate from the neuroepithelial wall of the neurotube during neurogenesis and gliogenesis in early development. Later there is also secondary gliogenesis in so-called nongerminal sites, as in the developing rodent forebrain and cerebellum (Hajós and Bascó, 1984) or in the adult optic nerve (Noble, 1990). Glial cells are subdivided into different types according to their birth date, distribution, immunocytochemistry, morphology, and function (Choi and Lapham, 1978; Choi, 1981; Levitt and Rakic, 1980; Ghandour et al., 1983; Bovolenta et al., 1984; Misson et al., 1988; Raff, 1989; Skoff, 1990). Two intermediate filaments, the glial fibrillary acidic protein (GFAP) and vimentin, are commonly detected in glial cells in both developing and adult mammalian central nervous systems (CNS).

During development of the nervous system, either GFAP or vimentin is sometimes localized in a particular group of glial cells whereas at other times both are found in the same cell (Sharp et al., 1982;

Wang et al., 1984). It is believed that immature vimentin-expressing glial cells differentiate into mature, GFAP-expressing astrocytes and/or myelin-forming oligodendrocytes during the late stages of development because it has been shown that 1) in some cases a decrease of vimentin expression in the developing brain is coupled with an increase in GFAP expression (Dahl, 1981), and that 2) there are "transitional" cells with cytological and immunohistochemical features intermediate between those of radial glia and astrocytes (Pixley and De Vellis, 1984; Hirano and Goldman, 1988), and between those of astrocytes and oligodendrocytes (Choi et al., 1983a,b). Such transitions have been clearly demonstrated in *in vitro* experiments (Fedoroff et al., 1983; Ogawa et al., 1985).

Neuroglia played important roles in neuronal cell migration and in directing axonal growth (Rakic, 1971; Silver et al., 1982). With GFAP immunostaining, Rakic and Levitt observed that glia and neurons were present concomitantly during neurogenesis and that the radial glia themselves later transformed into astroglia after completion of neuronal migration (Levitt and Rakic, 1980; Levitt et al., 1981). Glial processes were also shown to be involved in axon development and pattern formation. Double labeling with GFAP and neurofilament immunostaining revealed that in rodents, subpial axons were closely intermingled with astroglial processes which were well organized into a

"palisading pattern" (Bitner et al., 1987). Bonhoeffer's group reported that in the developing chick optic tectum, there also exists a palisading pattern of radial glia similar to that reported in the rodent brain and that retinal axons coursed through the area densely packed with highly organized radial glia which were oriented perpendicularly to the direction of axonal ingrowth (Vanselow et al., 1989).

Besides the axon inductive and promotive effect, much evidence from both *in vivo* and *in vitro* studies had also suggested a certain inhibitory or repulsive effect of glial cells to axon growth. Glia with such properties might direct axonal growth cones by so-called "negative guidance" (Schwab, 1990). For example, Silver and colleagues reported that, in mice and rats, the upper wall of the distal half of the primitive eye stalk was formed by a group of glia which transiently became pigmented prior to and during the passing of pioneer optic axons. It was observed that all the outgrowing axons avoided this melanotic tissue and preferred the pigment-free area (Silver and Sidman, 1980; Silver and Sapiro, 1981). In another study, Silver reported that a knotlike structure of glia was found at the mouse optic chiasm (Silver, 1984). It was suggested that the mechanism of the glial guidance was, at least to some extent, a blocking effect of the glial knot in preventing the ventronasal axons from growing rostrally. In other words, the glial cells served as a barrier to the

ventronasal axons. Recently, evidence from Mason's laboratory indicated that along the midline of the chiasm, crossed and uncrossed retinal axons responded to midline cues differently. The uncrossed axons seemed to have encountered some substrates which were not permissive to their growth (Godement et al., 1990). With antibody labeling, Mason and colleagues revealed a group of radial glia in the midline of the chiasm. They suggested that there might be a recognition mechanism between retinal axons and glial cells by which uncrossed axons were pushed to stay on the side ipsilateral to the eye of origin (Mason et al., 1990; personal communication).

In the present study, radial glia in the hamster superior colliculus were observed to change their distribution and morphology dramatically with development and these changes were correlated in time with target approaching and target invasion of the retinotectal axons in the optic tectum. The results also indicated that certain populations of glial cells might have a role in forming a midline glial barrier to help establish the laterality of retinal axon projection at the embryonic stage and of retinocollicular end-arbor elaboration at the postnatal stage.

## MATERIALS AND METHODS

Syrian hamster pups of various ages were bred in our colony or

purchased from the Charles River Laboratory (Wilmington, Massachusetts). For embryonic brain tissue, fetuses from E10.5 to E14 were fixed by immersion for 24 hours in 4% paraformaldehyde made in 0.1 M phosphate buffered saline, pH 7.4. For postnatal study, pups of ages between P0 and P14 were sacrificed using an overdose of Nembutal and perfused transcardially with an initial rinse of 0.9% saline containing 0.25% sodium nitrite followed by 4% paraformaldehyde made up in 0.1 M phosphate buffered saline (pH 7.4). Brains were dissected out and post-fixed in buffered 4% paraformaldehyde for three to five hours, cryoprotected in buffered 30% sucrose and sectioned at a thickness of 40 micrometers in the transverse plane using a cryomicrotome. Sections were collected in phosphate buffer containing 0.1% sodium azide.

Immunohistochemistry:

Monoclonal antibodies to GFAP and to vimentin were purchased from Boehringer and each used at a dilution of 1:30 in phosphate buffered saline (PBS) with 0.1% normal horse serum (Sigma), 0.3% Triton X 100 and 0.2% sodium azide.

Free-floating sections were rinsed in PBS and the non-specific binding blocked in PBS containing 20% normal horse serum. They were next incubated in primary antibody for 18 to 24 hours on a rocker at

room temperature (or for 96 hours at 4°C), rinsed in PBS and incubated in biotinylated horse anti-mouse IgG (1:200 dilution) for one hour at room temperature. The tissue was rinsed in PBS and placed in an avidin-biotin-HRP complex (Vector Labs, ABC kit). Finally, the HRP was visualized using DAB as the chromagen. Control sections were processed according to the same protocol except that the primary antibody was omitted in the first incubation. Sections were mounted on subbed slides, dried overnight, cleared in xylen,e and coverslipped.

DiI Tracing of Radial Glia Development:

Embryonic brains of various ages were fixed by immersion of fetuses in 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) for at least two days. Postnatal brains were fixed by transcardial perfusion with the same paraformaldehyde followed by at least two days of postfix. Brains were dissected out of the skull either after (for embryonic tracing) or before (for postnatal tracing) postfix. To label radial glia in the tectal area, a small crystal of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) was placed onto the lining of the dorsal side of the ventricle at the level of the cerebral aqueduct of Sylvius. Depending on the age, brains were then returned to the fixative and kept at room temperature for four to ten days. Brains were embedded with

agarrose before sectioning to preserve tissue integrity. 100μm thick transverse sections were cut on a vibratome. They were immediately collected in 0.1 M phosphate buffer, mounted onto microscope slides and coverslipped. Labeling of the tissue was observed and photographed with a Nikon epifluorescence microscope using a rhodamine filter.

## RESULTS

### 1. Radial Glia in the Embryonic SC

Two distinctive populations of radial glia were stained by anti-vimentin antibody during the embryonic stage. The first population was observed as early as E10.5 in the still primitive SC. With thick ventral attachments lining the ventricle and bulky endfeet in the pial membrane, the radial processes fanned out from the ventricular zone to the pia through the entire tectum (Figs. 1A, 1B). These radial glia were called "lateral radial glia" to contrast radial glia only exist in the midline at later ages. The second population was observed at E13 along the midline of the tectum. Initially, a vimentin staining-blank strip appeared at the tectal midline dorsal-ventrally around E11.5 (Figs. 1C, 1D) and E12 (Figs. 1E, 1F). Some staining of a few fibers were still seen within the strip close to the pia at around E12, but the rest of the blank strip in the midline area was completely vimentin-negative. Finally the midline

radial processes intensely stained for vimentin suddenly appeared in the whole midline area at E13 (Figs. 1G, 1H). By this time the radial glia in the midline had developed a morphology distinctly different from lateral radial glia: their long processes were straight and densely packed; they were much more intensely stained for vimentin than SC radial glia; and they were dorsoventrally oriented, only occupying the tectal midline. Therefore, they were called "midline radial glia". There was still some vimentin-negative areas immediately above the ventricle, below the newly formed vimentin-positive midline structure. These areas became vimentin-positive one day later at E14 (Fig. 2).

Both populations of radial glia had thick ventricular attachments lining the ventricle and enlarged endfeet embedded in the pia. However, at E13 and later, the morphological differences between the midline radial glia and lateral radial glia became obvious. The midline radial glia had longer, thicker ventral processes arising from either side of the tectum forming a dome- or bulb-like structure in the subventricular zone (Figs. 1G, 1H, 2). Their processes were tightly packed, elongating from the top of the dome structure to the midline pia. On the other hand, the lateral radial glia appeared as equally spaced slender strands spanning the entire thickness of the SC with smaller ventral processes lining the ventricle.

When radial glia of the embryonic SC were labeled with DiI by placing the crystal in the ventricle, the morphological features of these glia were observed to be similar to those revealed by vimentin-staining. In addition, cellular structures not expressing vimentin antigen were observed in the midline area around E12 (data not shown). Since these radial fibers were vimentin-negative at this age, it was likely that the midline radial glia were generated around E11 through E13. The cells originated in the ventricular zone and sent out processes dorsally, squeezing into the midline area originally occupied by the lateral radial glial processes. That these cells at this stage were vimentin-negative might reflect the fact that they were less differentiated. Presumably they then differentiate into intensely vimentin-expressing midline radial glia at around E13. From E14 the midline glial structure became similar to what was observed in postnatal brains.

Since both populations were similar in their immunohistochemistry and morphology to the radial glia in other developing systems (Levitt and Rakic, 1980; Hirano and Goldman, 1988; Misson et al.,1988; Vanselow et al., 1989), they were both classified here as radial glia. Nevertheless, due to the differences in their morphology, distribution, and intensity of vimentin expression, there were definitely two distinguishable populations. This was proven to be true when they were labeled by

different antibodies at later ages (see below).

2. Postnatal Changes of Lateral Radial Glia

From P0 to P3, vimentin-positive radial processes spanned the entire SCs of both sides, extending from the luminal surface of the ventricle to the pia (Fig. 3). Although the SCs on both sides had grown much thicker dorsal-ventrally at P3, morphologically these radial glia were quite similar to what they were at birth (Fig. 3).

By P5, the density as well as the staining intensity of the radial processes started to decrease. These decreases became obvious at P7 and P9 (Fig. 3). Also on P9, vimentin-positive astrocyte-like cells were first observed scattered in the tectum (Fig. 3, arrows). On P12 there were very few, if any, vimentin-positive radial processes in the SC and by P14 these processes were completely absent (Fig. 3). This disappearance of immunolabelling of radial glia could be due to either the disappearance of radial glia themselves or the shutting off of vimentin expression in the remaining radial glia processes at this stage. It was also possible that both processes contributed to this phenomena. At present, the disappearance of radioglial processes had been confirmed by DiI labeling (see below).

During the entire first week of postnatal life, lateral radial glia were attached to the pial surface by vimentin-positive enlargements, or

endfeet. The endfeet were most densely distributed in the pia before P3 (Fig. 4A). As the density of radial processes decreased significantly between P5 and P9, so did the density of endfeet shown by immunostaining (Fig. 4B).

While lateral radial glia were stained by anti-vimentin antibody during this perinatal period, they were not stained by anti-GFAP antibody. This was in contrast with midline glia. In fact, from P0 to P14 midline glial cells and their processes were seen to express both GFAP and vimentin, as described below.

3. Postnatal Midline Radial Glia and Processes in the Tectum

Throughout the first two weeks, the midline radial glia were consistently exhibiting positive staining for vimentin (Fig. 5). These cells occupied the midline of the optic tectum throughout the rostrocaudal axis of the SC. The processes of these cells spanning the thickness of the midline actually arose from two groups of midline glia located closely next to each other, one group on each side, at the ventricular surface. In the rostral SC, these cell processes could be seen to form two tufts on either side of the midline (Fig. 6A), only joined together at the more dorsal portion. At more caudal levels, the two tufts joined each other in the subventricular zone, forming a single midline septum (Fig. 6B).

While the processes of both populations of radial glia remained GFAP-negative during the embryonic period, the midline radial glia were first observed to become GFAP-positive at around birth (E16 = postnatal day zero, P0). At P0, all the cell bodies were GFAP positive. But the processes close to the pia were still GFAP-negative (Fig. 5A). From P3, all the midline processes were GFAP-positive except for a few light or blank areas namely, disruptions of the staining, in the rostral tectum. When results from immunostaining for vimentin and for GFAP were compared, staining patterns, cell distribution, and cell morphology indicated that the two antibodies were labeling the same group of cells at the midline.

The midline glia also differed from lateral radial glia in their life span and in their temporal aspects of vimentin expression, especially during the first two postnatal weeks. As stated above, from P5, the density as well as the staining intensity of the lateral radial glia decreased. In contrast, the midline glia were consistently positive for both GFAP and vimentin at least until the end of the first two postnatal weeks (Fig. 5E). With thickening of the tectum during this period, the glial processes were stretched longer and thinner, but their integrity remained.

4. DiI Labeling of Radial Glia

DiI labeling of both embryonic radial glia and early postnatal (P0 to P3) radial glia showed a pattern very similar to what was observed when the brain was immunostained by vimentin antibody at each respective age. Nonetheless, there was an astonishing difference: at P5 and on with vimentin staining, the density of labeled radial fibers in the SC parenchyma was found to diminish, but what caused the decrease was not clear. When DiI was used to label the radial glia from the ventricle, a population of radial glial processes were observed to be detached from the pia (Fig. 7). When this happened, the endfeet of these radial glia were still apparent at the end of the long glial processes. But these endfeet were located in various levels of the SC parenchma on a transversely cut section, instead of buried in the pial membrane (Fig. 7). This pulling back of radial processes was more and more extensive with age. By P9, the radial glia DiI-labeled from the ventricle only extended their processes to a zone around the ventricle, leaving the majority of the SC empty. This was consistent with the time frame of the disappearance of vimentin-stained radial glia in SC parenchyma.

On the other hand, DiI labeled midline radial glia maintained their integrity during the same postnatal period when SC radial glia were withdrawing from the tectum. Their endfeet were observed to gather

together in the midline pia, as in the early stages. This result was also observed with GFAP- and vimentin-immunostaining of these cells and their processes during the same period.

## DISCUSSION

### 1. Radial Glia in the Perinatal SC

Through immunohistochemistry, two populations of radial cells are observed. The first population is positive to vimentin immunostaining only, distrbuted in SC parenchyma with space between each other and spanning the tectum between ventricle and pia. The other population shows a distinct morphology and intense vimentin labeling in the midline during the embryonic period and is positive for both GFAP and vimentin immunostaining during the postnatal period. This group of glia appears just before the arrival of the first retinal axons at the SCs and remains in the midline until after P14 when retinal axons have formed adult-like terminal arbors in the target area.

