1988

Radial Glia and the Migration of Young Neurons in the Adult Avian Brain

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RADIAL GLIA AND THE MIGRATION OF YOUNG NEURONS IN THE ADULT AVIAN BRAIN.

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A thesis submitted to the faculty of
The Rockefeller University
in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy

New York, N.Y. 1988

The unique and magical approach to science of my advisor, Fernando Nottebohm, is the basis of the work presented here. His teachings, enthusiasm and friendship will be permanent lessons for me. Comrades at both Millbrook and the city branches of the Nottebohm laboratory have helped me with many aspects of this work. I owe special thanks to Marga Theelen who helped me with some of the tedious mapping presented in Chapter 3; collaborating with her in this work and others not presented here has been most enjoyable. David Vicario was always ready to talk, made useful suggestions for this dissertation and helped me put together the system described in Appendix A. Daniel Buskirk gave me antibody 40E-C and shared his sorcery about monoclonal antibodies. Judy Dye and Commander in Chief, Barbara O'Loughlin, kept the laboratory running smoothly and helped me with spelling and typing, a few of the many things I am bad at. Many thanks to Amanda Tolles and the bird keepers in Millbrook, Dawn Jackson and Sharon Sepe; their help and great care for the birds was fundamental for this thesis. The instrument shop with the help of Rudolf Franz made possible the construction of small gadgets that, much to Rudy's surprise, worked. Gianni Piperno's advice on Western blot technique was very useful for experiments presented in Chapter 2. The basic research training I received at the Instituto de Investigaciones Biomedicas of the Universidad Autonoma de Mexico has been most useful. Special thanks to Horacio Merchant who taught me so many things and to Jay Valinsky who first introduced me to the developing nervous system and to Rockefeller University. I am grateful to Nicole LeDouarin and Marie-Aimee Teillet for the things that I learned during the stay in their laboratory. My thesis committee: Torsten Wiesel, Bruce McEwen and Bernice Grafstein, read and made useful comments on my thesis. Many friends and professors have made my study at Rockefeller most enjoyable. I am thankful to them and to this Institution for this great opportunity. Truly valuable and challenging has been the informal learning from my sisters, grandparents and very good friends, Alejandro Garciarrubio and Enrique Escandon. Specially I would like to thank Ellen Prediger for her companionship and unlimited help, and my parents who were the first and very best of my friends and teachers.

Note to the reader:

The work described here is divided into units and these units presented as chapters. This is done to facilitate in the reading of the story I wish to tell, and to ease the access to specific information. The first chapter states the problem and gives general background on neurogenesis in development and adulthood. Chapters 2, 3 and 4 describe the main findings of this thesis and contain specific discussions for this data. In Chapter 5 the general conclusions are evaluated and future experimental alternatives with preliminary results are presented. In order to avoid repetition of experimental procedures used in different chapters, all material and methods are presented in chapter 6. This was also done so that any one interested in a specific protocol could easily find it. Appendix A is also part of methods and describes a new technique to map histological sections developed for this thesis. This technique is presented separately in detail because it can be of general interest for many other applications.

ABSTRACT

The adult bird brain continues to produce new neurons and integrate them into functional circuits. These cells are born in the ventricular zone (VZ) of the lateral ventricle, sometimes up to 5-6 mm away from the location were some of these neurons finally mature (Nottebohm, 1985). How do new neurons find their way through the adult parenchyma away from the ventricle and into their final location?

An antibody prepared against adult canary brain, 40E-C, stained ventricular zone cells that send long, unbranched processes into the telencephalon. Based on this morphology and their partial reactivity to GFAP we identified these cells as radial glia. The same antibody also stained a subset of brain astroglia and reacted with non-brain material such as mesenchyme, Sertoli cells and the Z-line of muscle. A weaker reaction was obtained with erythrocytes and some endothelial cells. 40E-C also reacted with radial glia of developing rat brain but failed to show any such glia in adult rodent brain. Western blot analysis showed that this antibody recognizes vimentin.

The position of radial glia was systematically mapped in adult male and female canary brain. Radial glia fibers were found to orient mainly in the mediolateral plane and were more abundant in some parts of the telencephalon (e.g. hyperstriatum, caudal neostriatum and lobus parolfactorius) than in others (e.g. anterior neostriatum, archistriatum and septum), which had few or no radial glia fibers.

Radial glia in mammals normally convert into adult astrocytes, their persistence into adulthood in birds probably relates to neurogenesis. This hypothesis is based on the following findings. A small, elongated cell type not previously described in adult avian brain was frequently seen to be associated with the long processes of the radial glia, oriented in the same direction and often in close apposition. Mapping the positions of these cells showed them to be almost unique to the telencephalon and therefore absent in regions where neurogenesis does not occur. It is then shown that the new elongated cells are the precursors of new neurons. At short survivals after [^{3}H]-thymidine (a tag for dividing cells) injections, many VZ cells are labeled only on the lateral walls of the lateral ventricles. After three days from the last [^{3}H]-thymidine injection, the number of labeled VZ cells drops as the number of labeled elongated cells increases. By day 20 the number of labeled elongated cells reaches a maximum and decreases thereafter as labeled neurons start to appear.

One day after the last [^{3}H]-thymidine injection, labeled elongated cells were present only very close to the VZ. Two days later the labeled elongated cells have moved laterally and by six days some of these cells are as far as 2.5 mm from the ventricle. Between days 15 and 20 these cells reach the most distant areas of the telencephalon. During the first week of migration most labeled elongated cells are found in regions rich in radial glia. The velocity of migration during the first 6 days is at least 20um per hour. Between days 6 and 15 the velocity drops to 8um per hour and we believe that this happens as many of the labeled elongated cells detach from the radial glia. Thus radial glia are used as fast dispersal pathways at least during the first week of migration.

Since the number and distribution of labeled glia does not change with survival time, we conclude that the labeled elongated cells are migrating cells that differentiate only into neurons: that is, they are young migrating neurons. The number of migrating cells exceeds by a factor of 3 the number of new neurons that form. Many labeled picnotic (dying) cells can be seen at the time when migrating cells start to decrease in number. This raises the possibility that new neurons attain their final position by the selective survival of migrating cells only at sites where new neurons are required.

New preliminary experimental approaches to adult neurogenesis are presented. These include, intraventricular injections of fluorogold to define the cytoplasmic anatomy of migrating neurons, the use of the antimitotic ARA-C to block neurogenesis and the precise mapping of discrete zones of cell proliferation in the ventricular walls of the adult canary brain.

The unique properties of neuronal migration in an adult brain provides a new model and new clues for the understanding of neurogenesis.

CONTENTS

CHAPTER 1

INTRODUCTION

For many years, it has been thought that vertebrate neurogenesis was a developmental phenomenon, terminating in early post-natal life. It seemed impossible that new neurons could correctly position and connect themselves in the relatively fixed matrix of the adult brain. The discovery of high rates of adult neurogenesis in canaries forces us to find ways in which this could happen: where and when are neuroblasts dividing? and how do young postmitotic neurons find their way to their final site of differentiation? This thesis explores how this might occur. The picture which emerges is that of a brain of dynamic structure, in which cells divide in the walls of the ventricular cavities, engage in long migrations guided by a complex system of long glial fibers to finally differentiate into neurons.

As with many other kinds of cells, neurons are not able to divide once they reach final differentiation. These cells form from precursor cells (stem cells) that exit the cell cycle to become terminally differentiated (Baserga, 1985). The stem cells that give rise to neurons (true *neuroblasts*, Jacobson, 1978 p. 28-29) divide only in specific proliferative regions of the brain. These regions are found adjacent to the ventricular cavities of the brain in what is known as the ventricular zone (Boulder Committee, 1970). Such proliferative zones are usually removed from the sites where neurons achieve their final anatomical and physiological identity.

Neurogenesis involves not only neuroblast division, but also migration and path-finding by the postmitotic cells from the neurogenic zone to their final site of differentiation. Thus, a description of neurogenesis requires identification of the neuroblast population and of migratory cells destined to became neurons, which I will call *young migrating neurons*.

The dogma has been that neurogenesis is a developmental phenomena. However, during the last three decades exceptions have been found and the pervasiveness of this rule has come under question. Not only has neurogenesis been documented in adult fish, which continue to grow after reaching sexual maturity (e.g. Leonard *et al.*, 1978), but it has also been reported in mammals (Altman, 1970). The best documented example of neurogenesis in adult vertebrate brain comes from the avian literature. It is this example that has established beyond doubt that new neurons can continue to be produced in adult nervous systems that have ceased to grow.

New neurons continue to be added to the adult canary brain (Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984; Burd and Nottebohm, 1985). The first report of this phenomenon concerned the higher vocal center (HVc), a specialized area of the telencephalon that is closely apposed to the floor of the lateral ventricle (Nottebohm *et al.*, 1976; Nottebohm, 1987). The new neurons, it was reported, were born in the ventricular zone overlying HVc, from where they migrated over distances no greater than 500 um to reach the sites of their final differentiation (Goldman and Nottebohm, 1983). Soon thereafter, however, it was reported that neurons born in adulthood took positions throughout the telencephalon, up to 5 mm away from the nearest ventricular wall (Nottebohm, 1985).

It is, perhaps, surprising that adult neurogenesis happens at all. For it to occur: 1) neuroblasts have to share the ventricular zone (VZ) with functioning adult ependymal cells; 2) young neurons have to move large distances from the VZ to their final destination, migrating through the dense network of fibers and cell bodies of the functioning adult brain parenchyma; 3) they have to locate their final position where, 4) they have to differentiate into mature neurons. A rich literature exists on how each of these steps occurs during development, but not a word on how this might happen in adulthood.

My Doctoral Dissertation identifies the location of proliferative zones within the VZ, it describes the morphology of young migrating neurons and the paths they follow during migration, their rate of displacement, and the time elapsed between birth and their assumption of an adult neuronal morphology.

As indicated above, previous knowledge of the processes of neurogenesis stems mainly from work on embryonic and fetal material. A brief general review of this knowledge follows. The chapter ends with a review of what was known about adult neurogenesis when my doctoral research commenced.

Neurogenesis in the developing brain.

The primordium of the vertebrate brain is a pseudostratified columnar epithelium in which spindle-shaped cells are packed together like cigarettes in a box. One end of the cell is in contact with the outer brain surface (limitant membrane), the other with the inner wall facing the brain ventricles. Between this palisade, distinctly more rounded cells can be seen and in these cells mitotic figures are common. It is from these cells that

all neurons and glia of the brain form. It is not known to this day if there are at these early stages of development predetermined populations of neuroblasts and glioblasts. (Jacobson, 1978). Wilhelm His (1887) thought that the round and the spindle-shaped cells belonged to two separate cell populations. He suggested that the first, mitotically active, were neuroblasts ("germinative corpuscles"), and the second, spindle-shaped, he called "spongioblasts". He thought that the spongioblasts formed a syncytium and were the precursors of glia. He failed to recognize an alternative interpretation which had in fact been suggested by a contemporary, Schaper (1897). What appeared to be two cell types was one cell in different stages of the cell cycle (F.C. Sauer, 1935).

The first signs of neuronal differentiation do occur at the level of the ventricular zone but slightly later during development. At the beginning of this century Ramón y Cajal, using silver impregnation techniques, described the genesis of neurons. His writing remains one of the most lucid on this subject and for this reason his initial four phases of neurogenesis will be briefly summarized (see review Ramón y Cajal 1929, pp 71-116).

1) Germinative Cell. This cell corresponds to the neuroblast (germinative corpuscle of His). It is the mitotically active cell in the ventricular zone.

2) Apolar cell; Is the earliest stage at which the immature neuron is capable of fixing silver. This cell is round and remains close to the VZ. The silver stained neurofibrillary network distinguishes it from the neuroblast. In a few cases, Ramón y Cajal found that some of these apolar cells start to orient their neurofibrillary tangle radially between spongioblasts. As shown below by more recent markers, these cells might still undergo mitosis.

3) Bipolar cells. The apolar cell transforms into a bipolar elongated cell by the formation of two prolongations. This happens as the young neurons migrate away from the ventricular zone. With the silver impregnation techniques used on embryonic chick spinal cord and the retina between days 2 and 4 of incubation, Ramón y Cajal found that the distal process was frequently larger and darker. He suggested that this was the axonal primordium. The bipolar cells migrate wedged between spongioblasts (also called "epithelial" cells by Ramón y Cajal). Initially the distal process follows the orientation of the "epithelial" elements. The growth cone then turns 90^{o} when it reaches the limitant membrane and starts growing tangentially (parallel to the neural tube). At these developmental stages the thickness of the neural tube is less than 100um and many of the young migrating neurons could transiently be in contact with both the outer limitant membrane and the inner ventricular surface. The anatomy of these bipolar cells as depicted in drawings, shows them spindle-shaped with an elongated nucleus often showing two nucleoli (e.g. Ramón y Cajal 1929, pp. 82).

4) Phase of Monopolarity. The bipolar cell, upon its arrival to the distal margin of the spinal cord and/or retina, loses its proximal process, becoming unipolar. The remaining process becomes the axon and the dendritic tree is then produced de novo.

This scheme, still accepted today, serves to illustrate the basic steps in neuronal genesis. The identity of the neuroblast (apolar cell of Cajal) fibrillary network stained by Ramón y Cajal remained unknown for many decades. Only recently immunofluorescence of the medium sized neurofilament protein (NF-M) has provided a similar neurofibrillary pattern in the neuroblasts of early developing chick embryos (Bennent and DiLullo, 1985). It is therefore likely that the reduced silver methods of Cajal were revealing the

incipient neuronal intermediate filament network. It is of interest that argentophilic methods (Sechrist, 1969) as well as staining with neurofilament antibodies reveal that the first cells to express this neuronal intermediate filament are still mitotic (Bennet and DiLullo, 1985) suggesting that neuroepithelial cells committed to form neurons might continue dividing. However, in some regions of the early chick embryo telencephalon all cells transiently express NF-M. Since this neuroepithelium later gives rise to glial cells, this observation suggests that glial precursors also transiently express NF-M (Bennet and DiLullo, 1985).

The introduction of [^{3}H]-thymidine autoradiography to the study of neurogenesis (Sidman *et al.*, 1959) has made possible the precise dating of the birth of neurons in the CNS. The radioactive nucleotide becomes incorporated into the DNA as a stable marker during S phase of the cell cycle. Cells carrying this marker, divide, migrate and differentiate, and are then visualized at any time by autoradiography. Many such studies (reviewed by Jacobson, 1978 pp.58-88) reveal three principles of nervous system development, 1) Phylogenetically older parts of the brain generate their neurons earlier than brain structures added more recently in evolution. 2) The larger 'long projection' neurons are born first, then come intermediate sized neurons and last, small neurons, generally interneurons. This last principle is best illustrated in the 'inside out' genesis of the cerebral cortex of mammals, where the large pyramidal long projecting neurons of layer VI are produced first while cells in more superficial layers are produced sequentially later and have to migrate past their older siblings (Angevine and Sidman, 1961). 3) Glial cells are born after neurons and unlike them, glial cells not only divide in the ventricular zone but can also incorporate [^{3}H]-thymidine within the brain parenchyma. Thus, the fact that post-mitotic young migrating neurons do not

incorporate [^{3}H]-thymidine defines them as neuronal in character in contrast to glia (Jacobson, 1978 p.44).

Cells that incorporate [^{3}H]-thymidine in the ventricular zone later appear as differentiated neurons deep in the central nervous system parenchyma. The distance that newly generated neurons have to migrate depends on the age at which they were born. As the brain increases in size so does the migratory path. Thus, initially young neurons might need little guidance to complete their migration, but at later stages, migration pathways are required. Ramón y Cajal (1929, pp. 88) first suggested that the leading process of a migrating neuron follows the orientation of the epithelial cells, though he never directly assigned a major role to the epithelial cells as 'migratory guides'. Recent work strongly suggests that this is the case.

There is considerable confusion in the terminology used to refer to the elongated cells that have their cell bodies at or near the ventricular zone and project one or several processes into the brain parenchyma (see also Chapter 2). I began referring to them as epithelial cells following the terminology of Ramón y Cajal (1911), however before reviewing their role as the scaffolding for the developing brain, the use of the term, *'radial glia'*, now in vogue to describe these cells, should be put into perspective.

By the end of the last century many scientists identified these elongated cells in Golgi stain-impregnated embryonic and fetal material. Magini (1888) was first to call them "radial" glia ("cellules radiees de la nevroglie"). Golgi (1885), Vignal (1888), Sala y Pons (1894), Thomas (1894) and Ramón y Cajal (1911) referred to them as epithelial cells. Other authors refer to them as fetal ependyma (Retzius, 1894) or spongioblasts (Kölliker, 1890). Almost a century elapsed until Rakic (1971a,1972) reintroduced the

term 'radial glia' into the literature. Now it is widely used by many doing research in developmental neurobiology.

The terminology used to describe cells in the developing brain suffers from basic flaws. This problem probably arises as a result of the assignment of static names to cellular elements that are constantly changing. It is likely that the group of cells we collectively call radial glia at later stages of development, include a mixture of cell types, all originating from the early neuroepithelial cells that have become greatly elongated due to increased thickness of the brain walls. Since these cells include neuronal and glial precursors, the glial character of radial glia in some cases is uncertain. Arguments in favor of the glial identity of radial glia include: 1) Their disappearance after most neurons have been generated and coincident with gliogenesis (see below); 2) Lamellate expansions and/or ending on blood vessels, which have been suggested to be diagnostic signs of glial phenotype (Schmeckel and Rakic, 1979); 3) The presence of glial intermediate filament protein (GFAP), (Bignami and Dalh, 1974 a,b; Levitt and Rakic, 1980); 4) Increased glycogen content in the pial endfeet, considered typical of glial cells (Peters and Feldman, 1973, Peters *et al.*, 1976). Radial glia that show these glial features may not include all elongated cells with cell bodies in the ventricular zone and long radial fibers ending in blood vesels or the surface of the brain. No single criterion identifies individual radial glia. For example, at early stages of fetal development (E48, Rhesus monkey), when elongated cells neither have lamellate expansions nor endings in blood vessels (Schmeckel and Rakic,1979) the glial phenotype of these cells is difficult to establish. In rodents, GFAP is difficult to detect in early radial glia (Bignami and Dahl, 1973, 1974), but instead these cells contain mainly vimentin, a marker for both neuronal and glial precursors. Thus, the brain starts with elongated cells reaching from the ventricle to the brain surface. As the brain increases in size by the addition of new

neurons some elongated cells remain, becoming greatly elongated and ending either on the brain surface or blood vesels, it is these cells that we call radial glia. In mammals radial glia disappear converting into astrocytes (see below).

All the confusion in the use of terminology in developmental neurobiology makes it imperative to define what names refer to. For the purpose of this thesis. I will use: *Neuroblast* as the proliferating precursor of neurons. *Ventricular zone*, as defined by the Boulder Committee (1970) but extrapolated to the adult brain, i.e. a group of highly packed cells (*Ventricular zone cell*) forming a pseudostratified epithelium and covering the walls of the lateral ventricle. This is the site of neuroblast proliferation. *Young Neuron* (or *young migrating neuron*), is equivalent to the bipolar cell of Cajal. This cell moves away from the ventricular zone and into the brain parenchyma to become a neuron. *Radial glia*, an elongated cell with its cell body in the ventricular zone and a long process that penetrates the brain parenchyma. (For an explicit discussion of Radial glia and tanycytes in the canary brain, see chapter 2).

The interest in radial glia during histogenesis is two-fold, 1) It has been claimed that their radially oriented fibers serve as guides for the migration of neurons and, 2) They are thought to transform into astrocytes. The evidence for these two inferences is reviewed separately below.

Radial glia as guides for young migrating neurons. Magini suggested in 1888 that the swelling observed on the shafts of radial glia might correspond to young developing neurons. Ramón y Cajal (1929, pp. 88) later showed how the leading process of the migrating neuron followed the orientation of the radial glia (epithelial cells). This observation was further substantiated by the ultrastructural studies of Rakic (1971a,b and

1972). The close association found between young migrating neurons and radial glia in the mammalian cerebellum and neocortex (Rakic 1971a,b, 1972) led to the suggestion that the young neuron moves along the surface of the radial glia fiber (Rakic 1972). In support of this interpretation are 3 other observations. 1) The orientation of radial glia is appropriate, establishing a bridge between the ventricular zone and distant regions were neurons finally mature, 2) In mammals, these cells are transitory and are present only during the period of neuronal migration, disappearing thereafter. 3) Young migrating neurons have been observed to move in vitro along glial fibers (Edmondson and Hatten 1987). While radial glia may play a central role in guiding neuronal migration under some conditions and in some regions of the brain, migration independent of glial guidance has also been demonstrated. The best example is in the formation of peripheral ganglia by neural crest cell migration (LeDouarin et al, 1984). In this case young migrating neurons move through the mesenchyme around and in between somites. In the CNS as well, it has been suggested that migration can follow cues other than radial glia (Zagon, *et al.*, 1985; Rakic, 1985). In the formation of the pontine gray nuclei, migrating cells move along axonal pathways in this case radial glia fibers are perpendicular to the migratory direction of the young neurons. (Rakic, 1985). In the case of the granular neuron in the cerebellum, part of the cell (leading process) follows the radial fiber of Bergmann glia towards the granular layer while the trailing process aligns with axons. Thus, migrating young neurons are highly mobile cells that can follow and transverse different terrains and perhaps use a diversity of pathfinding cues. The conditions that determine which cues the young migrating neuron will follow remain unknown.

During development it has been shown that neuronal migration occurs at a rate of 2-6 um/h (Jacobson 1978, pp 66 and 87). Altman (review, 1970) calculated a velocity of 2-3 um/h during postnatal neurogenesis in the hippocampus and olfactory bulb. None of these studies, though, have used sufficiently short intervals to follow closely the migration of young neurons. This becomes relevant in chapter 4.

Conversion of radial glia. In mammals, as neurogenesis and neuronal migration come to an end, radial glia disappear. The "astrocytic" ultrastructural morphology of radial glia and the observation that the disappearance of these cells coincides in time and position with the appearance of astrocytes, had led to the suggestion that radial glia convert into astrocytes (Ramón y Cajal 1911, Schmeckel and Rakic, 1980). Other mature macroglia like Bergmann glia (Rakic, 1971b; Choi and Lapham, 1978), and perhaps tanycytes (Millhouse, 1972) may also originate from radial glia. In contrast, radial glia-like cells continue to occur in the brains of adult reptiles, amphibians and fish (Ramón y Cajal, 1911, pp:628; Van Gehuchten, 1897; Studnicka, 1900; Retzius, 1894, 1898). Relevant to this thesis is the comment by Ramón y Cajal who thought that in birds, as in mammals, radial glia converted into macroglia (p. 834, 1911):

"De meme que chez les mammifères, L'épithélium consiste chez les oiseaux jeunes en cellules allongées, qui s'étendent depuis le ventricule jusqu'à la surface du cerveau. Cette disposition, sans aucun doute embryonnaire, doit disparaitre à l'âge adulte, et, comme chez les mammifères, le bout périphérique des ces éléments névrogliques doit s'atrophier."

Radial glia cells, more than any other cell type in the brain, show this clear phylogenetic tendency to change. To my knowledge the literature says no more about

the fate of radial glia in the developing avian brain and about whether birds and mammals differ systematically in this regard.

Neurogenesis in the adult brain.

Structural changes in the brain have been proposed to play a key role in plasticity and adaptive changes of nervous tissue. Ramón y Cajal (1911; V2, pp 807-820) suggested, for example, that "mental exercise" enhances the growth of axons and dendrites and in this way new neural pathways may be created. This view has been further developed by showing that major anatomical changes in the brain can follow modifications of the environment (Bennet *et al.*, 1966; Connor, *et al.*, 1981; Wiesel, 1982). Neurons are highly specialized terminally differentiated cells. They develop complex interconnections and hence, have a complex structure. A mechanism that meets the demands of learning and behavioral plasticity might, in some cases, require dramatic changes in both neuronal physiology and anatomy. Differentiated neurons might not be able to meet these demands while a new uncommitted cell could accomplish the task. Postnatal genesis of microneurons has been suggested as an important source of structural plasticity in circuits that control learned behaviors. Perhaps neurogenesis could be regulated by the environment (Altman, 1970).

The belief that neurogenesis is a prenatal or early postnatal phenomenon is still widespread. This idea is strongly influenced by the role that genetic factors are thought to play in premolding the structure of the brain. Adult neurogenesis is difficult to conceive based on the feeling that the adult brain is too big and complex to allow for the "embryonic mode of neurogenesis". Alternatively neurons could be dividing close to their final location within the brain parenchyma, this however, has never been shown to happen. Mitotic neurons or neurons that incorporate [^{3}H]-thymidine at short survivals

are never observed. The belief that neurogenesis is a developmental trait has also been strengthened by mammalian studies, where in fact adult neurogenesis may be poorly represented (Rakic 1985). When adult neurogenesis has been reported, it has been regarded as atypical and the result of special circumstances. So for example, olfactory epithelium of adult vertebrates (Graziadei and DeHan, 1973) has been attributed to the need to replace cells exposed to wear and tear. Neurogenesis in the CNS of adult fish (Johns, 1977; Leonard *et al.* 1978; Anderson and Waxman, 1985) has been attributed to the fact that the body and brain of fish continue to grow. Some regions of the adult rodent brain are reported to add new neurons in adulthood (Altman, 1963; Kaplan and Hinds, 1977; Bayer *et al.* 1982, Bayer, 1983). In this case, 1) neurons are added only in restricted areas of the brain (Hippocampus and olfactory bulb), 2) their small size makes neuronal identity difficult and frequently inconclusive, 3) the rate of suggested neuronal recruitment is relatively low, 4) rats also continue to grow after puberty.

In adult canaries, neurogenesis not only continues to happen, but it is a robust phenomenon (Nottebohm, 1985). Different species of birds not limited to songbirds generate new neurons in adulthood, e.g. budgerigars (Melopssittaues undulatus), ring doves (Streptopelia risoia) (Nottebohm, 1985) and Japanese Quail (Coturnix coturnix japonica) (Alvarez-Buylla and Nottebohm, unpublished). Thus neurogenesis seems to be an avian trait. Under the light microscope, labeled neurons have large round, pale nuclei with one or two basophilic nucleoli and irregular shapes of Nissl stained cytoplasm. This staining is typically neuronal and, unlike that of microneurons generated in postnatal hippocampus or olfactory bulb in mammals (Altman, 1970; Kaplan and Hinds, 1977; Bayer *et al.* 1982), they are easy to identify. New neurons in adult birds, look more like the macroneurons that are generated during late development of the basal ganglia in rats (Altman, 1970: p. 212). Agnostics would argue that the clear neuronal morphology

shown by [^{3}H]-thymidine labelled neurons is not sufficient to identify these cells as neurons. This criticism was elegantly met by Paton and Nottebohm (1984). Neurons impaled with microelectrodes and later shown to be labeled with [^{3}H]-thymidine produced action potentials. In addition, some of these cells responded to auditory stimuli and, when filled by peroxidase, they had the typical dendritic and axonal morphology of neurons. Furthermore, EM studies have shown synaptic endings on [^{3}H]-thymidine labeled cells with neuronal ultrastructure (Burd and Nottebohm, 1985).