### 2. Correlation of Radial Glia Differentiation with Retinal Axon Growth

Retinal axons grow out of the eye at E11. They first arrive at SCs around E13.5. Retinal axons start arborizing and sorting in SCs after P3 and form adult-like terminal arbors at P10 to P12. The correlation of growth of retinal axons with the development of glial cells in SCs are

shown as in the following table:

| | retinal axons | radial glia in SCs |
|---|---|---|
| **E11** | • start growing out of eye | • evenly distributed throughout SCs |
| **E11.5** | • arrive at optic chiasm | • low intensity vimentin staining starts |
| **E13.5** | • just arrived at SC | • form midline glial structure |
| **P3-P5** | • arborizing and sorting | • lateral radial glia withdrawing |
| **P12** | • form adult-like terminal arbors | • lateral radial glia disappear<br>• midline radial glia persist |

Retinal axons grow to lateral geniculate body (LGB) and SC in a precise retinotopic order. Since this ordered distribution of axonal terminals is established before the eye opening, cues provided along the path and by the target environment play important roles in guiding axons to topographically appropriate sites. This has been shown in experiments using antibodies raised against cell adhesion molecules like NCAM (Silver and Rutishauser,1984), and N-cadherin (Matsunaga et al.,1988; Letourneau et al., 1990) or molecules suggested for substrate pathways

like laminin (Cohen et al., 1987; McLoon et al., 1988). In Syrian hamsters, the first retinal axons reach the optic tectum at around E13.5 (So et al., 1977; Jhaveri et al., 1983). After a waiting period, these axons start arborizing and invading the target area at P3 (Frost et al., 1979; Schneider et al., 1985). The adult-like terminals appear at around P14, when the eyes open in hamsters.

In the present study, the peak of vimentin expression by SC radial glia occurs before P3, namely, before extensive elaboration and focalization of the terminal arbors of retinal axons are evident morphologically. All of these vimentin expressing radial glia disappear before P14, which is the time the final terminal arbors form in the optic tectum and the eyes open (Frost et al.,1979). Furthermore, retinal axons lose the ability to regenerate around P3 to P5 (So et al., 1981; Schneider et al.,1985), which is the time when vimentin expressing radial glia start withdrawing and differentiating into astrocytes (or vimentin expression is shut off) and astrocytes start to appear. It is likely that the changes in immunocytochemistry and morphology of these cells indicate the changes in the glial maturity and the changes in the environment in which the retinal axons grow. If this is true, the interactions between the glial cells and retinal axons may have dictated not only the pathfinding and terminal formation of retinal axons, but also regeneration during this period.

3. The Midline Radial Glia

The observation of midline radial glia and their processes is intriguing because of their unique distribution, time course and immunocytochemistry: Their dual expression of GFAP and vimentin indicates that the midline cells and their processes comprise a population of glial cells distinctive from lateral radial glia. They form a midline structure and they persist in the SC longer than lateral radial glia. What are the possible functions of these cells and processes?

(1) In the unilateral tectal lesion case, retinal axons cross the tectal midline only when the medial SC is sufficiently damaged (So and Schneider, 1978; Schneider,1981). Furthermore, it has been reported that retinal axons also cross the midline if the entire midline tissue is damaged by a dorsal to ventral knife cut through the pia at the neonatal period (Hsiao and Schneider, 1978; Jen and Lund, 1979). Such evidence has prompted the hypothesis that certain cellular structures in the midline functioned as an axon barrier to establish and maintain laterality during retinotectal innervation.

(2) Two interesting questions are raised: a) whether the midline glial cell processes form a retinal axon-repulsive barrier during development and b) if so, what kind of barrier is it. The existence of

non-neuronal cell barriers is suggested by Silver and his colleagues that retinal axons grow out of the optic stalk through a pigment-free extracellular tunnel and avoid certain axon-repulsive areas laid out by pigmented neuroepithelial cells (Silver and Sidman, 1980; Silver and Sapiro, 1981). These retinal axons then follow a substrate pathway mediated by surface molecules of glial cells (Silver and Rutishauser, 1984). Also, at the chiasm, the retinal axons confront a knot-like glial formation that directs crossing fibers to grow contralaterally. These axons are then separated by extraneuronal cells and guided towards their target zones (Silver, 1984). A similar situation may occur in the SC midline: the midline radial glia and their processes form a barrier separating axons on the two sides -- retinal axons grow within the SCs on either side of the barrier but are not permitted to grow to the SC on the other side. During the unilateral tectal ablation only when these glial cells and their processes are damaged are the retinal axons permitted to cross the midline.

(3) If there is a barrier at the midline formed by midline cell processes, what kind of barrier is it? Anatomically, there are intertectal axons between the two colliculi. Actually, DiI labeled intertectal axons are seen crossing the midline at E14.5, passing through the early midline glial processes (unpublished data). Therefore it seems possible that the

midline barrier is selective, at least during the period intertectal axons are forming. To some of the axons, the crossing is permitted. To others, it is not. Such a selective barrier might involve cell-cell recognition and interaction through membrane and/or extracellular molecules. Some axon growth inhibitory molecules like keratan sulfate proteoglycan (KSPG) (Snow et al., 1990a,b) or tenascin (Bronner-Fraser, 1988) can be candidates of molecular mechanisms of such a barrier. In fact, KSPG is localized in the tectal midline of P3 hamsters (Snow et al., 1990a). It is important to know whether this and other molecules are related to midline radial glia and their functions during retinotectal pathway formation.

Correlations of this type are intrinsically insufficient. Whether and how the midline glia interact with retinotectal axons cannot be shown from such correlations: one cannot decide whether the midline glia and the retinal axons actually recognize each other. It is even harder to determine whether they react to each other upon contact or recognition., if any. Further experiments have been designed (experiments and results reported in the next chapter) to explore the possibility that midline radial glia are directly involved in barrier mechanisms and retinal axons are restrained by this barrier during elongation and arborization in the tectum. This restraning results in the correct laterality of retinal projections in the SC during normal development.

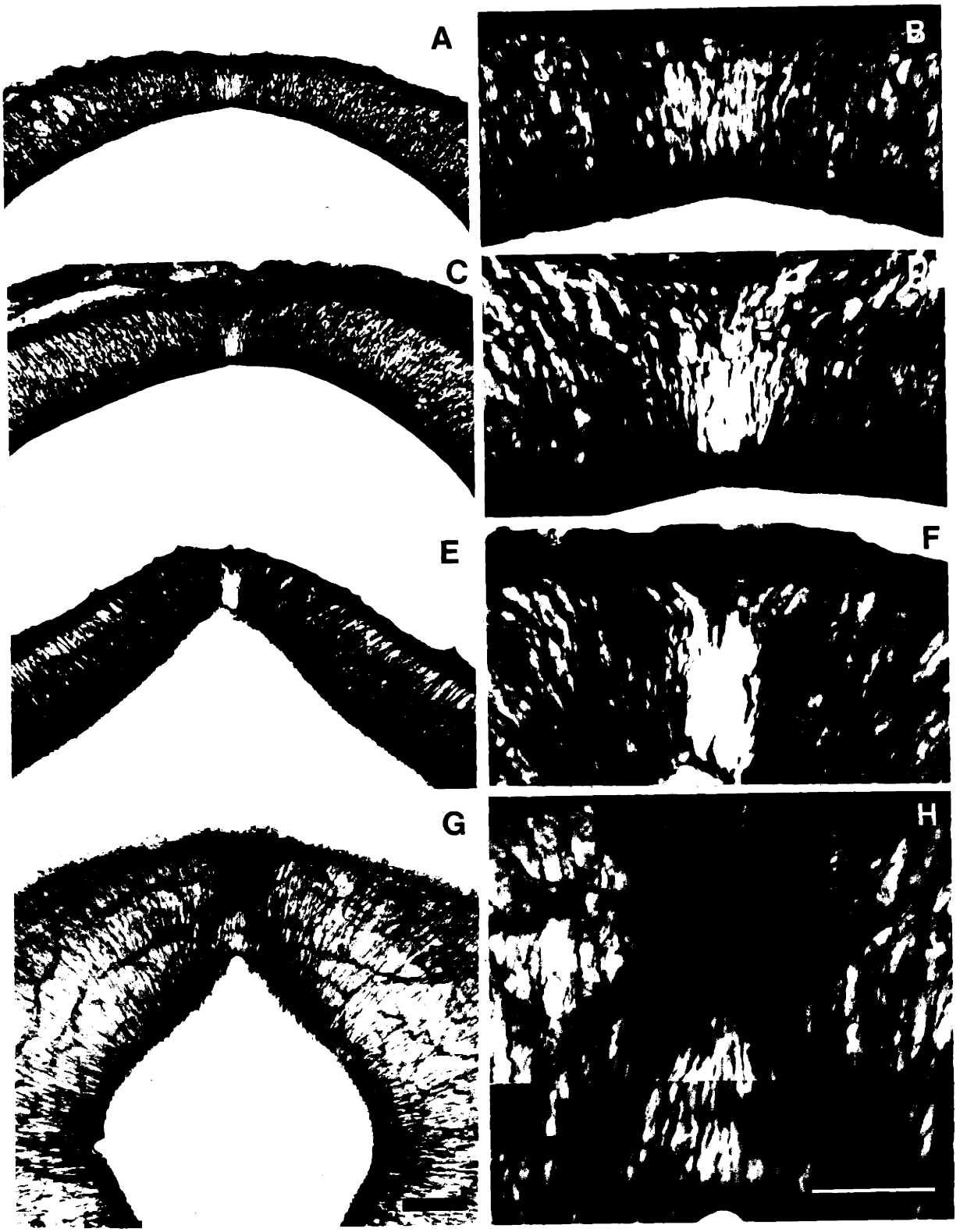

A
B
C
E
F
G
H

Figure 1: Vimentin staining of embryonic brains in the tectal area (A, C, E, G). Bar: 100μm. Higher magnification is on the right (B, D, F, H). Bar: 50μm.

(A, B) E10.5
(C, D) E11.5
(E, F) E12
(G, H) E13

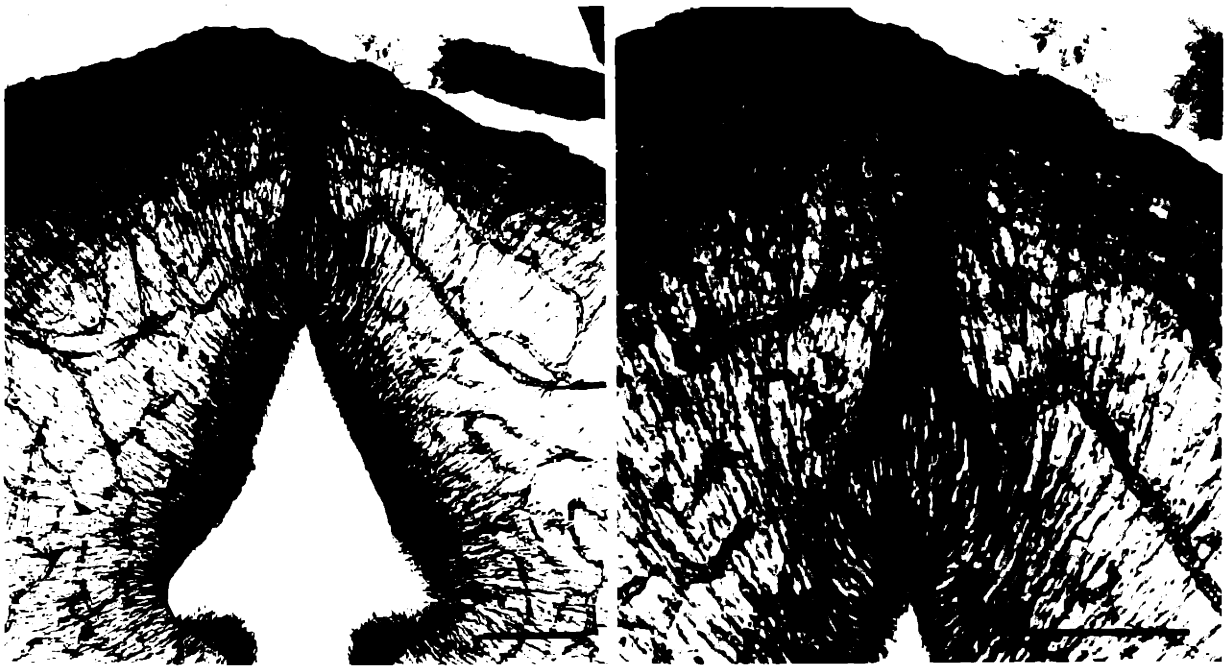


Figure 2: Vimentin staining of E14 tectum showing the largely established midline glial structure. Shown is the entire tectum (left) and the midline glial structure at higher magnification (right). Bar: 100µm.

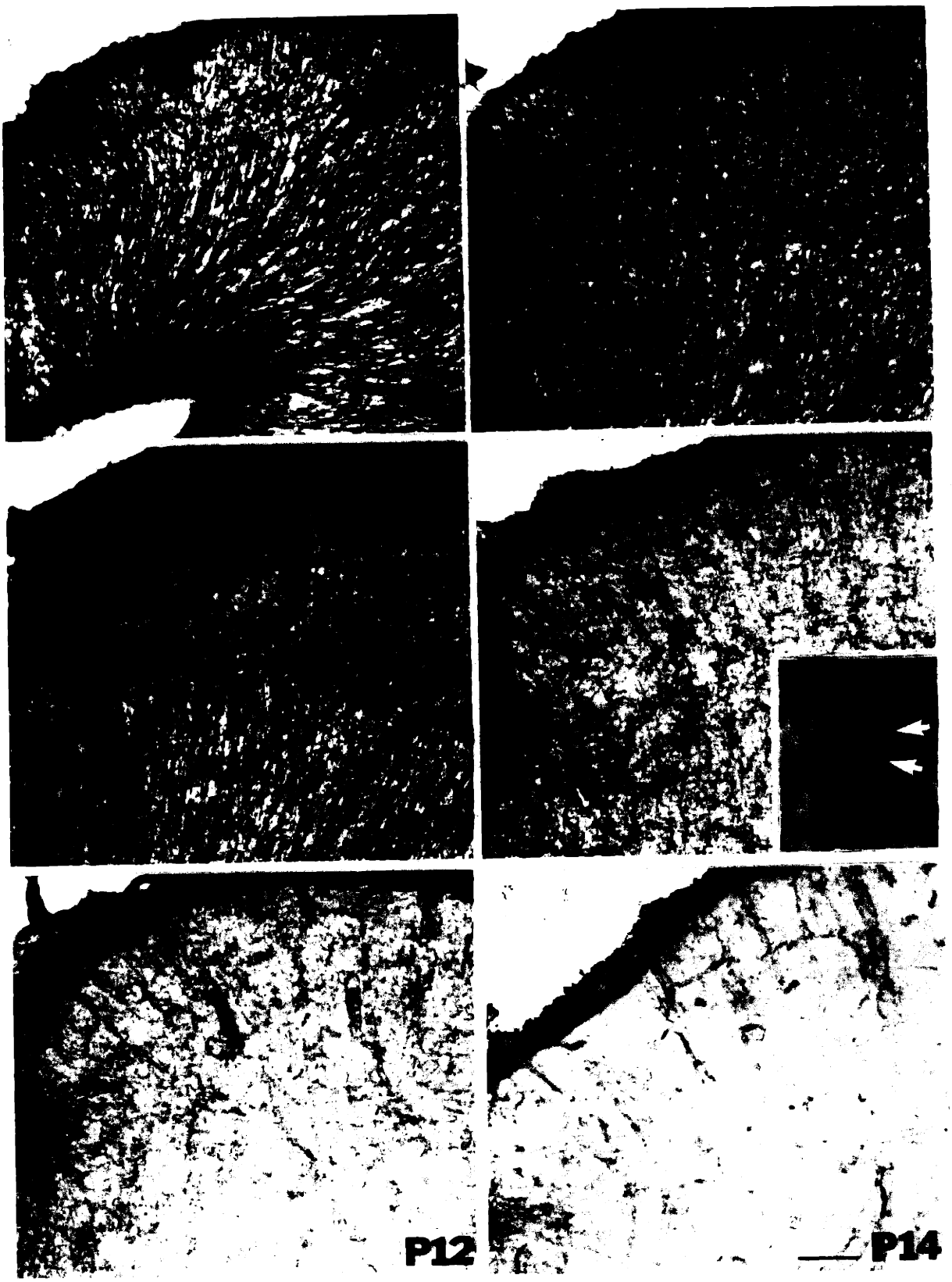

P12
P14

Figure 3: The lateral radial glial cells stained with anti-vimentin antibody at various post-natal ages. The diminishing of lateral radial cell staining is obvious by P7. Some astrocyte-like glial processes can be observed at P9 (arrows). Bar: 200μm.

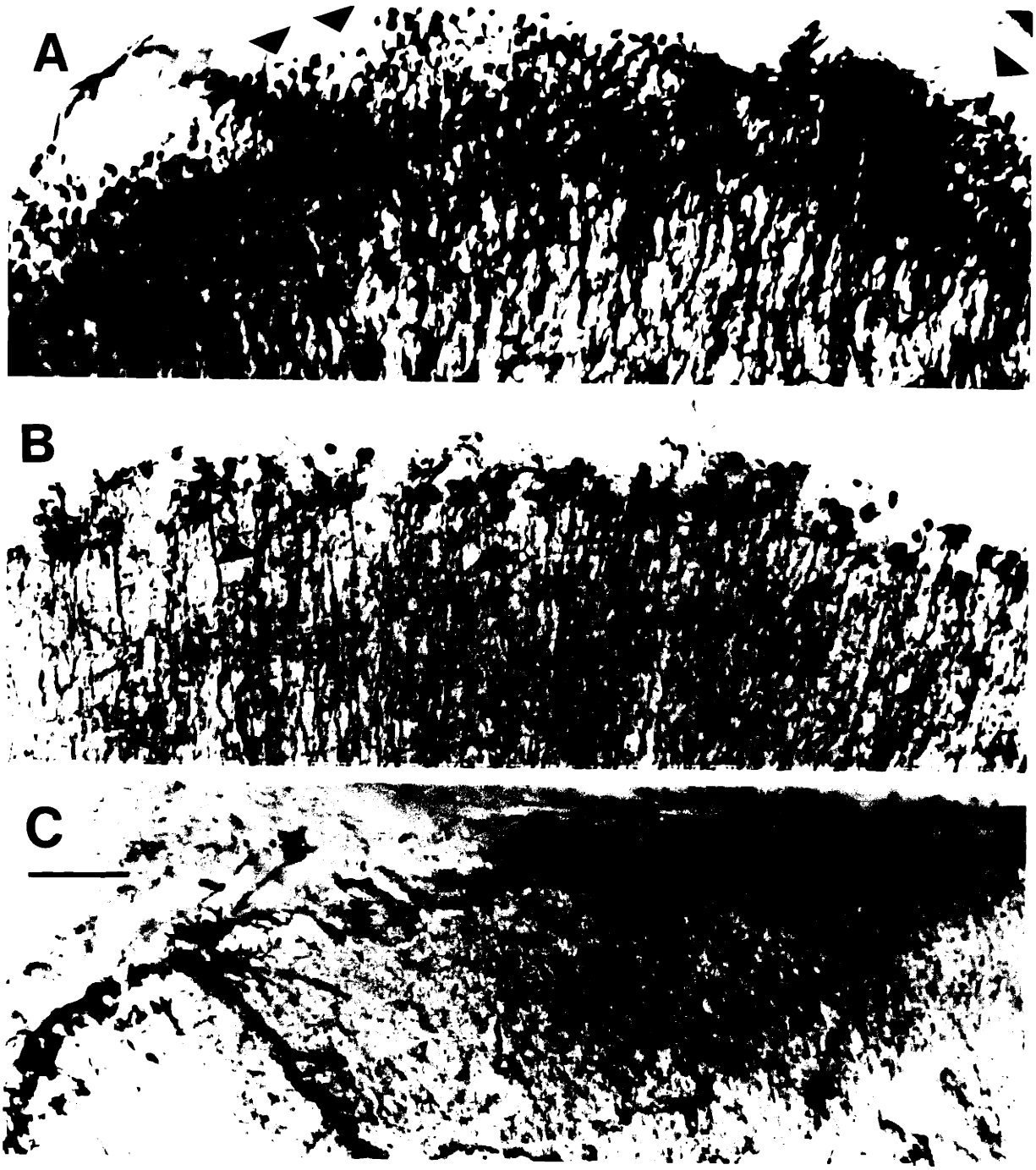

A
B
C

Figure 4: Vimentin-positive endfeet of lateral radial glial cells distributed in the pia. They diminish with age. Bar: 50μm.

(A) P1
(B) P5
(C) P14

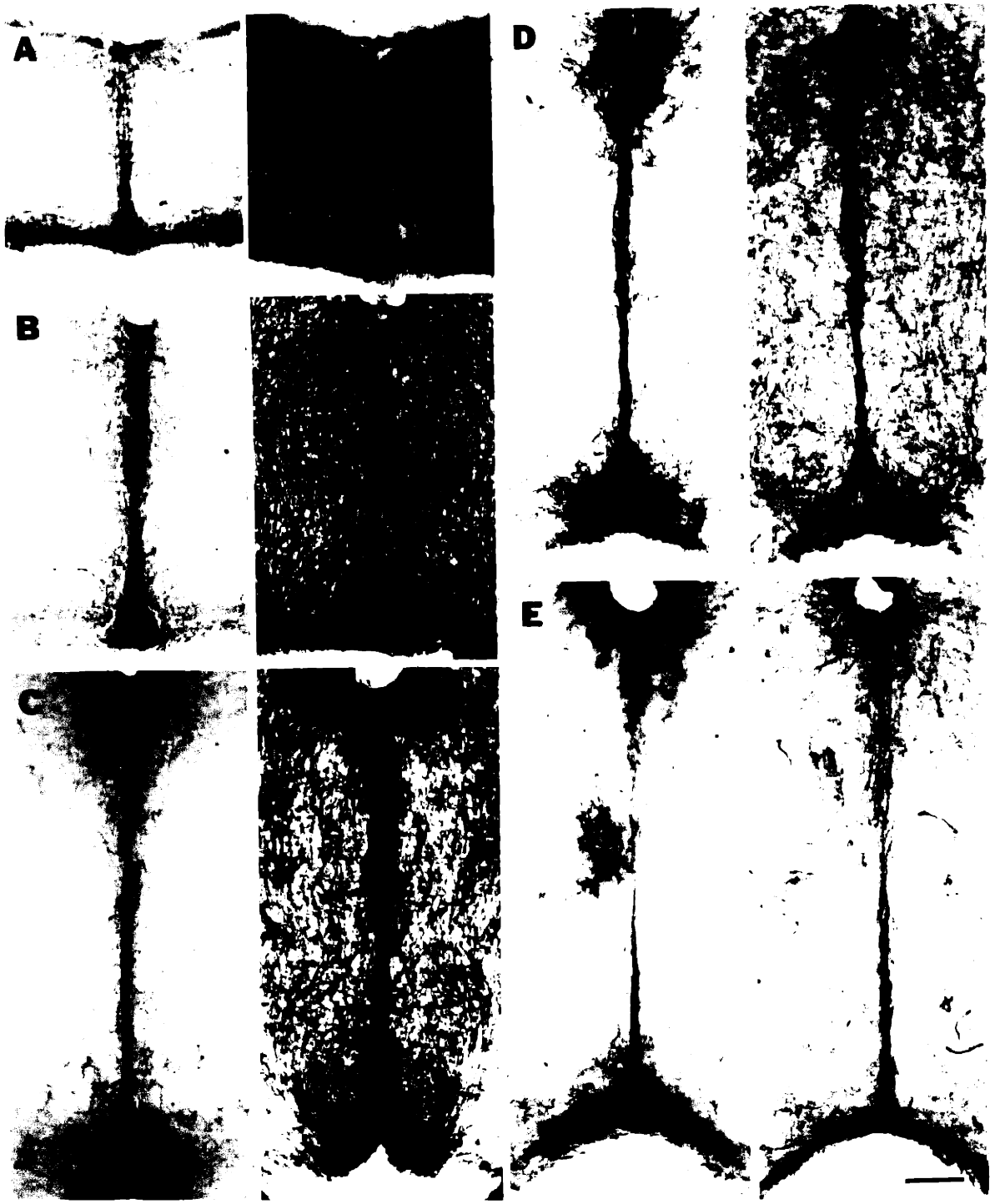

A
B
C
D
E

Figure 5: Midline radial glia stained by GFAP (left column) and vimentin (right column) antibodies. These midline radial glial cells are both GFAP- and vimentin-positive and persist till at least the end of the first two postnatal weeks while lateral radial glial cells disappear at that time. Bar: 200μm.

(A) P0
(B) P3
(C) P7
(D) P9
(E) P14

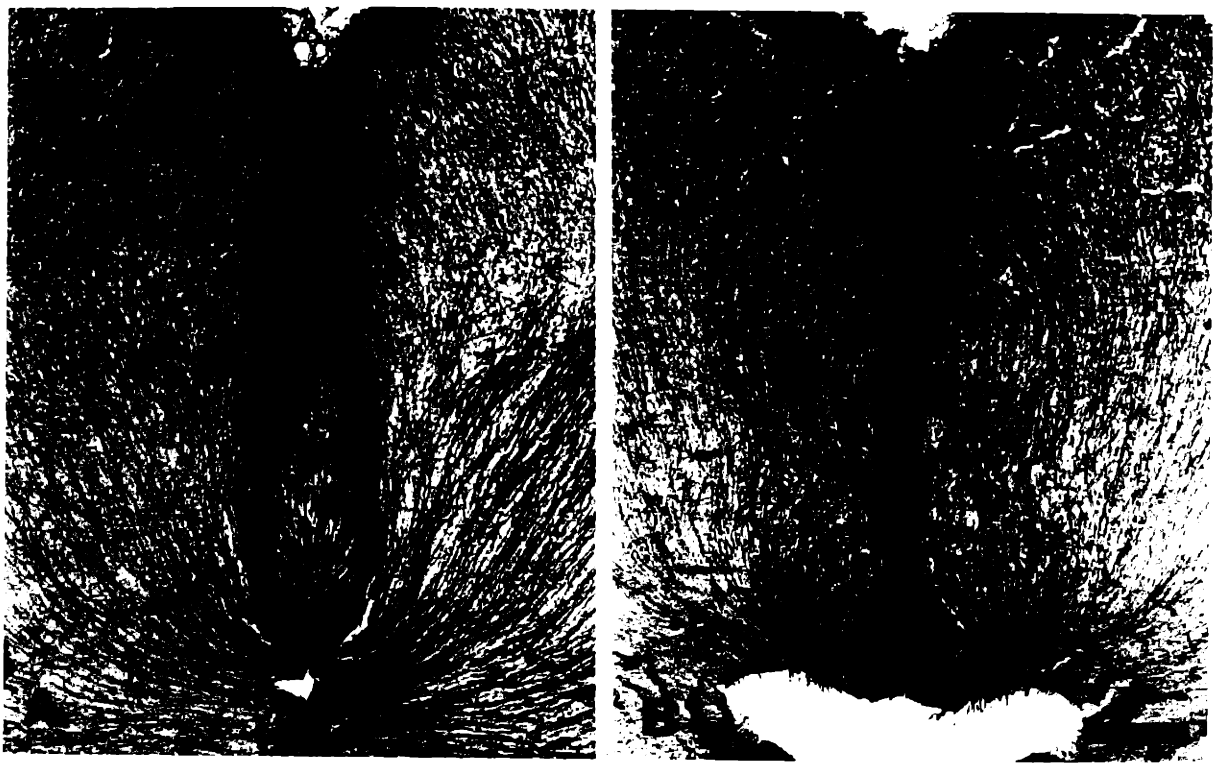


Figure 6: The difference between the rostral midline glial structure and the caudal midline glial structure at P3 using anti-vimentin antibody staining. Bar: 100μm.

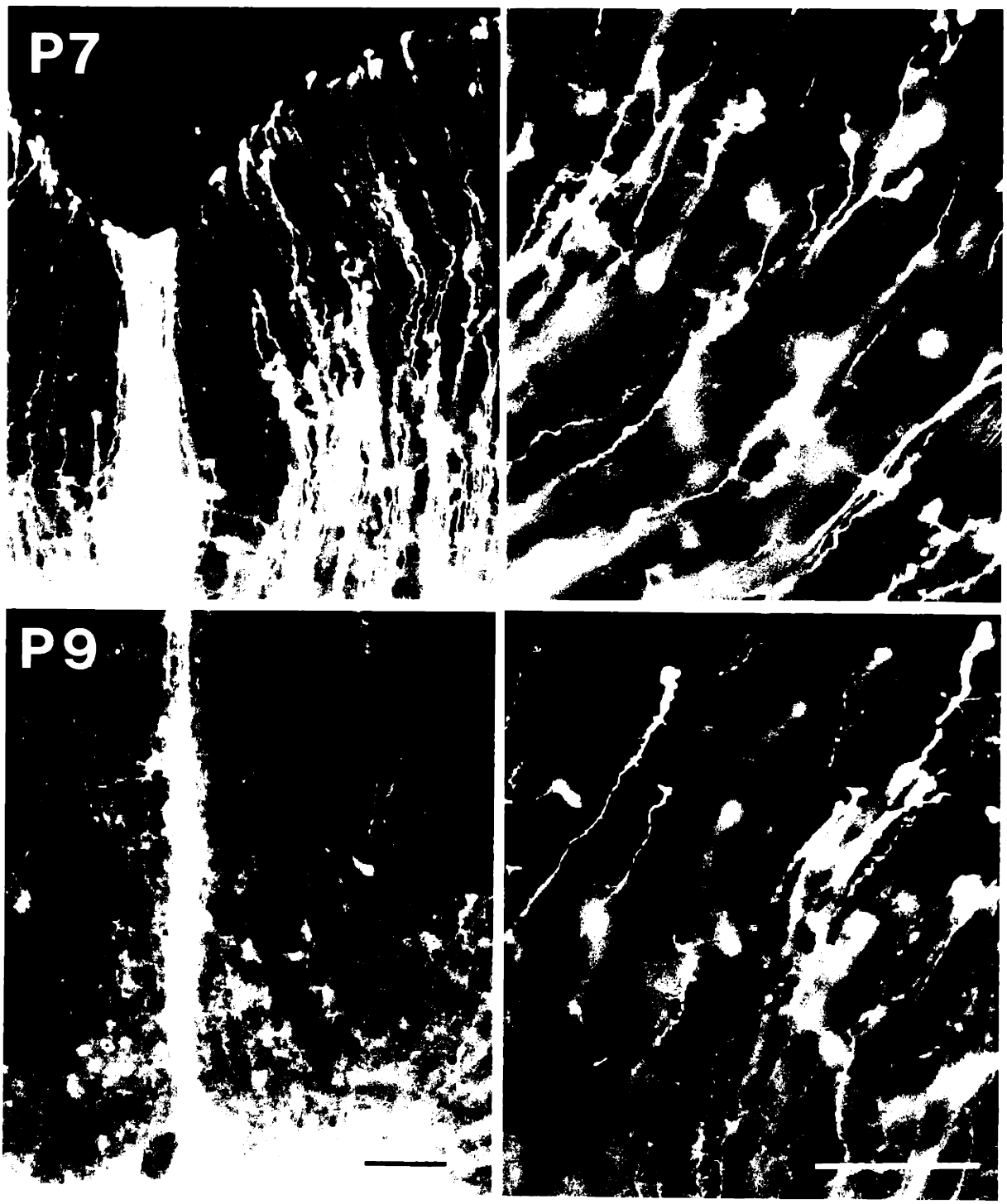

P7
P9

Figure 7: Withdrawing lateral radial glial cells shown with DiI labeling from the ventricle at P7 and P9. Higher magnification on the right. Bar: 100μm.