A further possible criticism is that the new neurons incorporated [^{3}H]-thymidine by a mechanism other than cell division (e.g. DNA repair). Two observations support the interpretation that these adult neurons are newly generated. 1) The number of grains per labeled cell is not significantly different in neurons than in glia or endothelial cells which are known to divide in adult brain (Nottebohm, 1985). 2) At short survival times after [^{3}H]-thymidine administration no labeled neurons are found, but labeled ventricular zone cells were observed (Goldman and Nottebohm, 1983). These observations suggest that, as during development, new neurons are generated at distant proliferative zones.

The connectivity of the newly generated cells has been studied in HVc by combining a retrograde label (or immunocytochemical stains) with [^{3}H]-thymidine. HVc projects to two other discrete brain nuclei: area X of lobus paraolfactorius and robustus archiestriatalis RA. Therefore, HVc contains at least three classes of neurons: X projecting, RA projecting and interneurons. By backfilling the long projecting neurons either from X or RA, it was found that most of the [^{3}H]-thymidine labelled neurons are interneurons (Paton, *et al.* 1985). The X projecting cells were recently confirmed to be produced early in development while RA projecting cells are added after hatching

(Alvarez-Buylla, *et al.* in prep). This together with the Paton *et al.* (1985) finding of a few RA projecting neurons labelled in adulthood, suggests that some RA projecting cells might be added in adulthood.

Neurogenesis, however, is not restricted to HVc but is widespread in the telencephalon (Nottebohm, 1985). Some of the new neurons added to the telencephalon stain positively for a dopamine associated receptor protein (Paton, et al., 1986). This suggests, together with the evidence presented for HVc, that the new neurons in the adult canary telencephalon are incorporated into existing circuits and thus achieve a functional status.

The significance of neurogenesis in adult brain remains otherwise untested. The size of the canary brain does not increase after one month of age. Sexual maturity is achieved at 8 months. Thus, addition of new neurons is presumably accompanied by cell death and, at least in the case of HVc, by the death of other neurons. The experimental evidence for this system is evaluated in a series of articles by Nottebohm (1984, 1985, 1987)

Summary.

Neurogenesis during development involves 1) the division of precursor cells in the ventricular zone, 2) the conversion of these cells into an elongated cell, 3) the migration of these elongated cells along radial glia or following some other cues and 4) final settlement and differentiation. In adult canaries many new neurons are added throughout the telencephalon, some times several mm away from the site were they were born. The adult brain was considered to large and complex for this to happen. Findings

in the following chapters address these questions: where exactly are new neurons born, and how are they distributed to regions distant from where they divide?

CHAPTER 2

RADIAL GLIA PERSIST IN ADULT AVIAN BRAIN

It is thought that in birds, as in mammals, radial glia disappear and convert into *astrocytes* (Chapter 1; Ramón y Cajal, 1911, pp. 834,V2). The possible relevance of radial glia to neurogenesis in adult avian brain was triggered by a chance observation. During a search for monoclonal antibodies that might be relevant to neurogenesis and neuronal migration in adult canary brain, one monoclonal antibody, 40E-C, was found to stain selectively cells which by their position and anatomy looked like radial glia (Nottebohm, Buskirk, Burd and O'Loughlin, 1985). In this chapter this antibody will be characterized in detail, I will describe these radial glia and other cell types positive to 40E-C and identify the antigen as vimentin (Alvarez-Buylla *et al.*, 1987).

Types of cells found in canary brain that are positive to antibody 40E-C.

I have used immunofluorescence and immunoperoxidase techniques (chapter 6) to study the types of cells that react with antibody 40E-C. Very few cells in the parenchyma of adult canary brain stain with this antibody. However, thin unbranched and relatively straight processes are seen throughout the telencephalon. The density of these fibers increases as one approaches the ventricular wall (also see chapter 3, Fig. 3-2), where many cell bodies and their processes are stained. At low magnification one gets the impression that a continuous layer of 40E-C positive cells lines the walls of the lateral ventricle. Positive cells are also found around blood vessels, on the surface of the brain and in large fiber tracks (Fig. 2-1). A detailed description and identification of these

cells follows.

Radial glia. 40E-C positive cells with their bodies in the ventricular zone of the forebrain project long unbranched fibers reaching into the forebrain parenchyma, sometimes over distances of several millimeters (Fig.2-1 and 2-2A and B). I will call these cells radial glia (more on this nomenclatural point in the Discussion). The 40E-C positive radial glia are interspersed with other ventricular zone cells that are negative for this antibody. The dense packing of cell bodies in the ventricular zone and the many 40E-C positive processes running in between the ventricular zone cells make difficult to estimate the proportion of positive and negative cells.

In some regions of the forebrain, such as the caudal hyperstriatum or the ventral neostriatum (Stokes, *et al.*, 1974), the radial glia processes travel close to or within the ventricular zone for some variable distance. In other areas the radial glia processes project directly out into forebrain (Fig. 2-2A, B and C). The initial segment of the process, in the periventricular parenchyma, is sometimes relatively straight and easy to visualize, but often it is difficult to follow. In the latter case this difficulty probably results from the fact that this part of the process has a corkscrew-like shape, coming in and out of the plane of section (Fig.2-2A). Further away from the ventricle the radial glia fibers straighten out and may travel for several millimeters.

The density of radial glial fibers stained by 40E-C decreases as one moves away from the ventricular zone, suggesting that in adult canaries there is a range of fiber lengths which end in a staggered manner (Fig. 3-2). There is also a range of fiber diameters that varies between 0.4 and 1.5 um.

It is not yet clear whether radial glia fibers end with an anatomical specialization and in association with a particular cell type. One often sees a thickening of the radial glial fibers. These thickenings in many cases are apposed "en passant" to capillary walls (Fig. 2-3 A and B). Rare but still of great interest are fibers that show periodic thickenings along their length, apparently in no particular association to blood vessels (Fig.2-3 C).

Though the above descriptions would fit most of the cells we call radial glia, in a few cases, restricted to ventral neostriatum, we have seen cells that have most of these properties yet whose cell bodies are in the parenchyma close to the ventricular zone (Fig. 2-2 C and D). The identification of such cell bodies has been confirmed by a nuclear counter-stain (Hoechst 33258, Aldrich). We believe that these "displaced" cells are also radial glia because their processes have a similar morphology, run parallel to other radial glia fibers and in some cases can be followed for a long distance (Fig.2C). Displaced radial glia have been described previously during development of the dentate gyrus of the hippocampus in the Rhesus monkey (Eckenhoff and Rakic, 1984), as well as in other systems (Horstmann, 1954; Stevenson and Yoon, 1982).

Tanycytes. Although these cells were originally defined as elongated glial cells (from the Greek, tanus= elongated; Horstmann, 1954), recently the term has been reserved for cells whose cell bodies are at or close to the ventricular wall and which have a process that extends into the periventricular parenchyma, frequently terminating on blood vessels (Millhouse, 1971; Seress, 1980; Bruni *et al.*, 1983). Tanycytes are most abundant in the ventral region of the third ventricle where their fibers form a rich plexus sometimes reaching the surface of the brain in the infundibular region (Bleier, 1971; Millhouse, 1971; Brawer, 1972; Joy and Sathyanesan, 1981; Flament-Durand and Brion, 1985).

Antibody 40E-C stains typical tanycytes in the ventricular wall of the third ventricle of adult canaries. These tanycytes form a rich plexus of fibers around the ventral part of this ventricle. This is shown in Fig. 2-4 D. The processes of adult canary tanycytes typically end at distances of 10 to 300 um from the ventricular wall. In periventricular regions where tanycytic processes are not as dense as near the ventral part of the third ventricle, they can be clearly seen to end on blood vessels (Fig. 2-4 C). In some cases, e.g. dorsal third ventricle, the diameter of the tanycytic fibers tapers down over a short distance, as these fibers course away from the ventricle.

Cells of tanycytic morphology are found in all ventricular walls. Typical tanycytes stained by 40E-C are rare in the ventricular zone of the lateral ventricle; however, even there we observed ventricular zone cells with processes ending on blood vessels 20-50 um away from the ventricle.

Bergmann glia. The fibers of these cells were partially stained as thin, unbranched, parallel processes in the molecular layer of the cerebellum. The processes were oriented perpendicularly to the cerebellar surface where in some cases the end feet were clearly stained. The label became fainter as one approached the Purkinje cell layer where presumably the unstained cell bodies are located. (Fig. 2-4 A).

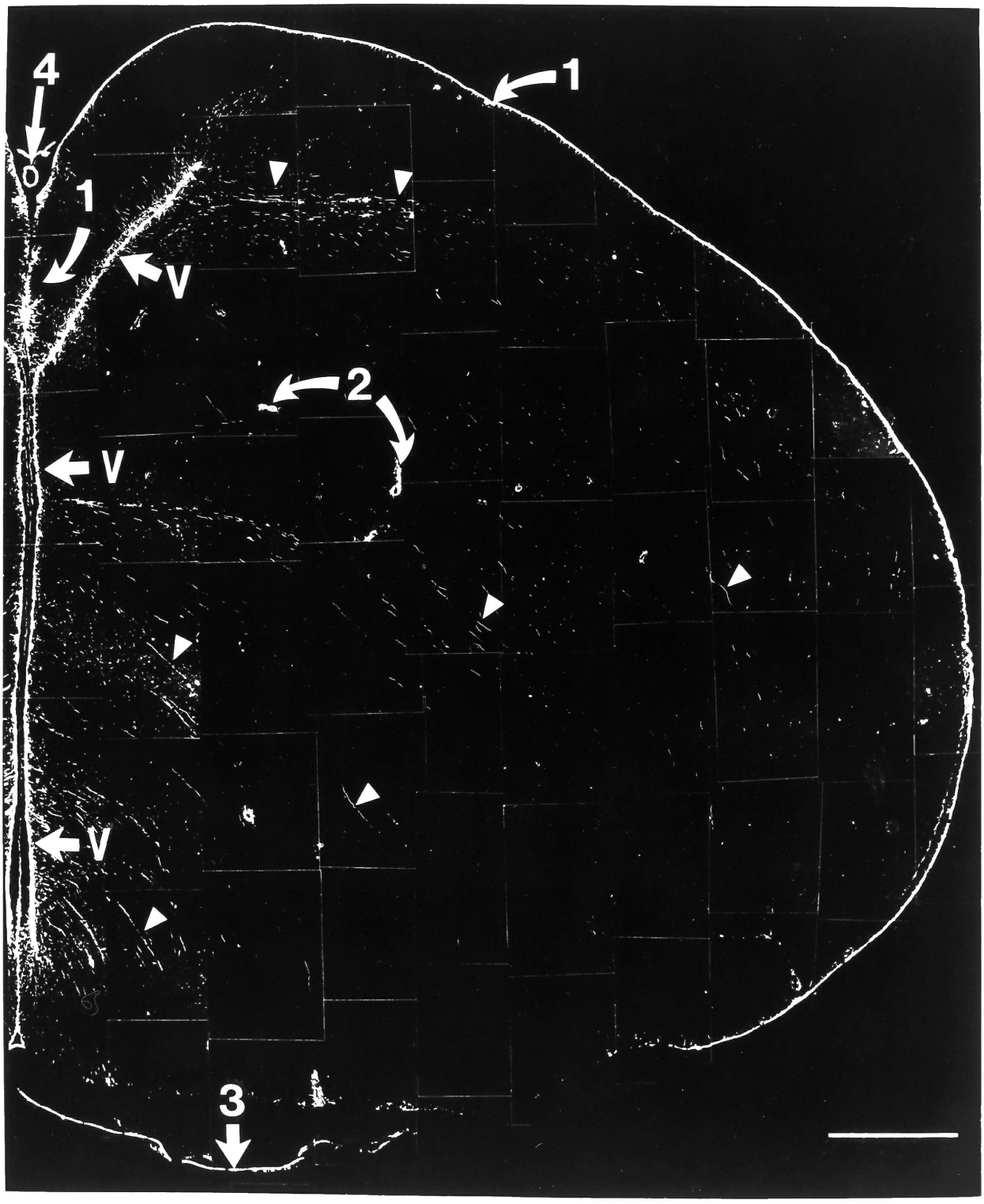
Astrocytes. The processes of four subsets of canary fibrous astrocytes stain with this antibody: 1) In fiber tracts, such as the anterior commissure and in the stratum opticum of the optic tectum, where these *fibrous astrocytes* are wedged between nerve fibers (Fig. 4B), (Linser, 1985). 2). On the brain surface, where 40E-C stains astrocytic processes and the glia limitans formed by their surface endings (Fig. 2-1, 2-4 E); in some brain surface regions one can see clusters of 3 to 5 astrocytic processes that project for 50 to

300 um into the brain parenchyma; the 40E-C positive processes of *brain surface astrocytes* are larger and particularly abundant in the medial surface of the telencephalon (Figs. 2-1 and 2-4 E). 3) *Perivascular astrocytes*, which have darkly stained, spider-like processes and are found around blood vessels (Fig. 2-3 F); interestingly, the greater part of brain parenchyma is devoid of 40E-C positive astrocytes and only a small fraction of the blood vessels are surrounded by positive perivascular astrocytes (Fig. 2-1). However, preliminary observations that will not be reported in detail here reveal numerous 40E-C positive astrocytes in and around brain lesions, and in this case we might be dealing with, 4) *reactive astrocytes* (Fig. 2-4 H) (Nathaniel and Nathaniel, 1981).

Many processes can be seen to stain with 40E-C in the medial habenula (Fig. 2-4 G). These processes do not show any polarized direction, as is the case for processes of radial glia, and therefore we believe that they belong either to tanycytes (Cupedo and Weerd, 1985), or to astrocytes or to both. The lateral habenula and elsewhere in the mid and hindbrain are practically devoid of these processes. Protoplasmic astrocytes and oligodendrocytes (Stensaas and Stensaas, 1968, Peters *et al.*, 1976) were never seen to stain with 40E-C and these two cell types probably constitute a majority of the canary brain glia.