# CHAPTER III

## Recrossing of Retinal Axons in the Optic Tectum After Selective Damage to the Midline Glial Processes

### INTRODUCTION

During nervous system development, neuronal cells extend axons to innervate their target in a highly ordered pattern. Among the mechanisms underlying normal development, interactions between neuronal and glial cells have been shown to be important and necessary in forming the correct neural network. Such mechanisms may involve cell adhesion molecules on cell membrane and/or extracellular matrix molecules from glial cells or neurons. This is especially well observed in development of the visual system. For example, during chick retinofugal pathway development, NCAM is found on neuroepithelial cells on the route retinal axons grow along before these axons leave the optic stalk (Silver and Rutishauser, 1984). Antibodies raised against NCAM injected into the eye cause retinal axons to deviate from their correct route (Rutishauser, 1985). N-cadherin, which is also expressed by embryonic glial cells in chickens (Hatta et al., 1987), is found to promote and guide neurite outgrowth from retinal explants or retinal ganglion cells (RGC) *in*

*vitro* (Matsunaga et al., 1988; Neugebauer et al., 1988; Letourneau et al., 1990). L1 is another cell adhesion molecule observed on chick Müller cells (Drazba and Lemmon, 1990) and rat Schwann cells (Kleitman et al., 1988) that has a promoting effect on retinal neurites. In addition to cell adhesion molecules, some extracellular matrix molecules, like laminin, are considered to form substratum pathways to induce and guide retinal axon growth in chicks (Cohen et al., 1986) and rats (McLoon et al., 1988). As is found in NCAM distribution, laminin is also localized on the endfeet of neuroepithelial cells that line the route of retinal axons in the early embryonic stage (Cohen et al., 1987).

Recently, inhibitory factors during development have been suggested to be another important mechanism in axon guidance (Patterson, 1988; Chiquet, 1989; Schwab and Schnell, 1991). During visual pathway development, some glial cells have been observed to exert an inhibitory and non-permissive effect on growing axons. Retinal axons avoid pigmented epithelial cells when they migrate out of the optic stalk (Silver and Sidman, 1980; Silver and Sapiro, 1981). Later, some of the axons that originated from the ventronasal retina, are steered towards the contralateral side by a glial knot at the optic chiasm (Silver, 1984). Remarkably, some glial cells in the midline of the brain and spinal cord have been reported to form major axon barriers during pathway

formation. For example, a vimentin-positive midline glial structure is found in prenatal and early postnatal stages in rat medulla oblongata and parts of the spinal cord. It has been suggested that this forms a barrier for corticospinal tract axons to prevent them from decussation in the wrong location during development (Joosten and Gribnau, 1989). The roof plate, composed of glial cells and located along the dorsal midline of the developing spinal cord, is likely another axon barrier for guidance of commissural and dorsal column axons (Snow et al., 1990a). Both midline barriers "guide" axons through an inhibitory or non-permissive effect during development. In addition, *in vivo* and *in vitro* experiments have provided evidence that keratan sulfate proteoglycan (KSPG) may be involved as the molecular mechanism in repelling growing axons (Snow et al., 1990a,b). Such an axon growth barrier is also suggested in the optic tectum because KSPG is found in the tectal midline in neonatal hamsters.

In previous chapters, a group of distinctive radial glia were shown to occupy the midline of the developing hamster tectum. These midline radial glia and their processes were immunohistochemically and morphologically different from radial glia in the tectal parenchyma, namely the superior colliculus (SC) radial glia. Since the midline radial glia were detected by a conventional glial marker (anti-vimentin antibody) immediately prior to the arrival of the first retinal axon in the

tectum in the embryonic stage and the glial structure persisted beyond the appearance of the adult-like terminal arbors (P14, see Frost et al., 1979) after birth, the differentiation of midline radial glia was seen closely correlated with the development of retinotectal axons.

Some earlier studies carried out in our laboratory (Hsiao and Schneider, 1978) and by Jen and Lund (Jen and Lund, 1979) found that if a knife-cut was made in the tectal midline of neonatal animals from the pia, the two tecta could fuse into one and retinal axons from one eye grew into both sides. Such a phenomenon might indicate the need of a form of compartmentalization during normal development. How and when this compartmentalization is achieved and what cellular and molecular structure(s) are involved was not clear.

As reported earlier, after unilateral tectal lesion, substantial amounts of regrowing retinal axons on the lesioned side were seen to cross the midline and terminate in the intact, opposite SC (Schneider 1973; Schneider et al.,1985). The crossing did not occur if the midline tissue was not damaged (So and Schneider, 1978). With both vimentin and GFAP immunostaining and DiI labeling, it was observed that in the case of unilateral tectal lesion, whenever the retinal axons crossed the midline, the midline radial glia had always retracted their processes from the lesioned midline pia. This retraction left a "gap" seen as a blank area

of immunostaining in the midline between the pia and the remaining proximal portion of the midline glial processes.

In the present study, a new surgical procedure was designed to specifically disrupt the processes of midline radial glia without significant damage to the SCs on either side. Most importantly, the midline pia was left intact to eliminate the possibility of tissue bridge formation. Thus the suggested barrier function of midline radial glia was directly tested.

## MATERIALS AND METHODS

Syrian hamster pups were bred in the MIT animal facility or were purchased from the Charles River Laboratory (Wilmington, MA). For lesioned brains, animals that had undergone midline undercutting surgery (see below) and had their left eye injected with horseradish peroxidase (HRP) were sacrificed using an overdose of Nembutal and perfused transcardially with an initial rinse of 0.9% saline containing 0.25% sodium nitrite followed by 4% paraformaldehyde made up in 0.1 M phosphate buffer (pH 7.4). The brains were not post-fixed so that HRP activity could be better preserved. Fixed brains were dissected out and cryoprotected in phosphate buffered 30% sucrose (pH 7.4). Transverse sections were cut at 40 micrometers on a cryomicrotome and sections collected alternately in 0.1 M phosphate buffer (pH 7.4) for HRP processing and for immunohistochemistry (described below).

### Surgical Procedures:

Surgery was done at the age of P0 for all pups. At this age, the dorsal surface of the pia was relatively flat from left to right. Hypothermia as anesthetic was quickly induced by removal of pups from the nest onto crushed ice for 5-7 minutes. The skull above the SC was exposed by opening the overlaying skin and removal of connective tissue

between the skin and skull with a cotton swab. With a Number 11 surgical blade, a small slit was made rostral-caudally at the lateral-most side of the skull next to the left SC. Undercutting of the midline was achieved when a hooked tungsten wire was inserted from the slit on the skull into the left SC and pushed towards the right to cut into the right SC below the midline pia. A lesion was thus made specifically onto the midline glial processes. With this procedure, only the midline separating the two retinal axon targeting areas, namely the two SCs, was damaged, while the pia and the meninges were left intact. The right eye was also removed at the same time to facilitate termination of the crossed retinal axons in the left SC. Animals were returned to the nest after they recovered from hypothermia and were sacrificed at later ages.

HRP Eye Injection and Retinotectal Axon Labeling:

HRP was injected into the remaining eye (always the left eye) 18 to 24 hours before the animals were sacrificed. Five lesioned animals at each of the ages P12 and P14 were used for labeling of recrossing retinal axons. In each case, one to three microliters of 40-50% HRP (Sigma) made in 2% dimethyl sulfoxide (DMSO, Fisher Scientific) were injected into the left eye through a micropipette after the animals were fully anesthetized with Chloropent. Animals were perfused as described above.

Sections from these brains were cut on a cryomicrotome and collected alternately into three groups. One group was used for HRP-filled axon labeling. The other two groups were used for vimentin and GFAP labeling. Sections with HRP-filled axons were incubated with tetramethyl benzidine (TMB, Sigma) as described in other publications (Jhaveri et al., 1988). They were then mounted onto microscope slides, counter-stained with neutral red, and coverslipped after alcohol dehydration and xylene clearing.

Immunohistochemistry:

Sections adjacent to those reacted for HRP were immunostained to observe their glial environment in the midline and were then analyzed together with the HRP-labeled regenerating retinal axons. Monoclonal antibodies to GFAP and to vimentin were purchased from Boehringer and each used at a dilution of 1:30 in phosphate buffered saline (PBS) with 0.1% normal horse serum (Sigma), 0.3% Triton X-100 and 0.2% sodium azide.

Sections were rinsed in PBS and the non-specific binding blocked in PBS containing 20% normal horse serum. They were next incubated in primary antibody for 18 to 24 hours on a rocker at room temperature (or for 96 hours at 4$^{o}$C). They were then rinsed in PBS and incubated in

biotinylated horse anti-mouse IgG solution (1:200 dilution) for one hour at room temperature. The tissue was rinsed in PBS again and placed in an avidin-biotin-HRP complex solution (Vector Labs, ABC kit) for an hour. Finally, the HRP was visualized using DAB as the chromagen. Control sections were processed according to the same protocol except that the primary antibody was omitted in the first incubation. Sections were mounted on subbed slides, dried overnight, cleared in xylene, and coverslipped.

DiI Labeling of Crossing Retinal Axons Combined with DiA Labeling of Midline Radial Glia:

Animals were lesioned on P0 to undercut the midline. To evaluate undercutting of the midline and observe the response of retinal axons to the surgery at an early stage, DiI was injected into left eye of live P1 pups (24 hours after surgery). Animals were allowed to survive for another two days. On P3 these pups were perfused as described above and DiA [4-(4-dihexadecylaminostyryl)-N-methylpyridinium iodide] was placed into the ventricle at the level of the cerebral aqueduct of Sylvius. The labeled brain was retured to paraformaldehyde for four days at room temperature. On fifth day 100μm thick transverse sections were cut on a vibratome. Sections were immediately collected in 0.1 M phosphate

buffer, mounted onto microscope slides and coverslipped. DiI labeling of the early growth of retinal axons in the tectum was observed and photographed with a Nikon epifluorescence microscope using a rhodamine filter. DiA labeled radial processes on the same section was also observed and photographed immediately with a FITC filter.

RESULTS

The damage inflicted by the tungsten wire on glial processes was much smaller than what was inflicted by heat lesion during unilateral tectal ablation. Even though the wire passed the left SC, both SCs looked similar in size during the first postnatal week. The tungsten wire inserted into the left SC severed the midline glial processes between their ventral attachment and the endfeet in the pia. The distal portion degenerated within 12 hours. This was observed when brains were fixed and immunostained for vimentin 12 hours after surgery (Fig. 1). In contrast, on the same immunostained section, the proximal portion of the glial fibers were seen well maintained. A similar situation was found 24 hours after surgery. The track of the cut left an elongated hole in the tectum. This made it easy to assess the surgery and determine where the knife entered and where the cut ended.

After the brain was fixed on P3, DiA was placed in the ventricle at the midbrain level to label radial fibers of both lateral radial glia and midline radial glia. When viewed under a microscope with an FITC filter, the labeling stopped abruptly at the cut because the severed distal portion of radial processes were not labeled in such cases (Fig. 2A). Although it had been three days after surgery when the brain was fixed, the severed proximal glial processes did not regenerat back into the cut

area. Therefore the part of the tectum dorsal to the flat track of the knife-cut across the left and right SCs appeared blank. The end of the proximal glial processes were well labeled and could be examined in detail. They lacked the usual enlargement -- the endfeet -- seen in normal SC radial glia. Instead their ends were hairy and branched as observed in the middle of unsevered radial processes. In some areas, the cut could be followed into the medial part of the right SC as well, after severing the midline radial glial processes. Since the non-severed lateral radial glial processes in the right SC were well labeled all the way into the pia where their endfeet were attached, the end of the cut during surgery on the left-right axis in a transverse section was obvious (Fig. 2).

When DiI was injected into the eye of lesioned animals on P1, it was picked up by retinal ganglion cells and actively transported to the axons up to their terminals. Since active transport was much faster than diffusion through the membrane, the entire axon was labeled within 48 hours (Fig. 3). Double labeling was achieved by combining DiI eye injection in live animals to label retinal axons for 48 hours with DiA labeling of midline radial processes from the ventricle after the brains were fixed. Through this approach retinal axon growth in the lesioned brain could be contrasted with the position of severed midline glial processes in the same transverse section.

At P3 most of the retinal axons from the left eye were still in the right SC. Axons were seen close to the midline before crossing. However, in a few cases, several single fibers were observed to have just grown across the midline (Fig. 2). The longest crossing axons had reached about one-third in the left SC from the medial side. There were two important differences between the crossing axons in the midline undercutting cases and the crossing in the unilateral tectal lesion cases. First, the crossing of retinal axons after midline undercutting was not by fascicules as was seen after unilateral tectal lesion. The first several axons seen here on P3 crossed the midline individually below the midline pia but at the superficial gray level, obviously not following any specific tissue bridge or substrate pathway (Fig. 2C). Second, if the crossing axons were traced back to the right SC it was found that the crossed axons were branches of those terminating in the medial tectum on the right side of the midline. Therefore retinal ganglion cells in this situation expanded their territory of projection instead of merely seeking novel target areas because the original target area was damaged as in the unilateral tectal lesion cases.

The level of crossing retinal axons was also intriguing. The first crossing axons were neither attached to the pia nor next to the proximal end of the severed midline glial processes. All of the early crossing axons

were at the stratum opticum level. After passing through the opening at the midline they then turned upwards, growing towards the areas immediately below the pia in the stratum griseum superficiale (superficial gray, Fig. 2). Further, crossing retinal axons were restrained within the zone destined for visual input. This phenomena possibly indicate that the crossed retinal axons are still guided by chemotropic factors or selective substratum, albeit on the wrong side.

With GFAP and vimentin antibody staining, the midline glia in the lesioned brain appeared to be relatively normal in the intact region after a week on P7 (Fig. 3A). When the lesioned area was examined with either the vimentin antibody staining or with the GFAP antibody staining, a blank area was found below the midline pia but above the detached midline glial processes. This blank area was similar to the immunostaining "gap" found after unilateral tectum lesion. Gliosis in the SC was rarely observed, although in some cases there was abundant gliosis next to the pia in the left SC at the area where the wire had entered for the undercutting.

On sections reacted for HRP visualization, retinal axons were seen to grow across the midline from the right SC into the left SC through those areas corresponding to the immunostaining negative area on adjacent sections stained by antibodies (Fig. 4). Axons crossing the

midline after undercutting did so in a fashion different from those in the unilateral tectal lesion cases. After the unilateral tectal lesion, retinal axons crossed the midline in fascicles either above the lesion along a tissue bridge or below the lesion in a few narrow streams, whereas in the midline undercutting cases the axons were not in fascicles. As was observed at the ages of P12 and P14, the crossing axons swept through the opening above the detached glial processes in a wide band into the left SC.

As expected, crossing was only observed in the part of the SC where undercutting of the midline radial processes was observed (Fig. 4B). With both SCs remaining, the quantity of retinotectal axons growing from the right SC into the left SC was often relatively smaller (Figs. 4B,4D) compared to the situation in the unilateral tectal lesion. This might be due to the fact that the original target area for these axons -- the right SC -- was not ablated. As was seen in the early crossing of retinal axons on P3, some of the crossing axons were likely to be long branches of axons also terminating in the right SC. Since axons arborizing in SCs after P3 are usually not fasciculated (Schneider et al.,1985), this might explain why these axons crossed the midline in a non-fasciculated form. If this was indeed the case, a further conclusion could then be drawn that the glial barrier was not only involved in the fast growing, elongation period of retinal axon development but was also involved in the period

when they were forming end arbors within the target zone. When the development of midline glia is studied it is observed that these glia maintain their integrity till at least two weeks after birth, much later than lateral radial glia in the SCs (see Chapter II), such a distribution fits the proposed barrier function of these midline cells.

## DISCUSSION

1. These results provide further evidence supporting the hypothesis that the midline glia play important roles in forming a retinal axon barrier in the developing optic tectum. They "guide" retinotectal axons during their target-approaching (elongation) and target-invading (arborization) periods by preventing these axons from growing into the SC of the wrong side. Damage to midline glia hinders the barrier and permits retinal axons to grow across the midline.

2. Recognition between the retinal axons and the target cells is shown not to be the decisive factor for correct axonal termination because whatever recognition mechanism functions in the normal brain should still function in the lesioned brain as the right SC is relatively intact in this case, yet retinal axons are not restricted to the right SC once the midline glial processes are disrupted. Therefore the so called "negative guidance" (Schwab, 1990) may also exist and be necessary in the rodent optic tectum

in ensuring the correct development of retinotectal axons.

3. Chemotropic or chemotactic mechanisms are most likely important in inducing axons to cross the midline and in determining where to go after crossing, because retinal axons are seen to always grow into the target area of the visual system, namely the optic tectum, after crossing the midline.

4. The competition for target territory between terminal arbors by axons from the two sides also constrains retinal axons relatively within their own target zone. In one of the contral experiments both eyes are left intact after the undercutting of the midline glial processes. The retinal axons from either eye still cross the midline into the opposite SC. But the crossing is minute and limited to the very medial portion of the SC on the other side. On the other hand, when one of the eye is taken out after undercutting so that the competition from the other eye is eliminated, the crossed retinal axons do not necessarily occupy the entire opposite SC. Usually some atrophy can be seen on the side contralateral to the enucleated eye. It is conceivable that there are some internal limitations on how much territory one axon can extend onto.

5. The barrier function of tectal midline radial glia may be an example of a general mechanism in neural development and system formation. Similar structures are reported in other animals evolutionally

distant to each other. Raedler and colleagues (Raedler et al., 1982) have found that in the rat SC area, a group of ventricular midline cells were generated during E12 through E15. These midline cells have short and broad ventral processes and long and thin dorsal processes which could be followed up to the pia basal lamina. Cavalcante and colleagues have also found that there is a midline glial structure that is unique in the developing opossum SC (Barradas et al., 1989). Although these studies suggest that the midline structure may have a role of mechanical support for the young tectum or provides guidance for migrating neurons in the SC, other functions of these cells are not ruled out. The present study suggests that such a midline structure may be a form of brain compartmentalization during development which establishes and maintains laterality in information processing. Similar barriers formed by midline glial cells have also been suggested both in the medula oblogata for corticospinal tract (Joosten and Gribnau, 1989) and in the spinal cord roofplate for the commissural and dorsal column axons (Snow et al., 1990) during development in rat.

It is difficult to elucidate the molecular mechanisms involved in the barrier with the present approach. Since KSPG has been located in normal hamster tectum, some work has been done in collaboration with Dr. Silver to detect the changes of KSPG distribution in animals with

midline undercutting. In the initial result it is found that after the midline glial processes are severed KSPG disappears at the lesioned area in the midline, although the soma of the midline radial glia are still KSPG positive (personal communication). This may provide some evidence of the involvement of inhibitory extracellular matrix molecules in the barrier. Other molecules, like chondroitin sulfate proteoglycan (Oohira et al., 1991) and tenascin (Wehrle and Chiquet, 1990), are also possible to play some roles in the compartmentalization of the developing brain.

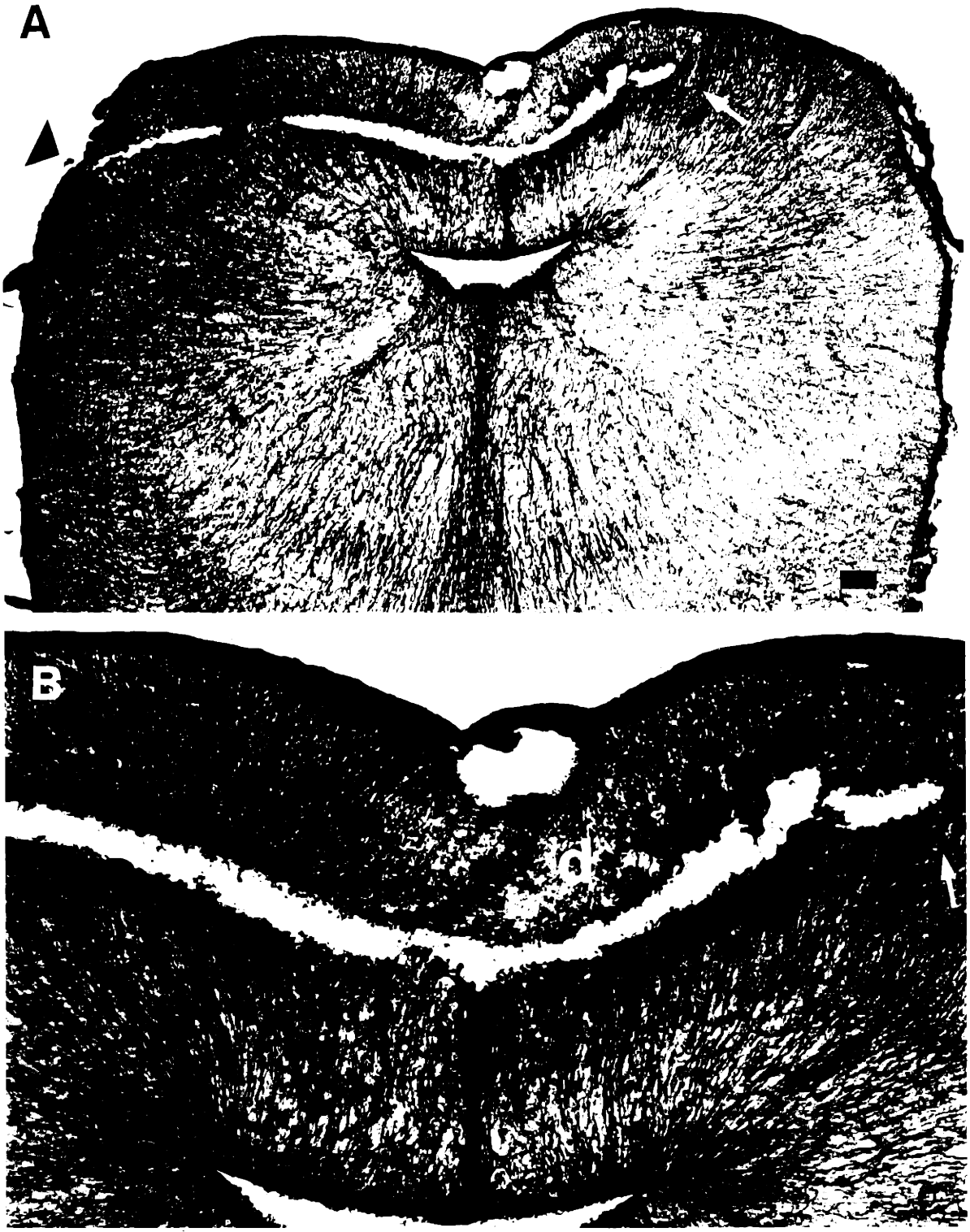

A
B
d

Figure 1: Anti-vimentin antibody stained section through the area lesioned with a tungsten wire. Knife entry point (arrowhead) and the end of the cut (arrow) can be traced clearly (A). Area showing degenerated midline processes after severing ("d") is shown at higher magnification (B). Bar: 100μm.

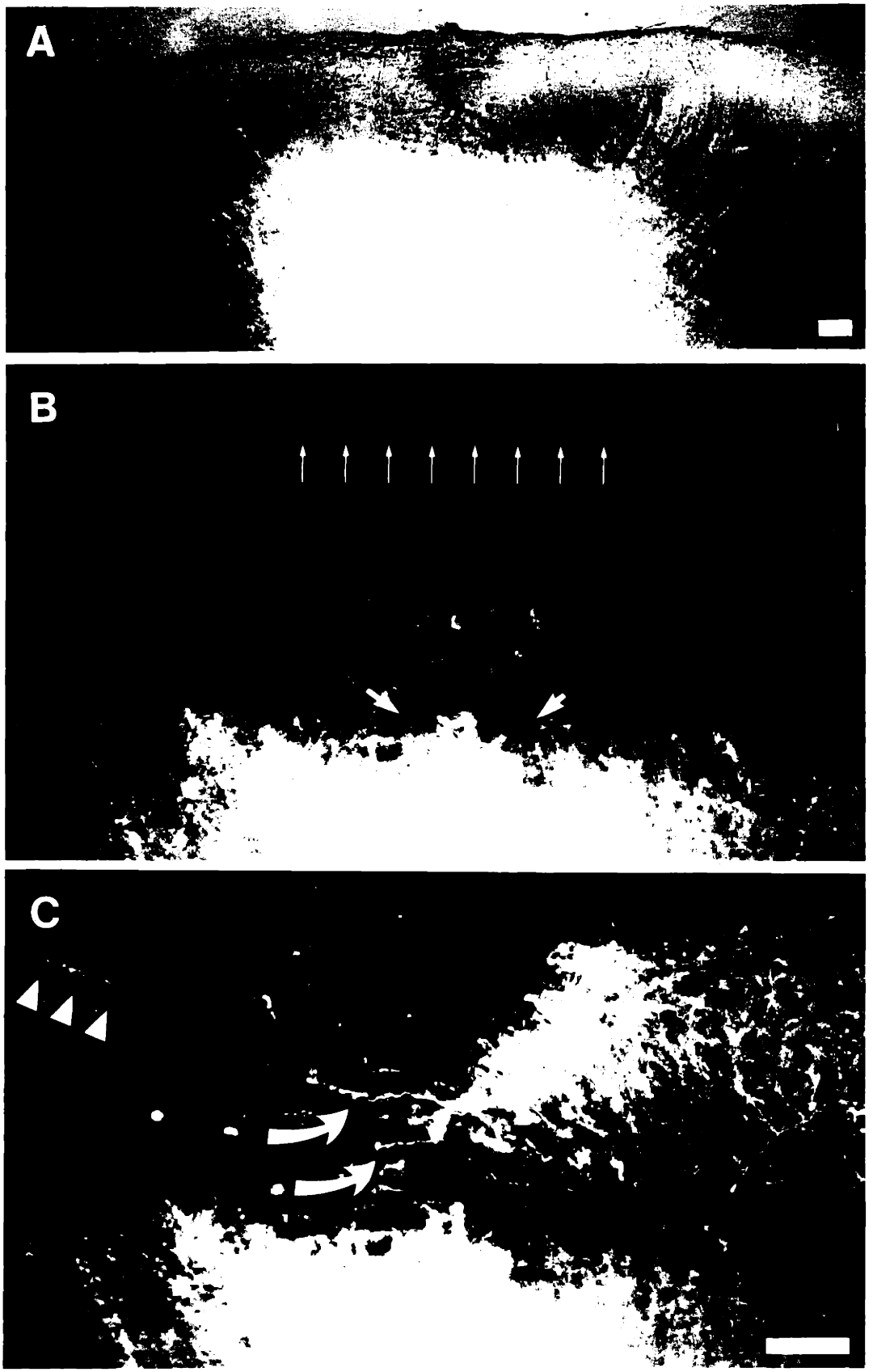

A
B
C

Figure 2: Double-labeling of radial processes and retinal axons in the same section. The severed radial processes and the intact lateral radial glial processes in the right tectum are labeled with DiA (A). The pia surface (small arrows) and the severed midline glial processes (large arrows) are shown (B). Crossing retinal axons at the midline (curved arrows) can be observed (C). Some of them reach the medial one-third of the opposite SC (arrowheads) (C). Bar: 100μm.