The area postrema, a specialized and highly vascularized surface organ that protrudes into the fourth ventricle close to where this ventricle leads into the central canal, has a great number of strongly 40E-C positive cells.

Figure 2-1. Photomicrographic reconstruction of 40E-C immunofluorescence in a frozen section (10 um thick) of anterior forebrain taken at AP level 3.0 of the canary atlas (Stokes *et al.*, 1974). The midline is close to the left edge of the photograph. The lateral ventricle (V) of the forebrain, here with both walls collapsed, can be seen close to the midline. Ventricular zone cells positive to antibody 40E-C line the walls of the lateral ventricle. Many thin segments of the radial glia processes can be seen throughout the section (arrow heads). In addition, the following structures, indicated by number on the photograph, are positive to antibody 40E-C: 1) processes of brain surface astrocytes; 2) processes of perivascular astrocytes; 3) pia, which has become detached at lower edge of section; 4) the smooth muscle in the large artery in the dorsal midline. Calibration bar: 1mm.


4
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1
V
2
V
V
3

Figure 2-2. Radial glia in adult canary forebrain stained with antibody 40E-C by the avidin-biotin-peroxidase technique and photographed with differential interference contrast optics.
A. Low magnification photomicrograph from a transverse 25um thick vibratome section at A 2.0 of the stereotaxic atlas of the canary brain (Stokes *et al.*, 1974). Radial glia fibers penetrating the hyperstriatum and coursing away from the wall of the lateral ventricle (V) where radial glia cell bodies are darkly stained. Calibration bar = 100um.
B. High magnification photomicrograph showing radial glial cell bodies at the ventricular zone of the lateral ventricle (V). These cells project a single unbranched process into the underlying hippocampus. 25 um vibratome section. Calibration bar = 10 um.
C. Low power photomicrograph of displaced radial glia (arrows) in the ventral neostriatum. V, ventricle lumen. 25um thick vibratome section. Calibration bar 50 um.
D. High power photomicrograph of the cell bodies of displaced radial glia (arrows) from the same region as C. 6um thick PEG section. Calibration bar 10 um.

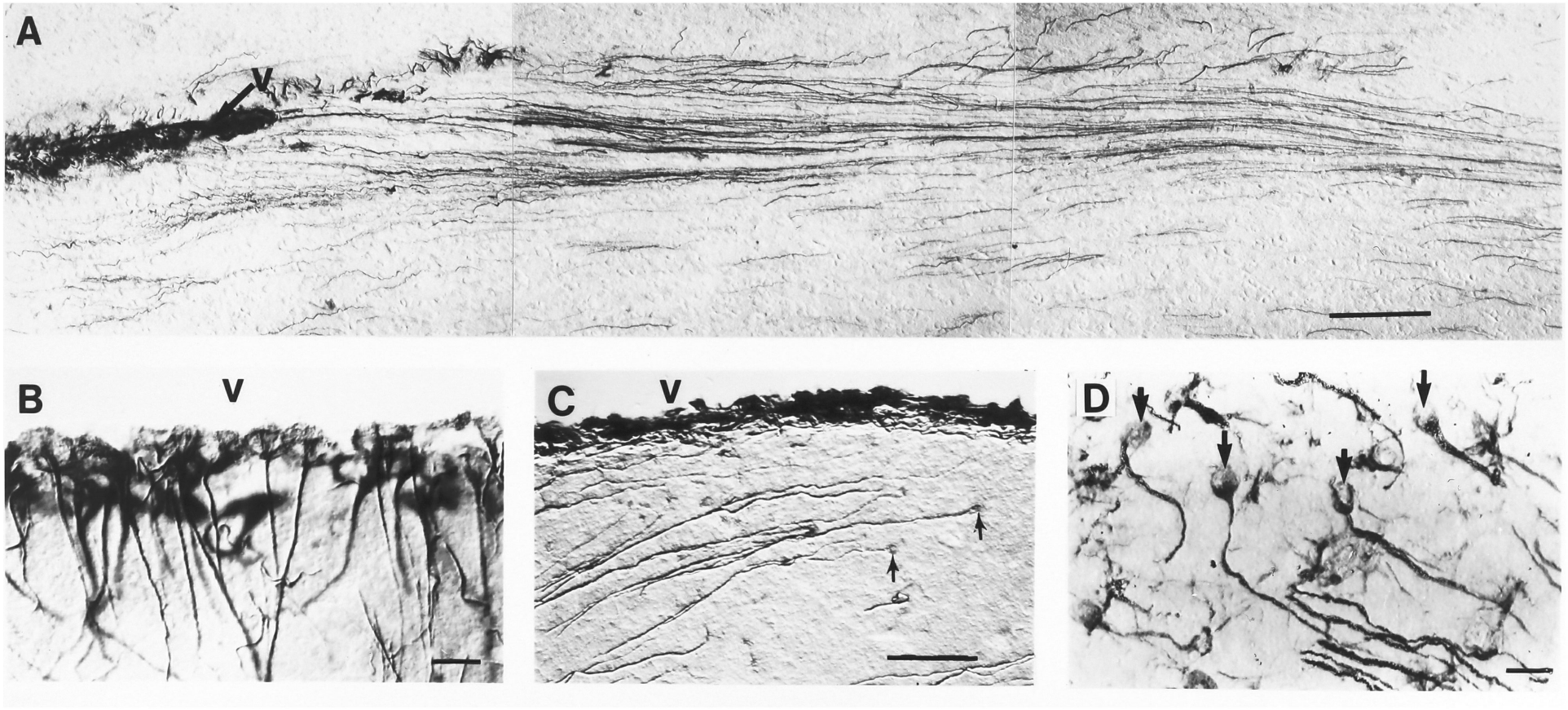

A
V
B
V
C
V
D

Fig 2-3. Photomicrographs of radial glia fibers thickenings using differential interference contrast optics. In A, two radial glia fibers contact the same capillary, one on each side. In B, a blood vessel that has been cut tangentially and runs diagonally from lower left to upper right, is apposed to thickenings of four radial glia processes (arrows). On some occasions one observes periodic thickening of radial glia (C). The arrows point to two such thickenings of several along this fiber. 6um thick PEG sections stained with the avidin-biotin-peroxidase technique. Calibration bar; A and B, l0 um; C, 50 um.

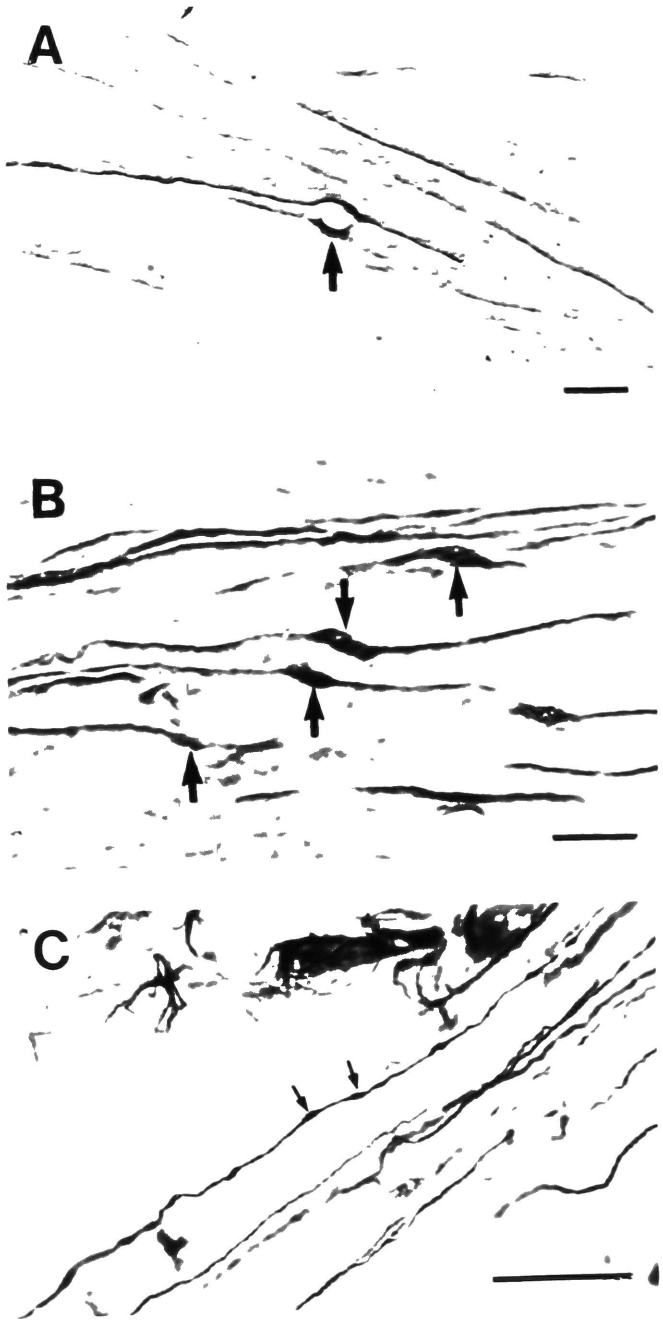
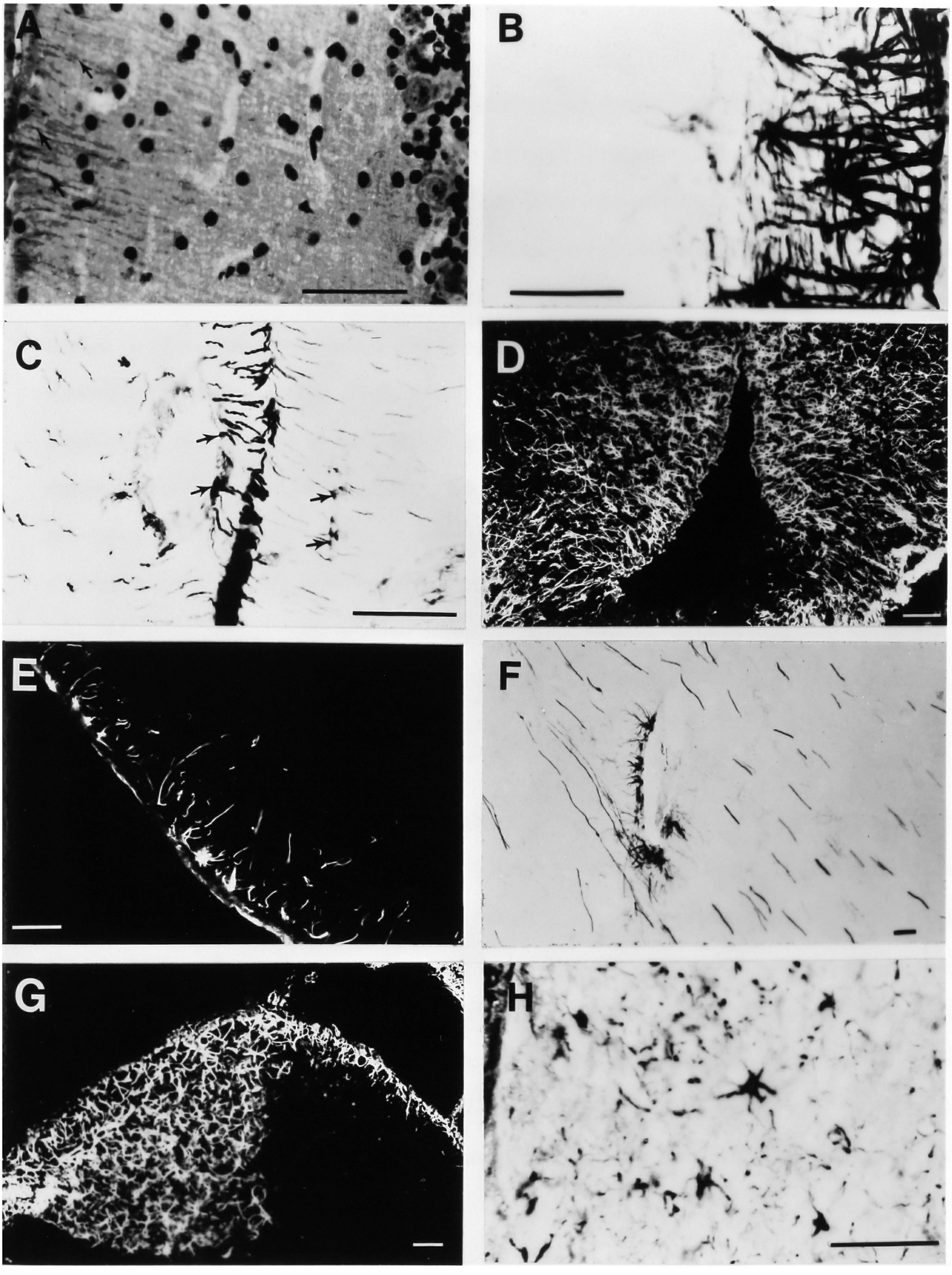

A
B
C

Figure 2-4. Photomicrographs of the other cell types stained by antibody 40E-C in the canary brain. A,B,C,F and H are 6 um thick PEG sections stained with the avidin-biotin-peroxidase technique. D,E and G are fluorescently stained 10 um thick frozen sections.
A. Distal segment of Bergmann glia (arrows) in the molecular layer of cerebellum. Counterstain is cresyl violet showing relative position of Purkinje cells on right (large, pale staining nuclei) and sparse glia on molecular layer.
B. Fibrous astrocytes on stratum opticum (right half of picture) of optic tectum. At the surface of the brain the end-feet of these astrocytes form a glia limitans.
C. Tanycytes with cell bodies on wall of collapsed optic tectum ventricle. Notice that some of the tanycyte fibers can be seen to end on the wall of a blood vessel (arrows).
D. Bed of 40E-C positive tanycyte fibers originating on wall of the ventral third ventricle.
E. Processes of brain surface astrocytes on the dorso-medial forebrain (dorsal is to the left, medial is bottom); the positive band overlying the brain surface is the pia mater.
F. Perivascular astrocytes in forebrain; notice radial glia fibers streaming by.
G. Habenula. Brain surface astrocytes stain over both medial (left) as well as lateral habenula, but a rich tangle of 40E-C positive fibers stains only in medial habenula.
H. Reactive astrocytes near small forebrain lesion produced seven days earlier.