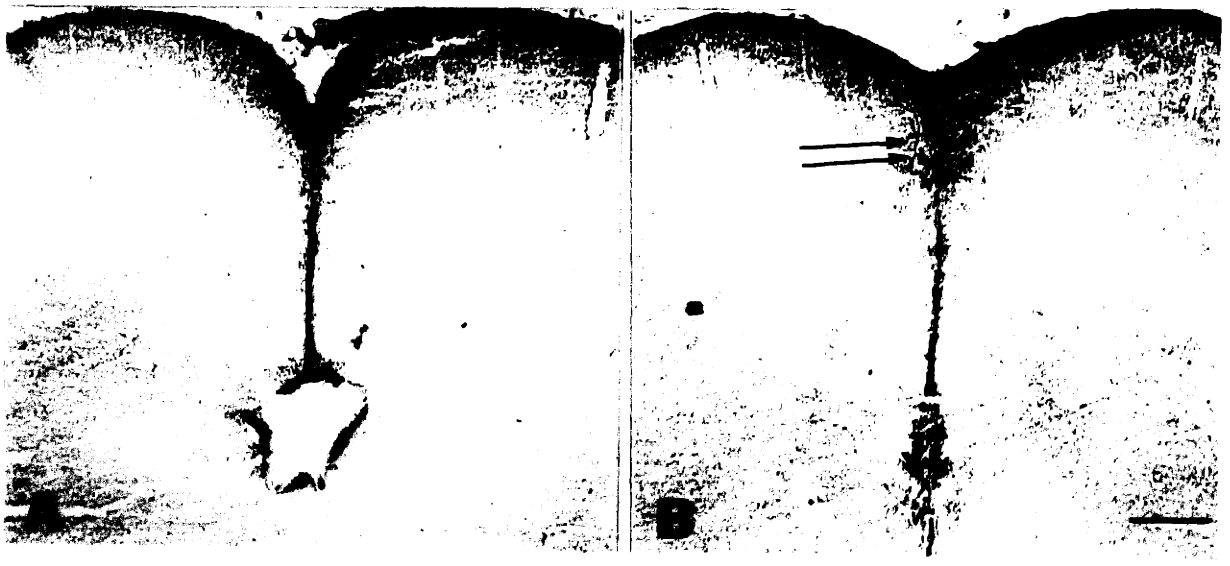


Figure 3: GFAP antibody staining of midline glial processes at P7 after undercutting. The intact area (A) and the lesioned area (B) is shown. The ends of the severed midline glial processes. Bar: 200μm.

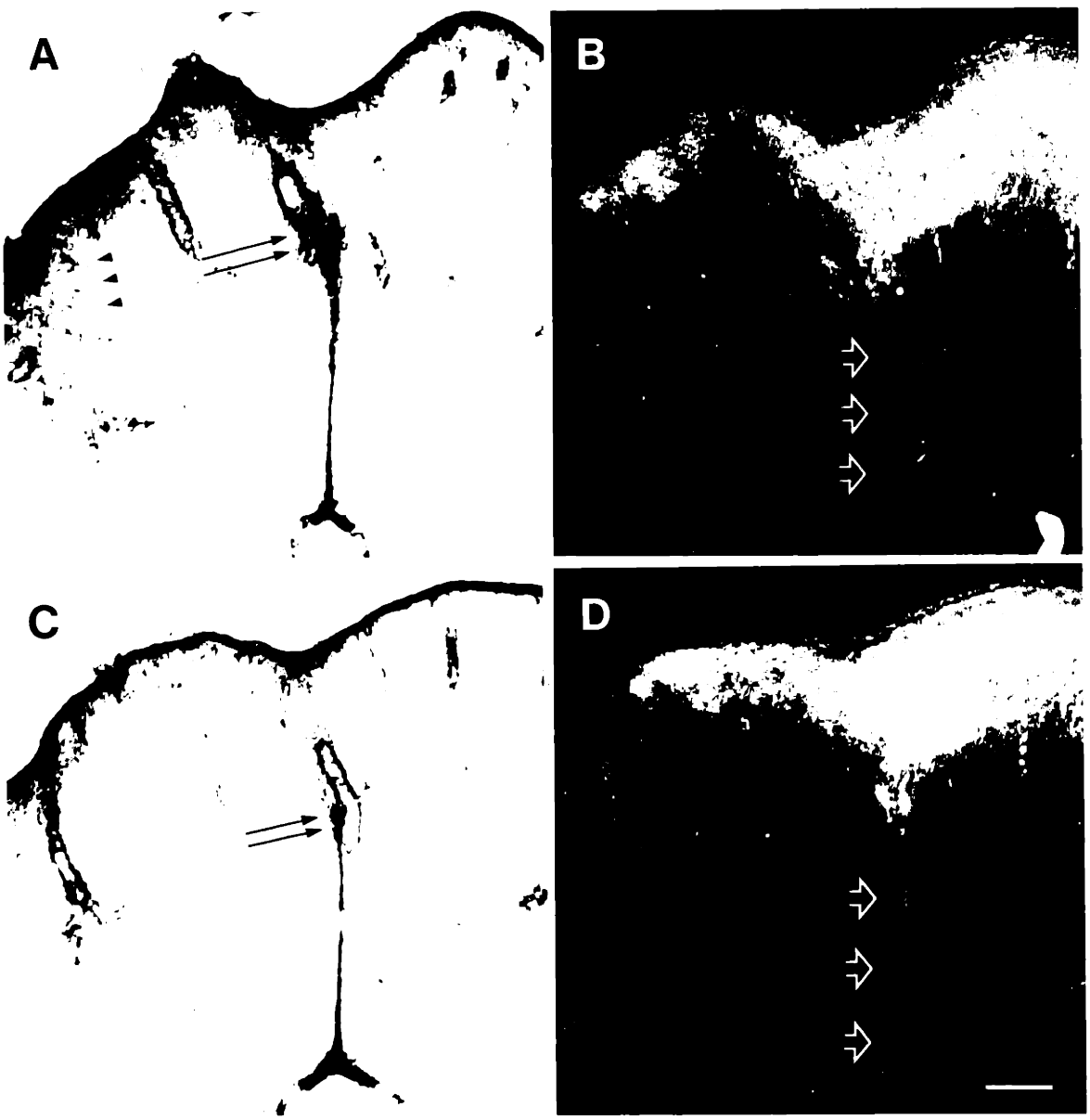
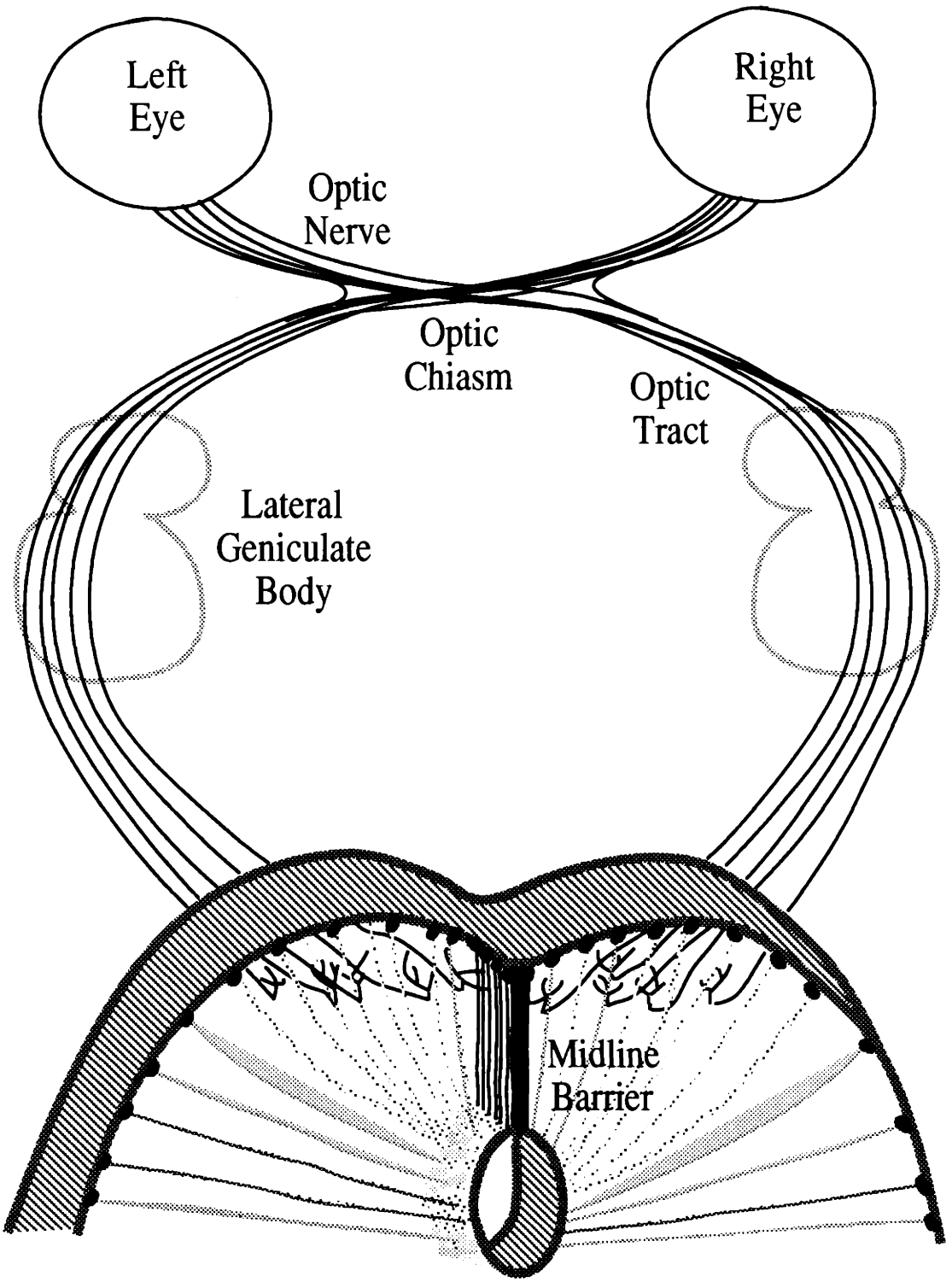

A
B
C
D

Figure 4: GFAP antibody staining at P14 after undercutting (A, C) compared with crossing retinal axons labeled with HRP (B, D). Adjacent sections (A/B, C/D) are examined using immunostaining and HRP labeling. The knife entry point (arrowheads in A,C), the ends of midline glial processes (arrows in A,C), and the midline (empty arrows in B, D) are marked. Bar: 200μm.

# CONCLUSION

The research presented here examines the glial environment in the optic tectum during development of retinal axon projections. First, the changes of radial glial cells, especially the radial glia in the tectal midline, has been investigated in relation to crossing of the midline by retinal axons after unilateral tectal lesion. Second, the development of the midline glial structure has been studied, covering the entire time course of retinal axon development from embryonic stages to early postnatal stages. Finally, specific anatomical and histological approaches have been designed to test directly the functions of the midline glial structure.

These experiments have been carried out to address an intriguing and significant hypothesis: During central nervous system development, specifically during retinotectal pathway development, the brain is compartmentalized by certain axon growth barriers in which glial cells take part. Such axon barriers have been suggested in spinal cord for different pathways (Joosten and Gribnau, 1988; Snow et al., 1990a,b). This form of compartmentalization is believed to be an important mechanism to ensure correct axonal pathfinding. In the optic tectum, the compartmentalization establishes and maintains the laterality of visual input. The normal pattern of retinal axon projection in relation to the midline barrier is diagrammed in Figure-1 on the next page.


Left
Eye
Right
Eye
Optic
Nerve
Optic
Chiasm
Optic
Tract
Lateral
Geniculate
Body
Midline
Barrier

The conclusions reached from this set of studies are as follows:

1) There is indeed a distinctive midline glial structure present during the formation of retinotectal projections. The timing of development of this midline glial structure closely correlates with the development of retinal axons in the optic tectum before and after birth.

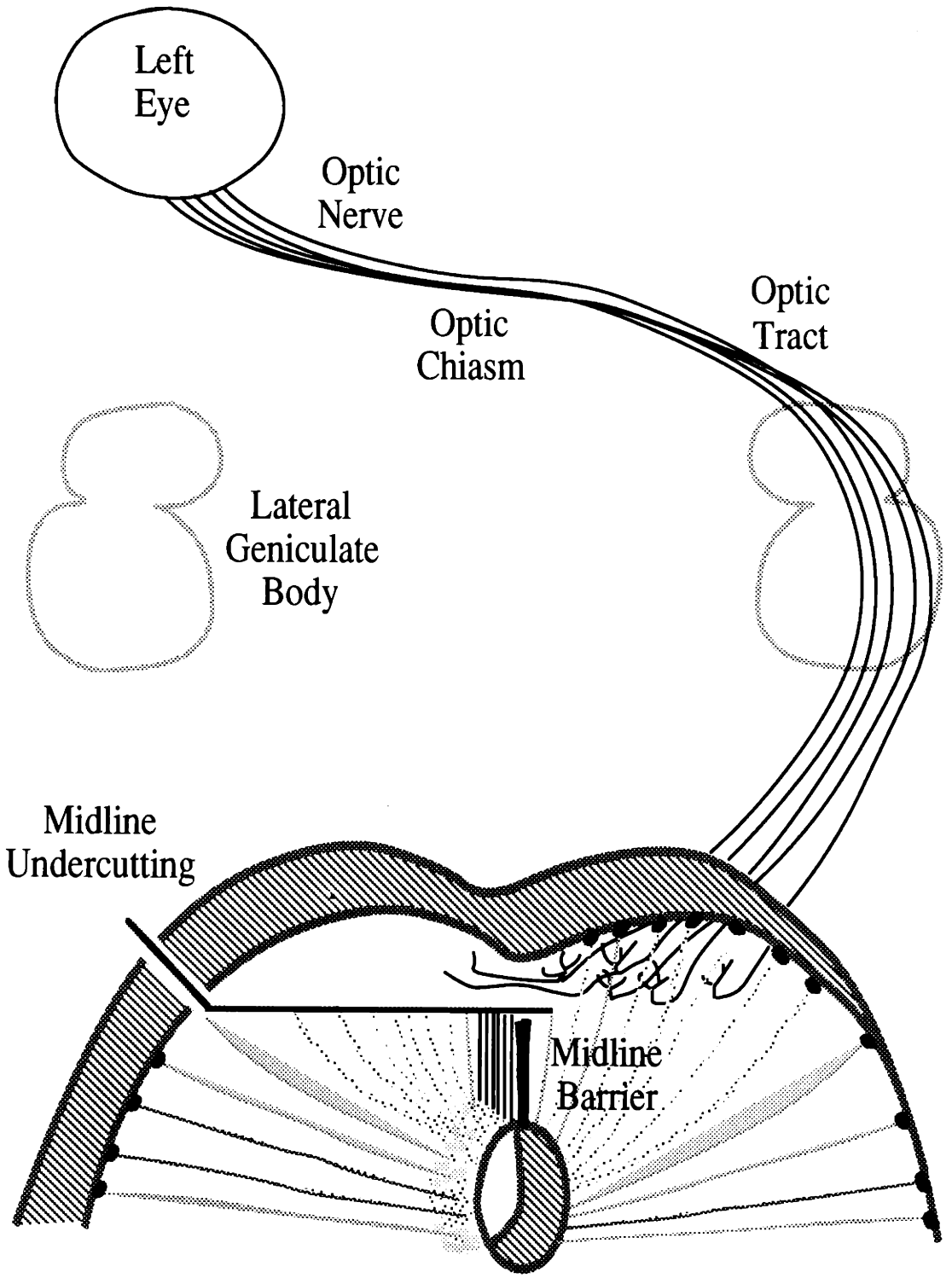
2) After the tectum is unilateraly ablated, retinal axons can cross the midline and terminate in the opposite side. The crossing of these axons is likely the result of the damage and withdrawal of midline glial processes. Exclusive severing of these midline glial processes under the midline pia at early postnatal age allows retinal axons to grow into the wrong side of the brain. This is evidence that these glial cells are involved in an axon barrier during development.