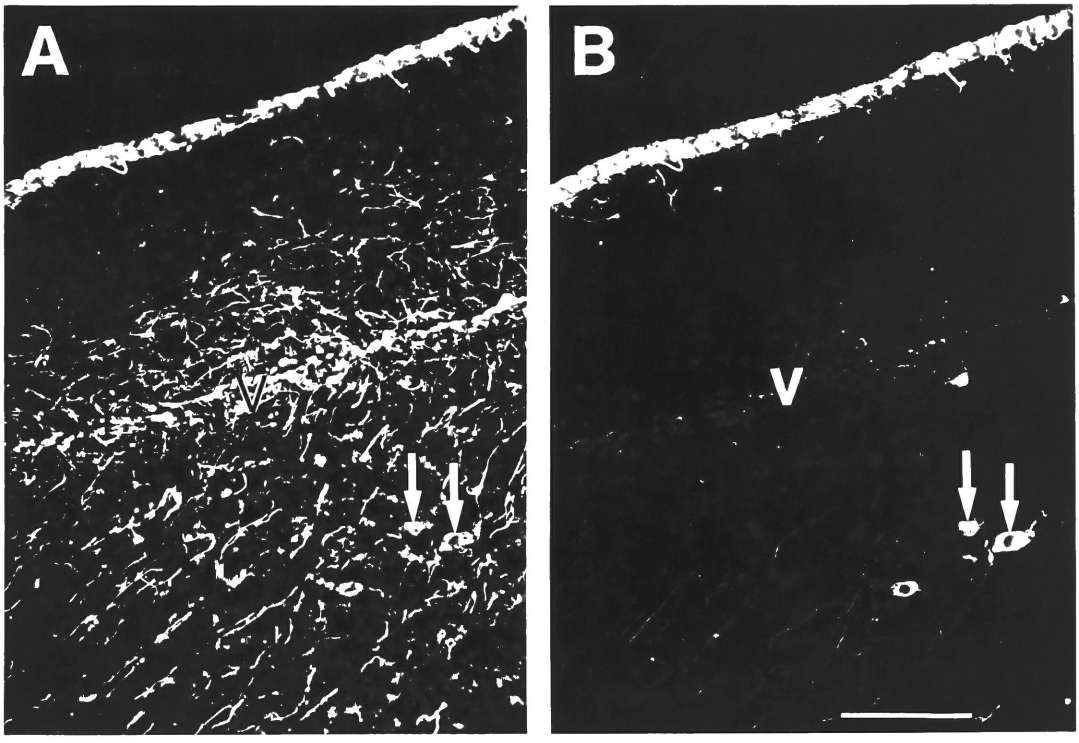

A
B
C
D
E
F
G
H

Other antibodies that stain radial glia in adult canary brain.

The observation of most interest, was the apparent presence of radial glia in the adult brain of a warm-blooded vertebrate. In order to further substantiate the finding and the identification of radial glia, the pattern of staining of antibody 40E-C with that of three other antibodies was compared. The following antibodies were used: 3A7, a monoclonal known to stain embryonic radial glia (Lemmon, 1985); a rabbit polyclonal antibody to vimentin (Eugenia Wang, Rockefeller University); and a commercially available antibody to mammalian vimentin (Labsystems). Antibody 3A7 stained radial glia in canary forebrain in a manner indistinguishable from that of 40E-C. The staining of other cell types described here was also similar. The rabbit polyclonal antibody to vimentin also stained radial glia; however the staining was not as bright as with 40E-C or 3A7. Also this vimentin antibody brightly stains all blood vessels partially masking radial glia. The commercially available antibody to mammalian vimentin gave a very weak signal, showing fragmented staining of radial glia.

In double-stained material, a rabbit polyclonal against glial fibrillary acidic protein (GFAP) (Eng and DeArmond,'83) brightly decorated all types of astrocytes that reacted with 40E-C, substantiating the identity of these cells as fibrous astrocytes (Linser,1985). Interestingly, anti-GFAP did not stain cells of the ventricular zone, though a small subset of the 40E-C positive radial glial fibers also stained weakly with anti-GFAP (Fig. 2-5). This suggests that a subpopulation of radial glia contains small amounts of GFAP. This was confirmed by staining only with GFAP antibodies, thus excluding the possibility of contamination from the 40E-C fluorescent staining (see Chapter 6).

Figure 2-5. Photomicrographs of double immunofluorescent stained section of lateral forebrain. A. Staining with 40E-C. B. Staining with antibody to GFAP. Processes of brain surface astrocytes (glia limitans) and perivascular astrocytes (arrows) stain with both antibodies. A segment of collapsed lateral forebrain ventricle (V) can be seen in A, with many positive radial glia bodies defining its walls. These cell bodies are negative in B. Notice many radial glia processes stain strongly in A and only a few stain faintly in B.

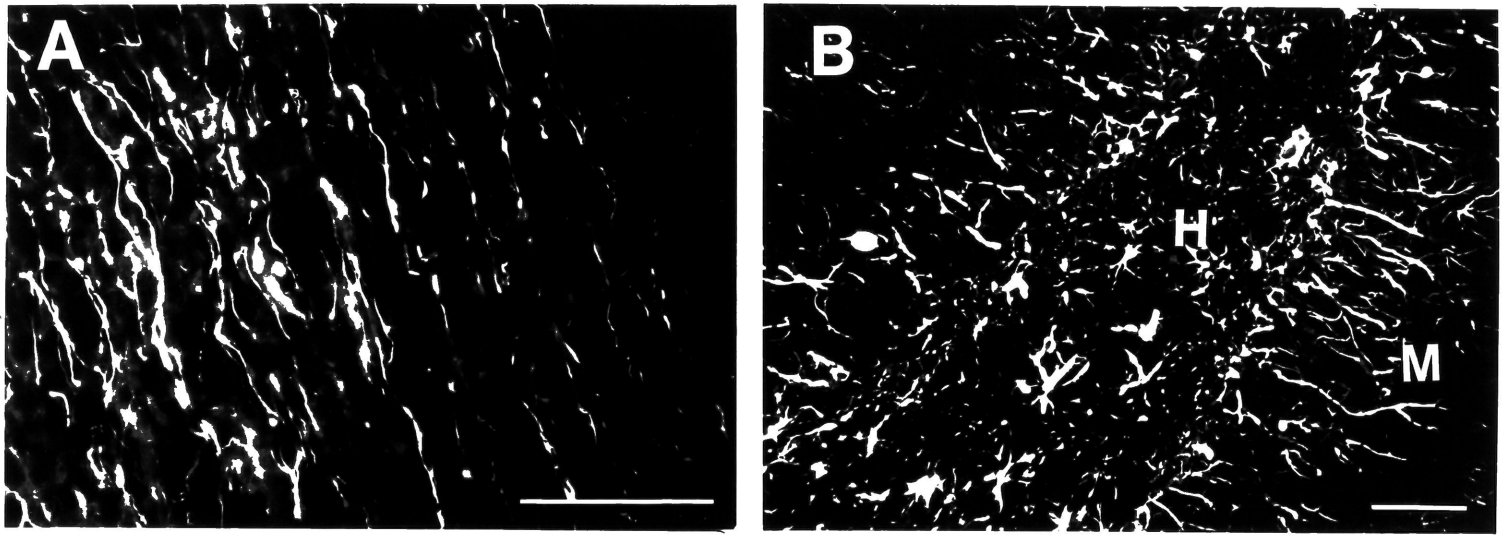

A
V
B
V

Comparison with other species.

Radial glia have been well described in the brains of young mammals (Rakic 1971a,b) but are thought to be absent from the brains of adult mammals (Schmeckel and Rakic, 1979). We hypothesized that if the cells we called radial glia in adult birds were similar to their counterparts in young mammals, then the latter cells might stain with our antibody, but no such cells should stain in adult mammals. Antibody 40E-C was tried with post-natal day 2 and adult rat material. Radial glia fibers were clearly revealed with 40E-C throughout the brain of the young rat (Fig. 2-6 A). In some regions they extended from the ventricular wall to the pial surface of the brain. These radial fibers were absent in the adult rat in which positive processes with an orientation reminiscent of radial glia could only be seen in the cerebellum (Bergmann glia) and as fibers from astrocytes in the molecular layer of the hippocampus (Fig. 2-6 B) (de Blas, 1984; Eckenhoff and Rakic, 1984). These observations are consistent with our identification of radial glia as one of the cell types stained by 40E-C in adult canary brain. In marked contrast with what we observed in canaries, antibody 40E-C stains many astrocytes in adult rat brain parenchyma (Fig. 2-6 B).

Antibody 40E-C stains radial glia in adult zebra finch, quail, and chicken, where they occupy positions similar to those described for canaries, that is, with cell bodies in the ventricular zone of the lateral ventricles of the forebrain and with fibers penetrating the forebrain parenchyma for distances of up to several millimeters.

Figure 2-6. A. Photomicrograph of radial glia fibers in postnatal day 2 rat forebrain. B. Photomicrograph of dentate gyrus of the hippocampus in adult rat; H refers to hilus and M to the molecular layer; notice astrocytes stained throughout, and that their more elongated processes reach into the molecular layer. In both cases the material was stained by immunofluorescent with antibody 40E-C. Calibration bar = 50 um.

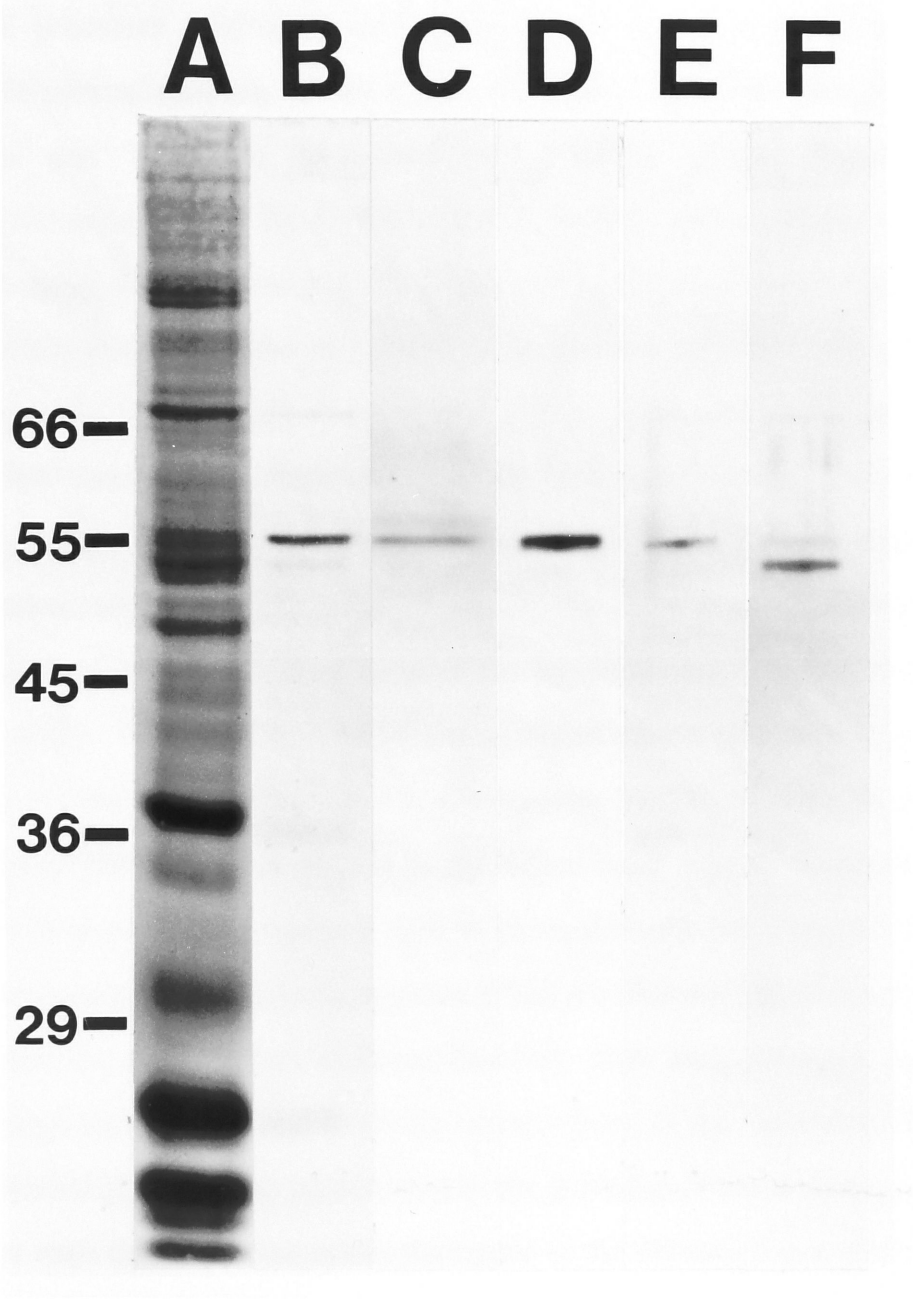

A
B
H
M

Characterization of antibody 40E-C.