3) When retinal axons seek a new target area, either after unilateral tectal ablation or after midline undercutting, they always prefer brain regions destined for visual input, even though it is sometimes on the wrong side. This indicate that retinal axons are under the influences of chemotropic factors or other recognition mechanisms in determining their growth direction.

The influence of chemotropic factors emitted from optic tectum in directing retinal axons is clearly observed by the transplant work in Lund's laboratory (Hankin and Lund, 1990). It was shown that in

anophthalmic mice, axons from a retina transplanted in the deep layer below the tectum in the midbrain grow into the SC successfully only if another retina is transplanted subpially. They conclude that innervation in the SC by axons from retina transplanted in the pia cause recipient cells in the tectum to emit diffusible factors, namely chemotropic factors, to other retinal axons. Such chemotropic factors attract retinal axons toward the tectum and are necessary for axons from the retina in the deep layer to find their target in the superficial gray layer of SC. Since the midline undercutting is done on P0, three days after retinal axons start innervating the optic tectum, similar chemotropic factors resulting from innervated SCs should have existed.

The abnormal retinal axon growth after midline undercutting is diagrammed on the next page.


Left
Eye
Optic
Nerve
Optic
Chiasm
Optic
Tract
Lateral
Geniculate
Body
Midline
Undercutting
Midline
Barrier

Besides these extrinsic factors stated above, there are also intrinsic factors governing axonal pathfinding and target seeking. Two aspects are closely related to and demonstrated in this study. First, axons have an intrinsic tendency for territorial expansion. When the midline is undercut, the distal portion of severed midline radial glial processes degenerate, leaving an opening in the midline barrier. This opening permits the crossing of the retinal axons. Meanwhile, in this manipulation the original target area, the right SC here, is largely intact and retinal axons terminate in it readily as seen both at P3 and at later ages. Nevertheless, it is found that retinal axons from one eye, the left eye in this case, not only sustain the entire original target zone in the right SC, but also expand into most of the left SC when they are unopposed. Such "filling in" of the empty space by developing axons has been reported in other studies. For example, when the retina is partially lesioned in newborn hamsters, the remaining retina expands its projections into the vacated area in the SC corresponding to the degenerated ganglion cells and their axons (Frost and Schneider,1979). The filling-in after midline undercutting is dramatic because retinal projections from the left eye almost doubled in their covered area. This must have been accomplished after massive axon terminal relocation at an age when axons normally do not regenerate if they are injured.

A second aspect of the tendency of axons to expand their termination area is that axons have an intrinsic limit on the size of their terminal arbors (Schneider, 1973). That means their expansion is not limitless. As is seen in P14 animals with midline undercutting retinal axons from the left eye do not expand into the entire left SC after they have grown out of the fully occupied right SC. A similar situation is reported from partial retina lesion experiments (Frost and Schneider, 1979). When a relatively large portion of retinal ganglion cells are damaged, the remaining retinal axons usually do not expand their terminal projection in SC to fully "fill in" in the vacated area. It is conceivable that the slight atrophy of the left SC after undercutting is related to the weak retinal innervation because the original target area in the right SC is fully available.

The mechanism of the midline barrier is not answered with the present research. For example, it is important to know whether the tightly packed cellular structure represents a "mechanical" factor responsible for the barrier function. However, since anatomically there are intertectal axons in the deep layer of the tectum, the mechanical mechanism seems unlikely to be essential for the barrier. If the barrier is selective some molecular mechanisms must be involved. Some initial results from Jerry Silver's laboratory indicate that proteoglycans secreted

by midline glial cells like keratan sulfate proteoglycan may be a good candidate for such a function. This area certainly deserves some further research. One possible approach is using enzymes to digest the proteoglycans found in the midline area so that cellular structure and molecular distribution can be examined separately. If molecular degradation alone can already permit retinal axon crossing, then cellular structure may not be important for the function of the barrier.

It is also possible that an artificial midline structure can be created by inserting polymer-like materials into the tectal midline. By manipulating the molecular content of the inserted polymer, the retinal axons can probably be either induced to grow across the midline when the molecule is axon growth promotive, or repelled from the midline when the molecule is inhibitory to axon growth.

## BIBLIOGRAPHY


Abney, E.R., Bartlett, P.P., and Raff, M.C., Astrocytes, ependymal cells, and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain, *Developmental Biology*, 83 (1981) pp. 301-310.

Alliot, F., Delhaye-Bouchaud, N., Geffard, M. and Pessac, B., Role of astroglial cell clones in the survival and differentiation of cerebellar embryonic neurons, *Developmental Brain Research*, 44 (1988) pp. 247-257.

Armson, P.F., Bennett, M.R. and Raju, T.R., Retinal ganglion cell survival and neurite regeneration requirements: the change from Muller cell dependence to superior colliculi dependence during development, *Developmental Brain Research*, 32 (1987) pp. 207-216.

Barradas, P.C., Cavalcante, L.A., Mendez-Otero, R. and Vieira, M.A., Astroglial differentiation in the opossum superior colliculus, *Glia* , 2 (1989) pp. 103-111.

Bignami, A., Eng, L.F., Dahl, D. and Uyeda, C.T., Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence, *Brain Research*, 43 (1972) pp. 429-435.

Bignami, A., Raju, T. and Dahl, D., Localization of vimentin, the nonspecific intermediate filament protein, in embryonal glia and in early differentiating neurons, *Developmental Biology*, 91 (1982) pp. 286-295.

Bitner, C., Benjelloun-Touimi, S. and Dupouey, P., Palisading pattern of subpial astroglial processes in the adult rodent brain: relationship between the glial palisading pattern and the axonal and astroglial organization, *Developmental Brain Research*, 37 (1987) pp. 167-178.

Bovolenta, P., Liem, R.K.H. and Mason, C.A., Development of cerebellar astroglia: transitions in form and cytoskeletal content, *Developmental Biology*, 102 (1984) pp. 248-259.

Bovolenta, P., Liem, R.K.H. and Mason, C.A., Glial filament protein expression in astroglia in the mouse visual pathway, *Developmental Brain Research*, 33 (1987) pp. 113-126.

Brunso-Bechtold, J.K. and Henkel, C.K., A possible role of glia in the decussation of hindbrain auditory fibers in the developing ferret, *Abstracts: Society for Neuroscience*, (1988) Abstract 300.9.

Caroni, P. and Schwab, M.E., Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter, *Neuron* , 1 (1988) pp. 85-96.

Caroni, P. and Schwab, M.E., Two membrain protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading, *The Journal of Cell Biology*, 106 (1988) pp.1281-1288.

Chiquet, M., Neurite growth inhibition by CNS myelin proteins: a mechanism to confine fiber tracts?, *TINS*, 12, 1(1989) pp. 1-3.

Choi, B.H., Radial glia of developing human fetal spinal cord: Golgi, immunohistochemical and electron microscopic study, *Developmental Brain Research* , 1 (1981) pp. 249-267.

Choi, B.H. and Kim, R.C., Expression of glial fibrillary acidic protein in immature oligodendroglia, *Science* , 223 (1983) pp. 407-409.

Choi, B.H., Kim, R.C. and Lapham, L.W., Do radial glia give rise to both astroglia and oligodendroglial cells? *Developmental Brain Research* , 8 (1983) pp. 119-130.

Choi, B.H. and Lapham, L.W., Radial glia in the human fetal cerebrum: a combined Golgi immunoflurescent and electron microscopic study, *Brain Research* ,148 (1978) pp. 295-311.

Chuong, C.M., Crossin, K.L. and Edelman, G.M., Sequential expression and differential function of multiple adhesion molecules during the formation of cerebeller cortical layers, *Journal of Cell Biology* , 104 (1987) pp. 331-342.

Cohen, J., Burne, J.F., McKinlay, C. and Winter, J., The role of laminin and laminin/fibronectin receptor complex in the outgrowth of retinal ganglion cell axons, *Developmental Biology,* 122 (1987) pp. 407-418

Crossin, K.L., Hoffman, S., Tan, S.-S. and Edelman, G.M., Cytotactin and its proteoglycan ligand mark structural and functional boundaries in somatosensary cortex in the early postnatal mouse, *Journal of Cell Biology* , 136 (1989) pp. 381-392.

Crossland, W.J. and Uchwat, C.J., Neurogenesis in the central visual pathways of the golden hamster, *Developmental Brain Research* , 5 (1982) pp. 99-103.

Dahl, D., The vimentin-GFA protein transition in rat neuroglia cytoskeleton occurs at the time of myelination, *Journal of Neuroscience Research*, 6 (1981) pp. 741-748.

Dahl, D., Rueger, D.C. and Bignami, A., Vimentin, the 57,000 molecular weight protein of fibroblast filaments, is the major cytoskeletal component in immature glia. *The European Journal of Cell Biology*, 24 (1981) pp. 191-196.

Dahl, D., Strocchi, P. and Bignami, A., Vimentin in the central nervous system *Differentiation*, 22 (1982) pp. 185-190.

Dixon, R.G. and Eng, L.F., Glial fibrillary acidic protein in the optic nerve of the developing albino rat: an immunoperoxidase study of paraffin-embedded tissue, *The Journal of Comparative Neurology*, 201 (1981) pp. 15-24.

Dodd, J. and Jessell, T.M., Axon guidance and the patterning of neuronal projections in vertebrates, *Science,* 242 (1988) pp. 692-699.

Drager, U.C., Edwards, D.L. and Barnstable, C.J., Antibodies against filamentous components in discrete cell types of the mouse retina, *Journal of Neuroscience*, 4 (1984) pp. 2025-2042.

Drazba, J., and Lemmon, V., The role of cell adhesion molecules in neurite outgrowth on Müller cells, *Developmental Biology* , 138 (1990) pp.82-93.

Edwards, M.A., Caviness, Jr, V.S., and Schneider, G.E., Development of cell and fiber lamination in the mouse superior colliculus, *The Journal of Comparative Neurology* , 248 (1986) pp. 395-409.

Edwards, M.A., Schneider, G.E. and Caviness, Jr., V.S., Development of the crossed retinocollicular projections in the mouse, *The Journal of Comparative Neurology*, 248 (1986) pp. 410-421.

Eng, L.F., Vanderhaeghen, J.J., Bignami, A. and Gerstl, B., An acidic protein isolated from fibrous astrocytes, *Brain Research*, 28 (1971) pp. 351-354.

Fedoroff, S., White, R., Neal, J., Subrahmanyan, L. and Kalnins, V.I., Astrocyte cell lineage.II. Mouse fibrous astrocytes and reactive astrocytes in cultures have vimentin- and GFP-containing intermediate filaments, *Developmental Brain Research*, 7 (1983) pp. 303-315.

Franke, W.W., Schmid, E., Osborn, M. and Weber, K., Different intermediate-sized filaments distinguished by immunofluorescence microscopy, *Proc. Natl. Aca. Sci. USA*, 75 (1978) pp. 5034-5038.

Frost, D.O. and Schneider, G.E., Plasticity of retinofugal projections after partial lesions of the retina in newborn syrian hamsters, *The Journal of Comparative Neurology*, 185 (1979) pp. 517-568.

Frost, D.O., So, K.F. and Schneider, G.E., Postnatal development of retinal projections in Syrian hamsters: a study using autoradiographic and anterograde degeneration techniques, *Neuroscience* , 4 (1979) pp. 1649-1677.

Ghandour, M.S., Langley, O.K., and Clos, J., Immunohistochemical and biochemical approaches to the development of neuroglia in the CNS, with special reference to cerebellum, *Int. J. Dev. Neurosci.*, 1 (1983) pp. 411-425.

Godement, P., Salaun, J., and Mason, C. A., Retinal axon pathfinding in the optic chiasm: divergence of crossed and uncrossed fibers, *Neuron* , 5 (1990) pp.173-186.

Gregory, W.A., Hall, D.H. and Bennett, M.V.L., Neuronal and glial gap junctions in the goldfish preoptic area, a thin section and freeze-fracture study, *Developmental Brain Research*, 44 (1988) pp. 9-19.

Gundersen, R.W., Response of sensory neurites and growth cones to patterned substrata of laminin and fibronectin *in vitro*, *Developmental Biology*, 121 (1987) pp. 423-431.

Hajós, F. and Bascó, E., *The Surface-Contact Glia* (Springer-Verlag, New York, 1984).

Hajós, F. and Gallatz, K., Immunocytochemical demonstration of radial glia in the developing rat olfactory bulb with antibodies to glial fibrillary acidic protein, *Developmental Brain Research*, 36 (1987) pp. 131-138.

Hammarback, J.A., Palm, S.L., Furcht, L.T., and Letourneau, P.C., Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin, *Journal of Neuroscience Research*, 13 (1985) pp. 213-220.

Hankin, M.H. and Lund, R.D., Induction of target-directed optic axon outgrowth: effect of retinae transplanted to anophthalmic mice, *Developmental Biology*, 138 (1990) pp. 136-146.

Hatten, M.E., Liem, R.K.H. and Mason, C.A., Two forms of cerebellar glial cells interact differently with neurons *in vitro, The Journal of Cell Biology*, 98 (1984) pp. 193-204.

Hatten, M.E., Fishell, G., Stitt, T.N., and Mason, C.A., Astroglia as a scaffold for development of the CNS, *The Neurosciences*, 2 (1990) pp. 455-465.

Heaton, M.B. and Paiva, M., The influence of target tissue age on neurite outgrowth from chick embryo trigeminal motor nucleus explants, *Developmental Biology*, 116 (1986) pp. 314-318.

Heaton, M.B. and Wayne, D.B., Specific responsiveness of chick trigeminal motor nucleus explants to target-conditioned media, *The Journal of Comparative Neurology*, 243 (1986) pp. 381-387.

Hirano, M. and Goldman, J.E., Gliogenesis in rat spinal cord: evidence for origin of astrocytes and oligodendrocytes from radial precursors, *Journal of Neurosciences Research* , 21 (1988) pp. 155-167.

Hsiao, K.K.-J. and Schneider, G.E., Neonatal hamster eye enucleation and tectal midline damage: effects of partial collicular ablation on retinotectal projections, *Society for Neurosciense Abstracts* , 1978, 4 : 474.

Jen, L.S. and Lund, R.D., Intertectal crossing of optic axons after tectal fusion in neonatal rats, *Brain Research* , 178 (1979) pp. 99-105.

Jhaveri, S., Carman, L. and Hahm, J.-O., Visualizing anterogradely transported HRP by use of TMB histochemistry: comparison of the TMB-SNF and TMB-AHM methods, *The Journal of Histochemistry and Cytochemistry*, 36 (1988) pp.103-105.

Jhaveri, S., Edwards, M.A., Erzurumlu, R.S. and Schneider, G.E., Elongation and arborization of CNS axons, *(Mal)Nutrition and the Infant Brain*, 1990 Wiley-Liss, Inc., pp. 111-125.

Jhaveri, S., Edwards, M.A. and Schneider, G.E., Two stages of growth during development of the hamster's optic tract, *Anat. Rec.*, 205 (1983) pp. 225.

Jhaveri, S., Schneider, G.E. and Erzurumlu, R.S., Axonal plasticity in the context of development, *Vision and Visual Dysfunction: Vol II, Development and Plasticity of the Visual System* , (J. R. Cronly-Dillon, Macmillan Press, London 1991), in press.