Antibody 40E-C reacts with a perinuclear filamentous network inside cultured fibroblasts. In kidney and testis our antibody stains connective tissue cells in a very conspicuous manner. There is also a corona of positive cells on the surface of the seminiferous tubules, with positive processes reaching towards the lumen of the tubule; we believe these to be Sertoli cells. In striated muscle (M. pectoralis) this antibody stains in bands; these bands in contiguous muscle fibers are commonly in register and seem to correspond to the Z-line (Lazarides,1980). In canary embryos our antibody stains mesenchyme. All the cells described up to here stain very strongly with antibody 40E-C. Other cells, such as endothelial cells in large blood vessels and red blood cells, stain in a fainter, though still recognizable manner.

In order to obtain more direct evidence, a crude extract containing the intermediate filament proteins of adult canary brain was separated by one-dimension gel electrophoresis, transferred to nitrocellulose paper and reacted with different antibodies. Lane A of Fig. 2-7 shows the staining of the proteins in the nitrocellulose strip; lane B shows a single band of molecular weight 55 Kd that reacted with antibody 40E-C; lane C shows a band of the same molecular weight which reacted with antibody 3A7; lane D and E show that this same band reacted also with the polyclonal and the monoclonal antibodies to vimentin. In lane F the same protein extract reacted with antibodies to GFAP shows a band of somewhat lower molecular weight (52-53 Kd). These results strongly suggest that vimentin is the antigen recognized by antibody 40E-C. Antibody 40E-C also shows reactivity with a single 55Kd band from intermediate filaments obtained from adult and embryonic chicken using the same methods as for canary material.

Figure 2-7. Western blot analysis of detergent insoluble extract from adult canary brain. Proteins were separated by SDS-electrophoresis and transferred to nitrocellulose paper. Lane A shows the amido black stain of total protein. Lanes B to F were stained with the avidin-biotin-peroxidase technique after reaction with the following antibodies: Lane B, 40E-C; C, 3A7; D, rabbit anti-vimentin; E, monoclonal anti-vimentin and F, anti-GFAP.


A B C D E F
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55
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36
29

Discussion

Antibody 40E-C reveals radial glia in adult avian brain.

Glial processes originating in the ventricular wall and reaching far into the telencephalic parenchyma are stained by the monoclonal antibody described here. The term "radial glia" has been used to describe cells of similar morphology during development (Magini, 1888; Rakic, 197la and b). At the earliest stages these cells seem to reach the brain surface. However, the same term is also used to describe cells at later stages of development when the end of their long fiber no longer reaches the surface of the brain (e.g. Fig. l4 in Eckenhoff and Rakic, 1984). Cells with radial glia morphology have also been described in adult cold-blooded vertebrates, where Horstmann (1954) called them "tanycytes", meaning elongated cells. Although radial glia, according to the latter definition, could be cataloged as tanycytes, the more recent use of this term has been restricted to ventricular zone cells of the hypothalamic region (Flament-Durand and Brion, 1985). Tanycytes frequently end at blood vessels and do not reach very far into the brain parenchyma (Raphols and Goshgarian, 1985). Also worth noting is that some tanycytes reach the brain surface in the infundibular region. Tanycytes have been associated with an endocrine function, and in particular with the transport of substances between the vascular and ventricular systems (Flament-Durand and Brion, 1985). Radial glia have been associated with a different function: their long processes are thought to act as pathways for the dispersal of young neurons born in the ventricular zone (Rakic, 197la, b). Radial glia may also be involved in the guidance of axons during development and in other ways determine the cytoarchitecture of the nervous tissue (Katz *et al.*, 1980; de Blas, '84; Singer *et al.*, 1979; Dupouey *et al.*, 1985).

Interestingly, there is nothing in the literature that makes the putative radial glia and tanycyte functions mutually incompatible, and at least one study has suggested that radial glia play a role in the transport of substances (Grafe and Schoenfeld,'82). Clearly there is a need for an explicit and mutually exclusive set of criteria that distinguishes between radial glia and tanycytes.

Reasons to identify the elongated cells described here as radial glia include: 1) Their long fibers extend over distances of several millimeters as compared with the shorter distances typically associated with tanycytes. 2) Their fibers may contact blood vessels, but even when they do so, they can often be seen to reach beyond that vessel, and frequently are not seen to contact any blood vessel over long distances though blood vessels course in the vicinity. 3) We know that they are used in adult birds as routes for dispersal by young neurons born in the ventricular zone (Chapter 3 and 4; Alvarez-Buylla *et al.*, 1988; Alvarez-Buylla and Nottebohm, in prep.). 4) No such cells occur in the brain of adult mammals, where radial glia are known to have been transformed into astrocytes. Stensaas and Stensaas (1968), using Golgi stained material, reported a wide array of ventricular zone cells with long processes in adult avian brain. Their drawings included cells of typical tanycytic morphology as well as cells very similar to the ones we call radial glia. They called all of these cells "typical ependymal cells". They did not remark that some of these cells, by mammalian standards, were very atypical for an adult warm blooded vertebrate. Neurogenesis in adult avian brain had not yet been described, nor had Rakic (197la,b) reported on the relation between glial fibers and migrating neurons in mammals. The present chapter shows that cells with the above listed characteristics, conforming to the radial glia definition, occur in adult avian brain.

Monoclonal Antibody 40E-C recognizes cells that contain vimentin.

The reactivity of antibody 40E-C was restricted to a subclass of brain glia. Only radial glia, tanycytes, Bergmann glia and astrocytes associated with blood vessels, fiber tracts, the brain surface and lesions stained with antibody 40E-C. Outside the brain, this antibody stained mesenchyme, smooth and striated muscle, Sertoli cells, endothelial and red blood cells. These results suggested that vimentin, an intermediate filament protein, was the antigen recognized by 40E-C. Vimentin has been described in radial glia (Tapscott *et al.*, 198l; Bignami *et al.*, 1982), fibrous astrocytes (Yen and Fields, 198l; Linser, 1985), reactive astrocytes (Dahl *et al.*, 198l), Bergmann glia (Shaw *et al.*, 198l), mesenchyme (Franke *et al.*, 1978), endothelial cells (Lazarides, 1980), Sertoli cells (Franke *et al.*, 1979) and smooth and striated muscle (Lazarides, 1980).

In protein blots antibody 40E-C recognized a single band of a molecular weight of 55 Kd, which runs indistinguishably from bands stained by other well-characterized vimentin antibodies. Although it was previously thought not to react with vimentin, antibody 3A7 (Lemmon, 1985) also stained the 55 Kd band. Interestingly, the staining pattern of all these antibodies in brain tissue was not the same. Whereas 40E-C and 3A7 stained radial glia particularly well and endothelial cells poorly, Dr. E. Wang's polyclonal to vimentin was a better stain for the endothelial cells, though it also recognized the radial glia. The Labsystems monoclonal antibody to vimentin stained the radial glia in a weak and fragmented manner.

The failure of antibody 3A7 to stain endothelial cells has previously been suggested to indicate that it recognizes an antigen other than vimentin, possibly an intermediate filament-associated protein (Lemmon, 1985). The biochemical evidence

presented in this paper gives strong evidence that the antigen for both 3A7 and 40E-C is vimentin.

The difference between the vimentin antibodies in their staining intensity of the various cell types could result from cell-specific post-translational modifications or conformational differences of vimentin. After the extraction procedure all these forms of vimentin would probably run together under the denaturing condition of the protein gel, hence giving us the similar pattern of staining shown by Fig. 2-7.

Vimentin is present in radial glia during avian and mammalian development (Tapscott *et al.*, 1981; Bignami *et al.*, 1982). In agreement with this, the radial glia of the young rodent brain stain with antibody 40E-C. In mammals radial glia can convert into astrocytes at the end of development (Ramón y Cajal, 1911; Scmeckel and Rakic, 1979) and we have shown that there are many astrocytes in brain parenchyma of adult rodents that stain with 40E-C. The paucity of 40E-C positive astrocytes in avian brain parenchyma might be related to the persistence of radial glia into adulthood. Might the seeming mutual exclusiveness of 40E-C positive radial glia and parenchymal astrocytes be related to their respective functions?

Functions of radial glia in adulthood.

Glial cells, as indicated by their name (from the greek glia=glue; cement; Virchow, 1860) have been assigned static supportive functions (Ramón y Cajal, 1911), such as filling the space between neurons, and keeping cells glued together. In contrast radial glia play, during development, a key role in morphogenesis (chapter 1). In the fully developed avian brain, neurons continue to be added (Nottebohm, 1985); could radial glia in adulthood play a role in neuronal migration? A young neuron born in the

ventricular zone could happen to contact the nearest radial glia fiber, finding a tract that would take the young neuron directly away from the ventricle.

In the tectum of the adult goldfish, Stevenson and Yoon (1982) distinguish radial glia from the more retracted, process-bearing ventricular zone cells identified as tanycytes. It is of interest that after transection of the adult optic nerve of this species mitotic radial glia appear in the optic tectum (Stevenson and Yoon, 1981) suggesting that even in adulthood these cells might retain the potential to be involved in tissue remodeling.

The presence of radial glia in the brain of adult birds and cold-blooded vertebrates and their absence in the adult brain of mammals raises a question about physiological differences between the brains of mammals and those of other vertebrates. If radial glia, as has been suggested for tanycytes, play a role in secreting and transporting substances, then this role would be absent in the adult mammalian brain, or it would be discharged by some other cell types. For example, Mugnaini (1986) suggests that gap junctions could establish a functional syncytium of astrocytes that could stretch from the ventricle to the pial surface. The homeostasis of the neuronal microenvironment is regulated by glial cells in mammals. The increase for example of extracellular K+ during neuronal depolarization is thought to be buffered by astrocytes (Ransom and Carlini, 1986 and references therein). Recently this function has also been suggested for radial glia in adult turtles (Connors and Ransom, 1987), and therefore such a role might also be played by radial glia in adult birds. It should also be kept in mind that the glial vs. neuronal character of what we call radial glia is not established (see chapter 1). If radial glia were to become neurons, being themselves the neuroblast, then this could be

the reason why they are still present in the adult canary. This possibility is under investigation.

Summary.

This chapter shows that radial glia persist in adult avian forebrain where they stain with an antibody, 40E-C, that recognizes vimentin. I discuss the various possible roles that radial glia might have in adulthood, as conveyors of substances, as guides to neuronal migration, as buffers for electrolytes and maybe as neuroblasts. Subsequent chapters explore further the role that radial glia might play in neuronal migration.

CHAPTER 3

A NEW CELL TYPE IN ADULT CANARY BRAIN AND ITS RELATION TO RADIAL GLIA.

Long unbranched fibers revealed by a monoclonal antibody to vimentin penetrate the canary telencephalon parenchyma. These fibers originate from cell bodies present on the walls of the lateral ventricles (Chapter 2). Since radial glia may guide the migration of young neurons during adult neurogenesis as described for development (Chapter 1), it seemed important to study the relation of radial glia fibers and other cells in the adult bird brain. In this Chapter I describe a new cell type for adult avian brain, its relation to radial glia and its nuclear staining properties. The distribution of radial glia and of the 'new cell' type is then mapped in the adult canary brain (Alvarez-Buylla, *et al.* 1988).

Elongated cells attached to radial glia: A new cell type.

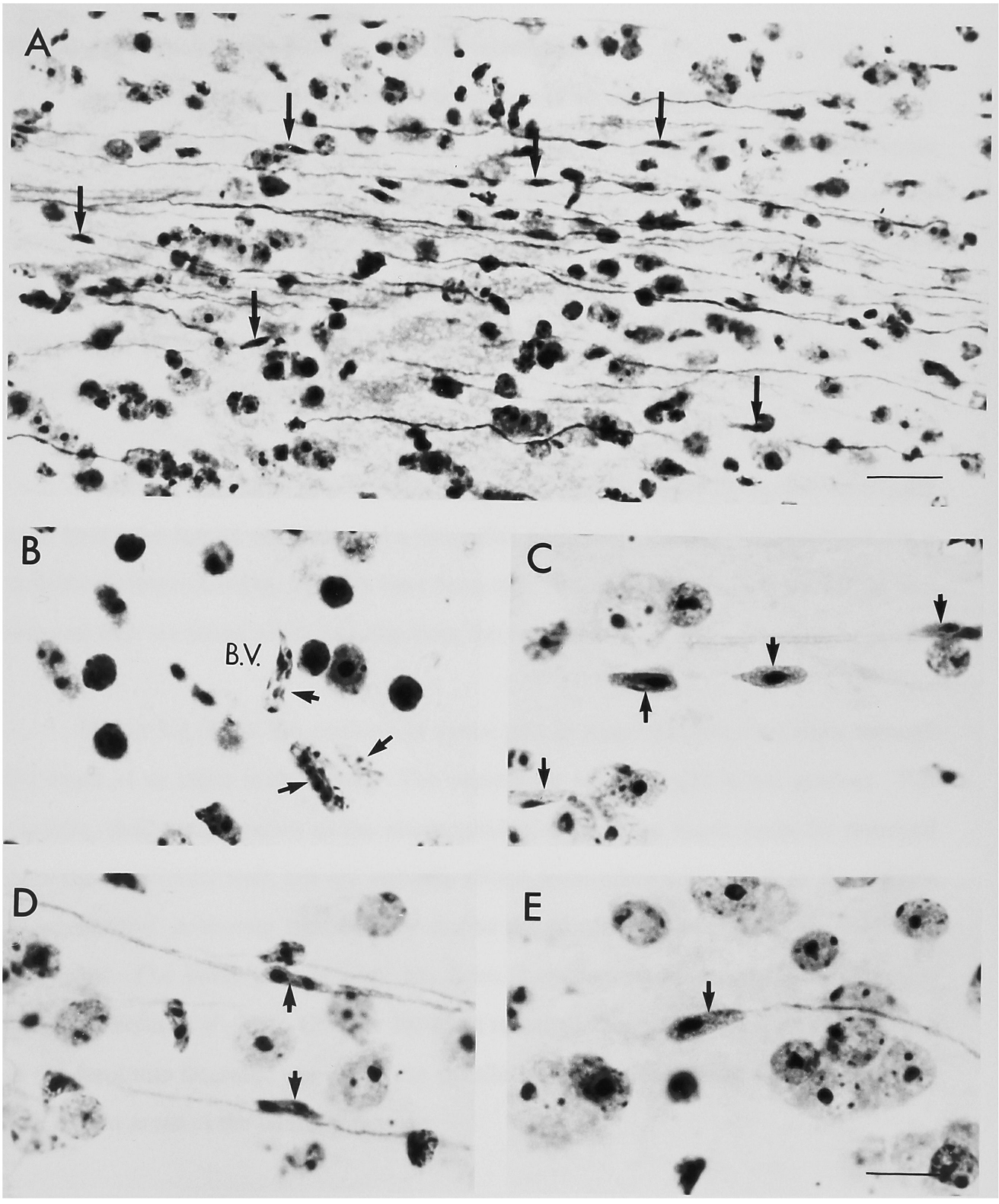
When brain sections are stained with antibody 40E-C and the cell nuclei are counter stained (Chapter 6) the relation of radial glia to other cell types can be studied. There is no clear evidence of mature neurons or glia organized in any special way with respect to radial glia (Fig. 3-1). However an elongated cell was often seen in association with the radial glia fibers (Fig. 3-1). These cells show very little anatomical overlap with any other cell type of the adult avian brain. For now I will refer to these cells as the new

cell type or simply as elongated cells. Typically they have an elongated nucleus that is 10 um long and 3-4 um across. This nucleus shows one or two relatively large basophilic nucleoli (Fig. 3-1 C, D, E). The cytoplasm is not clearly visible under these conditions. These cells do not react with the intermediate filament antibody that stains radial glia (40E-C), nor do they react with anti-GFAP antibodies. The only other cell type with which they could be confused is endothelial cells. However the nuclei of these two cell types stain differently. The nucleus of endothelial cells shows a mottled distribution of basophilic substance, while that of the elongated cell shows one or two darkly stained nucleoli (compare Fig. 3-1 B and 2C-E). In addition, the relation of endothelial cells and a vascular lumen is usually sufficiently clear to avoid confusion between these two cell types. Except for their relation to vascular lumina, endothelial cell nuclei show no particular orientation.

The orientation of the nuclei of the elongated cell is unusual in that it lies parallel to the long axis of radial glia fibers and often shows close apposition to such fibers (Fig. 3-1). Despite this frequent association with radial glia fibers, many cells with the elongated cell morphology were not associated with radial glia.

The nuclear histology of the new cell type is reminiscent of the bipolar cells of Cajal in embryonic chick brain (chapter 1), they also show an elongated nucleus with one or two nucleoli. The orientation and association to radial glia suggests that this new cell type could correspond to young migrating neurons (chapter 4). If these elongated cells were to be following radial glia, then the distribution of radial glia fibers could determine the pattern of dispersal of the young neurons. For these reason it was important to learn about the overall pattern of radial glia fibers in the adult bird brain. I constructed an atlas of where radial glia are and how are they oriented.

Figure 3-1. Photomicrographs of 40E-C stained radial glia and cresyl violet counter-stained cells.
A. 25um Vibratome section at the level of the hyperstriatum dorsalis. Note the parallel arrangement of radial glia fibers and the many elongated cells oriented in the same direction (some indicated by arrows). Calibration bar 20um.
B-E. 10um PEG sections; in B, nuclei of endothelial cells (arrows) on a tangentially cut blood vessel (B.V.). In C, D, and E examples of the nuclei of the new cell type (arrows). Note how some are closely apposed to radial glia fibers. Calibration bar 10um.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Radial glia are selectively distributed in the telencephalon.

The position of radial glia in 10um sections of an adult male and an adult female canary brain was mapped in detail with the aid of a computer yoked microscope (Chapter 6 and appendix A). The anatomy and distribution of radial glia were very similar in the two birds studied in detail. They were also very similar in 13 other male and female adult canaries used in this study. The abundance of radial glia in certain regions and their absence in others is consistent for all birds studied. The drawings presented here are those for the male.

Typically, the radial glia of adult avian brain has its cell body in the ventricular zone lining the lateral ventricle and a fiber that penetrates the brain parenchyma for a variable distance (Chapter 2). We have been able to follow individual fibers for up to 2 mm and they are found as far as 5 mm from the ventricle.

Figure 3-2 shows the position of radial glia in three transverse sections through the brain of an adult male canary. The occurrence of radial glia is not random. For example, they are abundant in the telencephalon, where they reach areas far removed from the ventricular wall, but are virtually absent from other areas, such as the septum and midbrain. A shorter type of fiber can be found coursing away from the midbrain ventricles. The latter kind of fiber has been characterized as belonging to tanycytes (Alvarez-Buylla *et al.*, 1987, Chapter 1). Most radial glia course from the lateral ventricle of the forebrain laterally, and many run parallel to the surface of the brain penetrating into distant areas of the lateral forebrain.

There are some regions of telencephalic parenchyma that receive more radial glia fibers than others (Fig. 3-2). Areas rich in radial glia fibers are: 1) hyperstriatum accessorium, dorsalis and ventralis; 2) lobus parolfactorius; 3) ventromedial part of caudal neostriatum; 4) dorsolateral caudal neostriatum. Areas with few if any radial glia fibers are: 1) Rostral neostriatum; 2) nucleus HVc, thought to be part of neostriatum (Nottebohm *et al.*, 1982); 3) archistriatum; 4) septum. In some areas discrete arrays of radial glia fibers seem to follow major anatomical lamina. For example, many fibers leave the lateral ventricle to travel above the lamina hyperstriatica (LH), which forms the dorsal boundary of the neostriatum. Radial glia fibers also seem to group in the dorsal border of LPO just under the lamina medullaris dorsalis (Fig. 3-2 A and B).

Since radial glia fibers running in the rostro-caudal plane would be difficult to identify in transverse sections. I prepared a series of sections in the coronal plane. The main medio-lateral orientation of radial glia fibers was confirmed. For example, the orientation of hyperstriatal fibers can be readily seen in a section cut very close to the roof of the brain (Fig. 3-3 A). In deeper sections (Fig. 3-3 B) a small subset of fibers is observed arising from the rostral edge of the lateral ventricle and coursing forward into the rostral tip of the telencephalon (hyperstriatum accessorium). Fibers from the caudolateral edge of the lateral ventricle also travel forward, in this case into lateral neostriatum. In summary, most radial glia fibers travel in the medio-lateral plane while a smaller number reach the most rostral and lateral forebrain traveling in the rostro-caudal plane.

Figure 3-2. Maps of the positions of radial glia (right panels) and cells of the new type (left panels) reconstructed from 10um PEG sections of a male canary brain. Cells of the new type that appear attached to radial glia are indicated by a cross and unattached ones by a dot. The number above each pair of sections indicates the position along the rostro-caudal plane corresponding to the canary atlas (Stokes *et al.*, 1974). A key to this position is on the lower left hand corner of the figure. On the right panels we show the position of radial glia on the same section. See text for details. Major laminae are indicated by dashed lines. *A, archistriatum; B, nucleus basalis; Cb, cerebellum; E, ectostriatum; FA, tractus fronto-archistriatalis; HA, hyperstriatum accessorium; HD, hyperstriatum dorsalis; HP, hippocampus; HVc, hyperstriatum ventralis, pars caudalis; IPC nucleus isthmi, pars parvocellularis; LPO, lobus parolfactorius; MLd, nucleus mesencephalicus lateralis, pars dorsalis; N, neostriatum; NC, neostriatum caudalis; PA, paleostriatum augmentatum; RA, nucleus robustus archistriatalis; Rt, nucleus rotundus; S, septum; TeO, optic tectum; V, ventricle; X, area X.* Calibration bar, 1mm.

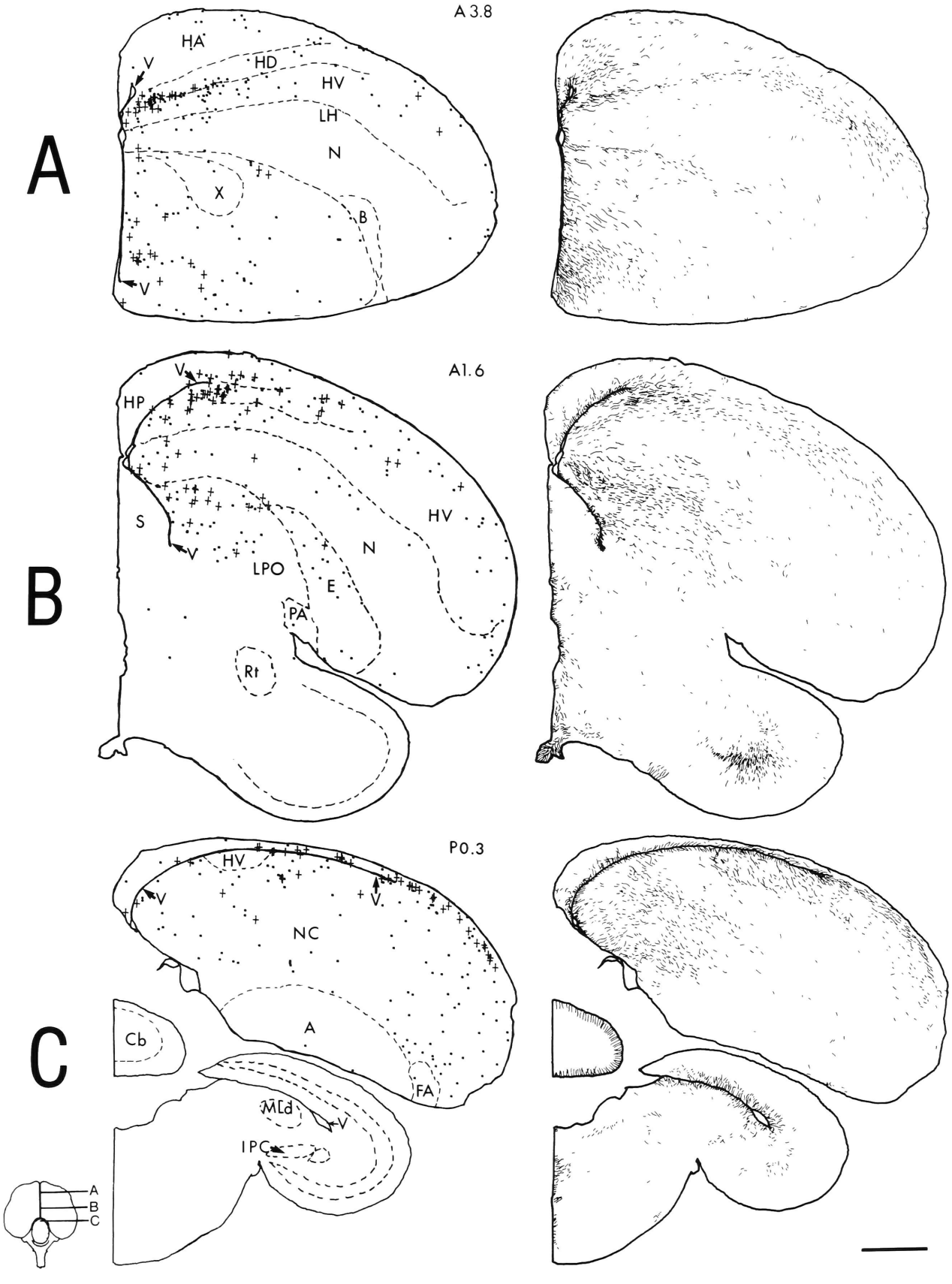
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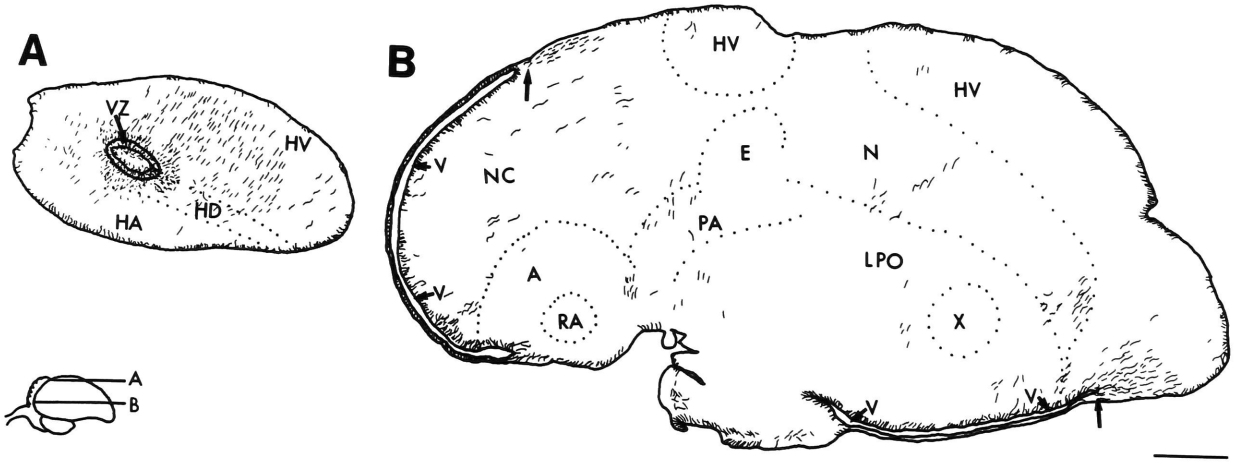
Figure 3-3. Maps of radial glia reconstructed from horizontal 10um thick PEG sections at the levels indicated on the lower left key. Up is lateral and right is frontal. Notice the anterior and posterior ends of the lateral ventricle where sets of radial glia fibers (arrows) originate and orient in a rostro-caudal direction. Major laminae are indicated by dotted lines. VZ, ventricular zone, other abbreviations are as in Fig. 3; Calibration bar, 1mm.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Relative distribution of radial glia and the elongated cells.