Joosten, E.A.J. and Gribnau, A.A.M., Astrocytes and guidance of outgrowing corticospinal tract axons in the rat. An immunocytochemical study using anti-vimentin and anti-glial fibrillary acidic protein, *Neuroscience*, 31, 2 (1989) pp. 439-452.

Kandel, E.R. and Schwartz, J.H., *Principles of Neural Science* (2nd edition, Elsevier, New York, 1985)

Kleitman, N., Simon, D.K., Schachner, M., and Bunge, R.P., Growth of embryonic retinal neurites elicited by contact with Schwann cell surfaces is blocked by antibodies to L1, *Experimental Neurobiology*, 102 (1988) pp. 298-306.

Lander, A.D., Molecules that make axons grow, *Molecular Neurobiology* , 1 (1987) pp. 213-245.

Letourneau, P. C., Madsen, A. M., Palm, S. L., and Furcht, L. T., Immunoreactivity for laminin in the developing ventral longitudinal pathway of the brain, *Developmental Biology*, 125 (1988) pp.135-144.

Letourneau, P.C., Shattuck, T.A., Roche, F.K., Takeichi, M., and Lemmon, V., Nerve growth cone migration onto Schwann cells involves the calcium-dependent adhesion molecule, N-cadherin, *Developmental Biology*, 138 (1990) pp. 430-442.

Levitt, P., Cooper, M.L. and Rakic, P., Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: an ultrastructural immunoperoxidase analysis, *The Journal of Neuroscience*, 1 (1981) pp. 27-39.

Levitt, P. and Rakic, P., Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain, *The Journal of Comparative Neurology*, 193 (1980), pp. 815-840.

Lund, R.D and Lund, J.S., Development of synaptic patterns in the superior colliculus of the rat, *Brain Research*, 42 (1972) pp. 1-20.

Maggs, A. and Scholes, J., Glial domains and nerve fiber patterns in the fish retinotectal pathway, *The Journal of Neuroscience*, 6 (1986) pp. 424-438.

Mason, C.A., Misson, J.P., Blazeski, R., and Godement, P., Retinal axon navigation in the mouse optic chiasm: midline cues and cell-cell relations, *Society for Neuroscience Abstract*, 16, (1990), 463.2.

Matsunaga, M., Hatta, K., Nagafuchi, A. and Takeichi, M., Guidance of optic nerve fibers by N-cadherin adhesion molecules, *Nature*, 334 (1988) pp. 62-64.

McLoon, S. C., McLoon, L. K., Palm, S. L., and Furcht, L. T., Transient expression of laminin in the optic nerve of the developing rat, *The Journal of Neuroscience*, 8 (1988) pp.1981-1990.

McDermott, K.W.G. and Lentos, P.L., The distribution of glial fibrillary acidic protein and vimentin in postnatal marmoset (*Callithrix jacchus*) Brain, *Developmental Brain Research*, 45 (1989) pp. 169-177.

Misson, J., Edwards, M.A., Yamamoto, M. and Caviness, Jr., V.S., Identification of radial glial cells within the developing murine central nervous system: studies based upon a new immunohistochemical marker, *Developmental Brain Research*, 44 (1988) pp. 95-108.

Mori, K., Ikeda, J. and Hayaishi, O., Monoclonal antibody R2D5 reveals midsagittal radial glial system in postnatal developing and adult brainstem, *Proc. Natl. Acad. Sci. USA* , 87 (1990) pp. 5489-5493.

Neugebauer, K.M., Tomaselli, K.J., Lillien, J., and Reichardt, L.F., N-cadherin, NCAM, and integrins promote retinal neurite outgrowth on astrocytes *in vitro*, *The Journal of Cell Biology*, 102 (1988) pp. 1177-1187.

Noble, M., Fok-Seang, J. and Cohen, J., Glia are a unique substrate for the *in vitro* growth of central nervous system neurons, *The Journal of Neuroscience*, 4 (1984) pp.1892-1903.

Ogawa, H., Sato, Y., Takeshita, I., Tateishi, J. and Kitamura, K., Transient expression of glial fibrillary acidic protein in developing oligodendrocytes *in vitro* , *Developmental Brain Research* , 18 (1985) pp. 133-141

Oohira, A., Matsui, F., and Katoh-Semba, R., Inhibitory effects of brain chondroitin sulfate proteoglycans on neurite outgrowth from PC 12D cells, *The Journal of Neuroscience*, 11 (1991) pp. 822-827.

Patterson, P.H., On the importance of being inhibited, or saying no to growth cones, *Neuron*, 1 (1988) pp. 264-267.

Pixley, S.R. and De Vellis, J., Transition between immature radial glia and mature astrocytes studies with a monoclonal antibody to vimentin, *Developmental Brain Research* , 15 (1984) pp. 201-209.

Placzek, M., Tessier-Levigne, M., Jessell, T., and Dodd, J., Orientation of commissural axons *in vitro* in response to a floor plate-derived chemoattractant, *Development*, 110 (1990) pp. 19-30.

Poston, M.R., Jhaveri, S., Schneider, G.E. and Silver, J., Damage of a midline boundary and formation of a tissue bridge allows the misguidance of optic axons across the midline in hamsters, *SN Abstract* , 14 (1988) pp. 594.

Raedler, E., Raedler, A. and Wegener, G., The median ventricular formation, *Anatomy and Embryology*, 165 (1982) pp. 377-387.

Raff, M.C., Glial cell diversification in the optic nerce, *Science* , 243 (1989) pp. 1450-1455.

Raff, M.C., Fields, K.L., Hakomori, S.I., Mirsky, R., Pruss, R.M. and Winter, J., Cell-type-specific markers for distinguishing and studying neurons and the major classes of glial cells in culture, *Brain Research* , 174 (1979) pp. 283-308.

Raff, M.C. and Miller, R.H., Noble, M., A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium, *Nature* , 303 (1983) pp. 390-396.

Raff, M.C. and Miller, R.H., Glial cell development in the rat optic nerve, *Trends in Neuroscience* , December 1984, pp. 469-472.

Rakic, P., Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A golgi and electronmicroscopic study in Macacus Rhesus, *Journal of Comperative Neurology* , 141 (1971) pp. 283-312.

Rakic, P., Neuronal-glial interaction during development, *Trends in Neuroscience*, 7 (1981) pp. 184-187.

Rickmann, M., Amaral, D.G. and Cowan, W.M., Organization of radial glial cells during the development of the rat dentate gyrus, *The Journal of Comparative Neurology* , 264 (1987) pp. 449-479.

Rousselet, A., Autillo-Touati, A., Araud, D. and Prochiantz, A., *In vitro* regulation of neuronal morphogenesis and polarity by astrocyte-derived factors, *Developmental Biology*, 137 (1990) pp. 33-45.

Rutishauser, U., Influences of the neural cell adhesion molecule on axon growth and guidance, *Journal of Neuroscience Research*, 13 (1985) pp. 123-131.

Schneider, G.E., Early lesions of superior colliculus: factors affecting the formation of abnormal retinal projections, *Brain, Behavior and Evolution* , 8 (1973) pp. 73-109.

Schneider, G.E., Jhaveri, S., Edwards, M.A. and So, K.F., Regeneration, re-routing, and redistribution of axons after early lesions: changes with age, and functional impact, *Recent Achievements in Restorative Neurology 1: Upper Motor Neuron Functions and Disfunctions* (Karger, Basel, Switzerland 1985) pp. 291-309.

Schnell, L., and Schwab, M. E., Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors, *Nature* , 343 (1990) pp.269-272.

Schnitzer, J., Franke, W.W. and Schachner, M., Immunocytochemical demonstration of vimentin in astrocytes and ependymal cells of developing and adult mouse nervous system, *The Journal of Cell Biology* , 90 (1981) pp. 435-447.

Schnitzer, J., Immunocytochemical studies on the development of astrocytes, Müller (glial) cells, and oligodendrocytes in the rabbit retina, *Developmental Brain Research* , 44 (1988) pp. 59-72.

Schwab, M. E., Myelin-associated inhibitors of neurite growth, *Experimental Neurology* , 109(1990) pp.2-5.

Schwab, M. E., and Caroni, P., Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading *in vitro* , *Journal of Neuroscience* , 8 (1988) pp. 2381-2393.

Schwab, M.E. and Schnell, L., Channeling of developing rat corticospinal tract axons by myelin-associated neurite growth inhibitors, *The Journal of Neuroscience*, 11, 3 (1991) pp. 709-721.

Sharp, G., Osborn, M. and Weber, K., Occurence of two different intermediate filament proteins in the same filament *in situ* within a human glioma cell line, *Experimental Cell Research* , 141 (1982) pp. 385-395.

Skoff, R.P., Gliogenesis in rat optic nerve: astrocytes are generated in a single wave before oligodendrocytes, *Developmental Biology*, 139 (1990) pp. 149-168.

Silver, J., Studies on the factors that govern directionality of axonal growth in the embryonic optic nerve and at the chiasm of mice, *The Journal of Comparative Neurology* , 223 (1984) pp. 238-251.

Silver, J., and Ogawa M. Y., Postnatally induced formation of the corpus callosum in acallosal mice on glia-coated cellulose bridges, *Science* , 220 (1983) pp. 1067-1069.

Silver, J. and Rutishauser, U., Guidance of optic axons *in vivo* by a preformed adhesive pathway on neuroepithelial endfeet, *Developmental Biology* , 106 (1984) pp. 485-499.

Silver, J. and Sapiro, J., Axonal guidance during development of the optic nerve: the role of pigmented epithelia and other extrinsic factors, *The Journal of Comparative Neurology* , 202 (1981) pp. 521-538.

Silver, J. and Sidman, R., A mechanism for the guidance and topographic patterning of retinal ganglion cell axons, *The Journal of Comparative Neurology* , 189 (1980) pp. 101-111.

Smith, G.M., Miller, R.H. and Silver, J., Changing role of forebrain astrocytes during development, regenerative failure, and induced regeneration upon transplantation, *The Journal of Comparative Neurology* , 251 (1986) pp. 23-43.

Smith, G.M., Rutishauser, U., Silver, J. and Miller, R.H., Maturation of astrocytes *in vitro* alters the extent and molecular basis of neurite outgrowth, *Developmental Biology*, 138 (1990) pp. 377-390.

Snow, D. M., Lemmon, V., Carrino, D. A., Caplan, A. I., and Silver, J., Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro*, *Experimental Neurology* , 109 (1990b) pp.111-130.

Snow, D. M., Steindler, D. A., and Silver, J., Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier, *Developmental Biology*, 138 (1990a) pp.359-376.

So, K.F., Development of abnormal recrossing retinotectal projections after superior colliculus lesions in newborn Syrian hamsters, *Journal of Comparative Neurology* , 186 (1979) pp. 241-258.

So, K.F. and Schneider, G.E., Abnormal recrossing retinotectal projections after early lesions in Syrian hamsters: age-related effects, *Brain Research* , 147 (1978) pp. 277-295.

So, K.F., Schneider, G.E. and Ayres, S., Lesions of the brachium of the superior colliculus in neonate hamsters: correlation of anatomy with behavior, *Experimental Neurology* , 72 (1981) pp. 379-400.

So, K.F., Schneider, G.E., and Frost, D.O., Normal development of the retinofugal projections in Syrian hamsters, *Anat. Rec.*, 187 (1977) abstract, pp. 719.

Sommer, I., Lagenaur, C. and Schachner, M., Recognition of Bergmann glial and ependymal cells in the mouse nervous system by monoclonal antibody, *The Journal of Cell Biology* , 90 (1981) pp. 448-458.

Sotelo, C., Maturation of the nervous system , *Progress in Brain Research* (Elsevier, Amsterdam, 1978) 48, pp.149-170.

Steindler, D.A., O'Brien, T.F., Laywell, E., Harrington, K., Faissner, A. and Schachner, M., Boundaries during normal and abnormal brain development: *in vivo* and *in vitro* studies of glia and glycoconjugates, *Experimental Neurology*, 109 (1990) pp. 35-56.

Suarez, I., Fernandez, B., Bodega, G., Tranque, P., Olmos, G. and Garcia-Segura, L.M., Postnatal developmnent of glial fibrillary acidic protein immunoreactivity in the hamster arcuate nucleus, *Developmental Brain Research* , 37 (1987) pp. 89-95.

Thanos, S., Bonhoeffer, F., and Rutishauser, U., Fiber-fiber interaction and tectal cues influence the development of the chicken retinotectal projection, *Proc. Natl. Acad. Sci. USA*, 81 (1984) pp. 1906-1910.

Tomaselli, K. J., Neugebauer, K. M., Bixby, J. L., Lilien, J., and Reichardt, L. F., N-Cadherin and integrins: two receptor systems that mediate neuronal process outgrowth on astrocyte surfaces, *Neuron* , 1 (1988) pp.33-43.

Uyeda, C.T., Eng, L.F. and Bignami, A., Immunological study of the glial fibrillary acidic protein, *Brain Research* , 37 (1972) pp. 81-89.

Vanselow, J., Thanos, S., Godement, P., Henke-Fahle, S. and Bonhoeffer, F., Spatial arrangement of radial glia and ingrowing retinal axons in the chick optic tectum during development, *Developmental Brain Research* , 45 (1989) pp. 15-27.

Wang, E., Cairncross, J.G., and Liem, R.K.H., Identification of glial filament protein and vimentin in the same intermediate filament system in human glioma cells, *Proc. Natl. Acad. Sci. USA* , 81 (1984) pp. 2102-2106.

Wehrle, B. and Chiquet, M., Tenascin is accumulated along developing peripheral nerves and allows neurite outgrowth *in vitro*, *Development*, 110 (1990) pp. 401-415.

Wolswijk, G., Riddle, P.N., and Noble, M., Coexistence of perinatal and adult forms of a glial progenitor cell during development of the rat optic nerve, *Development*, 109 (1990) pp. 691-698.

## ACKNOWLEDGEMENTS

I thank my advisor, Professor Gerald E. Schneider, for his highly appreciated guidance. His insights in determining research goals and his deep knowledge of the neurosciences not only have benefited me as his student but will lay a strong foundation for the future. I thank Professor Arthur D. Lander, Professor Ann M. Graybiel and Professor Carol A. Mason for their thoughtful and enlightening advice and discussions. Their distinguished research, deep perception in addressing scientific questions, and strong interest in teaching are the most treasured parts of my training and research.

I specially thank Dr. Sonal Jhaveri for her constant, valued advice over the years. Her mastery of the neurosciences and thoroughness in research have been, and will continue to be an example for my research career. The time she spent during my preparation of the thesis is much appreciated.

I also thank Dr. Josef Kapfhammer and Dr. Reha Erzurumlu for their precious advice, help, and friendship. Their outstanding knowledge and accomplishments in neuroscience have been an indelible part of my study. Their trust and friendship have always brightened my day.

I thank Professor William G. Quinn, Jr. and Janice Ellertson for their exceptional administration of student training in the department, for their deep concern, and for their generous help whenever needed.

My special thanks to Laurie L. Collier for her constant assistance and willingness to help in day to day matters allowing me to concentrate on my research. I am lucky to have a colleague who is also a wonderful friend.