Based on the nuclear morphology described in this chapter for the elongated cells, I mapped the position of this new cell type in the same sections used to map radial glia. A discrete pattern of distribution emerges: the elongated cells are virtually unique to the telencephalon. For example, Fig. 3-2 (left panels) shows the position of 629 cells of the new type mapped in three brain sections. Of these, 625 were in the telencephalon, the remaining 4 were in the thalamus. In these three sections, plus in two others not shown, the areas of telencephalon and non-telencephalon mapped were, respectively, 70.2 mm^2 and 25.4 mm^2 and the total number of elongated cells of the new type, 843 and 6 respectively. Thus, per unit area, elongated cells were 51 times more common in telencephalon than in non-telencephalon. There were no instances of the new cell type in septum, optic tectum, cerebellum or medulla. Recognition of exemplars of the elongated cells is much aided by using sections of the brain that are parallel to the long axis of these cells. It could be argued that the failure to see the new cell type in non-telencephalic regions resulted from the transverse plane of section used. However I also looked at coronal sections and found that cells of the new type were present in the telencephalon and absent in the rest of the brain.

Of the 629 elongated cells that were mapped in the three sections of Fig. 3-2, 158 were seen to be in close association with radial glia fibers. Those that were not seen to be apposed to radial glia fibers but were in regions rich in this fibers had, in most cases, the same orientation as fibers coursing in their vicinity. Despite this frequent association with radial glia fibers, the new cell type described here was also found, in smaller numbers, in parts of the telencephalon, such as ectostriatum and lateral parts of neostriatum, where there were few if any radial glia fibers (Fig. 3-2). Thus the new cell

type is in the telencephalon where it is more concentrated (but not unique) to regions rich in radial glia fibers.

DISCUSSION

The two main findings presented in this chapter are: 1) The presence of a small elongated cell type not previously described in adult birds, and, 2) the selective distribution of radial glia and the new cell type in the adult canary brain.

A new cell type and its relation to radial glia.

Histology books pay little attention to birds, as is the case when classical anatomists describe the cell types in the adult brain. The elongated cells described here are very easy to see with most common stains. They are widespread in the telencephalon and even without the counter-stained radial glia their coherent orientation is self evident. Stensaas and Stensaas (1968, Fig. 1F, p. 318) show what looks like one these new cells. They pay little attention to this cell, which they catalog as an "elongated microglia", stating that the nucleus of this cell is not as dark as that of other microglia (ibid, Fig. 1B,C,D,J and K) because of its "longitudinal orientation". At that time they did not know that new neurons continued to be added to the adult bird brain, and had no reason to suspect the correct identity of this cell (Chapter 4).

The distribution of exemplars of the "new cell type" tends to coincide with that of the radial glia fibers, to which they are often (at least 25% of cases) apposed. Radial glia and the "new cell type" occur in the telencephalon, but rarely if at all, in the rest of the brain. Neurogenesis in adult avian brain is also thought to be a telencephalic property (Nottebohm, 1985). Furthermore, indirect evidence suggests that neurogenesis in adult

avian brain occurs in the walls of the lateral ventricle of the forebrain (Goldman and Nottebohm, 1983), where radial glia fibers have their origin and from where the young neurons presumably migrate. The latter inference, together with the observations reported here, suggest that there is in the adult avian brain a functional relation between neuronal migration and radial glia. This is supported by observations in the developing brain of mammals, where elongated young migrating neurons are seen apposed to radial glia (Rakic 197la, b, and 1972, see Chapter 1). This hypothesis will be tested in the next chapter.

Pattern of fibers from radial glia.

It is common to describe the internal organization of the adult brain in terms of clusters or lamina of neurons or the position of their fiber tracts. The arrangement of glia in adulthood is frequently ignored. The present chapter attempts to give a comprehensive account of the distribution of radial glia fibers in an adult vertebrate brain. Such fibers have their origin in the walls of the lateral ventricle, they orient mainly in the medio-lateral plane and are common in some parts of the telencephalon, but rare in others. Radial glia with long fibers are absent from regions outside the telencephalon where tanycytes are common (Chapter 1, and Alvarez-Buylla *et al.*, 1987).

The distribution of radial glia may prove important in delineating the major migratory pathways that young neurons use in adult brain. Such migratory routes may also reveal the most likely site in the ventricular zone from where a differentiated new neuron originates ("glial coordinate system", Smart, 1978). Given that most radial glia are oriented in the mediolateral plane (except for the rostral and caudal telencephalon, Fig. 3-3) it is possible to predict that, if they act as a distribution system for young neurons moving away from the ventricular zone (Chapter 4), then most new neurons

must originate from the ventricular zone within the same rostro-caudal level. Likewise, the trajectory of radial glia fibers suggests that neurons born in adulthood which take positions in the lateral hyperstriatum originate from ventricular zone lining dorsal reaches of the lateral ventricle. Neurons in the ventral telencephalon (e.g. LPO, Fig. 3-2) probably originate from ventricular zone lining the ventral walls of the lateral ventricle. The differential clustering of radial glia may also reflect local differences in neurogenic potential within the ventricular zone.

The number of stained radial glia fibers decreases with distance from the ventricular wall. It is not possible to tell from the material used whether this is because 1) the fibers get thinner at greater distances from the ventricle, 2) the amount of antigen decreases with distance, or 3) the fibers end in a staggered manner. The representation of these fibers, e.g. in Fig. 3-2, does not try to choose between these different interpretations.

Perhaps one of the more puzzling observations turned up by the present report is that the distribution of radial glia throughout the telencephalon is so uneven, with radial glia-rich and radial glia-poor areas. It would be interesting to know to what extent the complement of radial glia seen in adult avian brain differs from that seen in embryos. The distribution seen in adulthood could be a consequence of an initial radial organization in the embryo shifted by the differential growth of certain regions of the brain. Interestingly, the fetal brain of mammals also shows local clustering of radial glia processes (Astrom, 1967; Sturrock and Smart, 1980).

The local distribution of radial glia fibers may be relevant to their possible role in substance transport from the ventricle to brain parenchyma and vascular system and vice versa. If there is such a role (e.g. Ivy and Killackey, 1978; Grafe and Schoenfeld, 1982) then it is unevenly distributed in the various parts of the telencephalon of birds. Recent research in turtles suggests that radial glia act as cellular mediators of K^+ redistribution following neural activity (Connors and Ransom, 1987). It is not known if this role also occurs in birds, but if it does there may be interesting physiological differences between areas that are rich or poor in radial glia.

Summary

An elongated new cell type in the canary brain is often associated with radial glia. These cells are found almost exclusively in the telencephalon where radial glia orient in a medio-lateral plane. Larger numbers of these cells in radial glia-rich regions suggest that the new cell type may correspond to young migrating neurons using radial glia fibers for their dispersal. With the information generated in this chapter about the expected plane of migration and the regions where this might be happening, we test in the next chapter the hypothesis that the new cell type moves away from the ventricle and converts into neurons. We will explore the sites of origin and overall pattern of migration of young neurons (Alvarez-Buylla and Nottebohm, in prep.).

CHAPTER 4

MIGRATION OF YOUNG NEURONS IN ADULT CANARY BRAIN

Chapter 1 raises the problem of how neurons that are born in the adult brain might reach parts of the telencephalon far from the ventricular zone. Unlike neuronal and most glial processes, radial glia are positioned with the cell body in the ventricular zone and a long process that penetrates the adult canary telencephalon (Chapter 2 and 3). This orientation could guide the migration of young neurons from the ventricular zone where they are thought to be born into the brain parenchyma. Associated with these radial glia fibers are elongated cells that are only present in the telencephalon (Chapter 3), the site of adult neurogenesis in canaries (Nottebohm, 1985). In this chapter the elongated cells are shown to correspond to young migrating neurons, and the paths and timetable of neuronal dispersion are described in the adult canary brain (Alvarez-Buylla and Nottebohm, in prep.).

Experimental approach.

In order to show that the new, small and elongated cell type was born in adulthood and that cells of this type migrate away from the ventricle, twenty-one 13-15 month-old male canaries received two IM injections of [^{3}H]-thymidine (for detailed methods see Chapter 6). They were sacrificed in 7 groups of three, 1, 3, 6, 15, 20, 30 and 40 days after the last injection. An additional group of three males received just one injection of [^{3}H]-thymidine and was killed 1 h later. Sections 10 um thick were stained with the 40E-C monoclonal antibody to vimentin, incubated for autoradiography and

counterstained to show cell morphology (see chapter 5 for experimental details). The number and position of labeled cells was counted and mapped in 4 non-adjacent sections of forebrain for each bird, taken at the level of the anterior commissure (Appendix A). Based on the information generated in chapter 3, this level of the brain was chosen because: 1) Many radial glia processes could be seen parallel to the plane of the sections, and 2) their medio-lateral orientation was away from the lateral ventricle (Fig. 3-2). It was thus expected that cells would move away from the ventricle instead of traveling for some distance parallel to the ventricular zone (see Fig. 3-2C).

The proportion of different labelled cell types changes with survival.

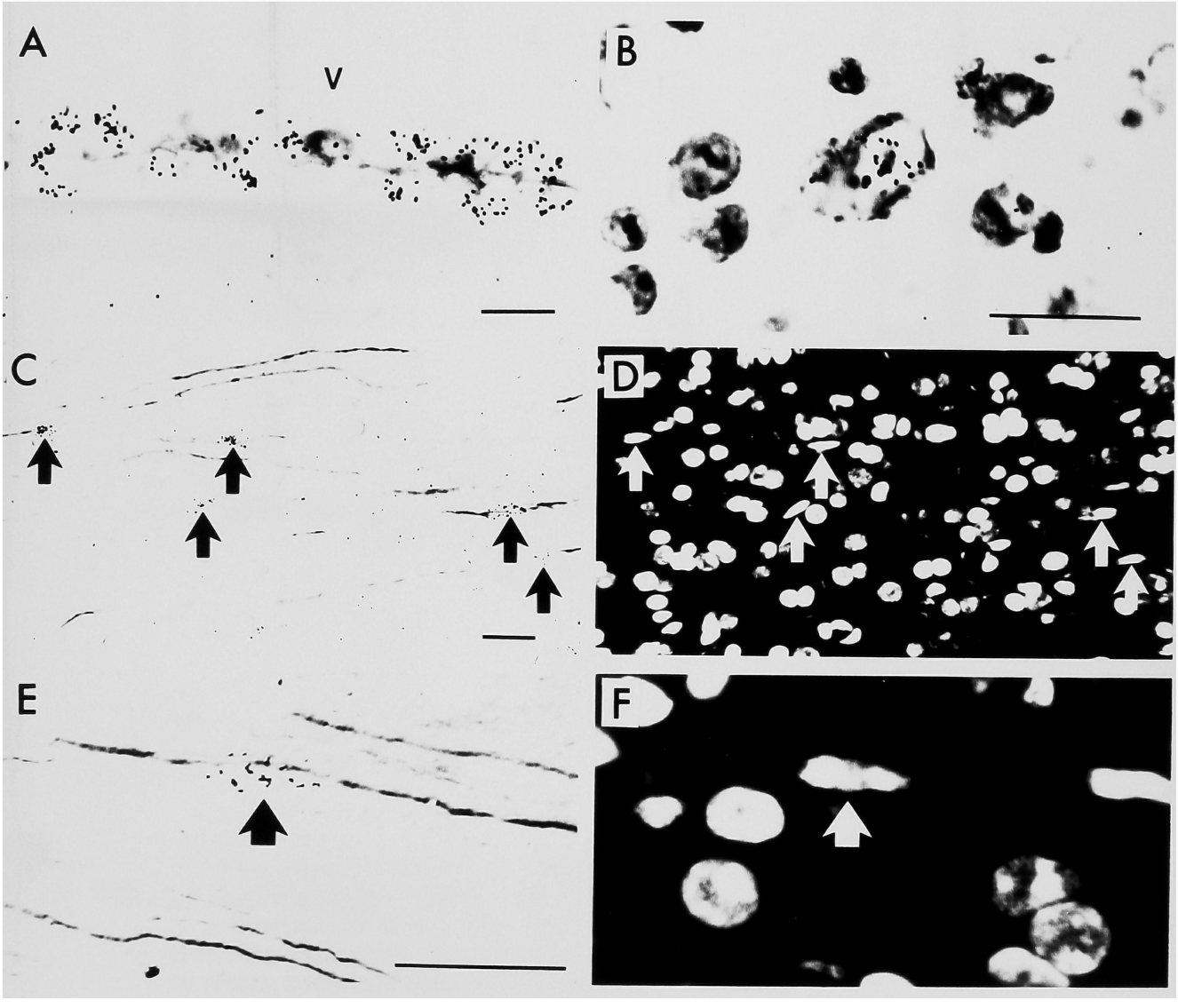
Seventy-two percent of all labeled cells in the sections of birds killed 1 h after their last injection were found in the ventricular zone lining the lateral ventricle of the forebrain (Fig. 4-1 A and 3); The small size of these cells and their high density did not allow one to distinguish between cell types in the ventricular zone. The remaining labeled cells were distributed throughout the brain parenchyma and were either glia, endothelial cells or cells of uncertain identity, in that order of abundance. At this survival time there were no labeled neurons, and no labeled small elongated cells that could correspond to the new cell type described in chapter 3. The distribution of labeled ventricular zone cells was very similar in birds killed 1 h or 1 day after the last injection of [^{3}H]-thymidine.

There was a 63% increase in the number of labeled ventricular zone cells between survival days 1 and 3. This increase may be due to the division of some of the cells labeled on day 1. The number of labeled ventricular zone cells decreased thereafter. By

days 20, 30 and 40 there were only 5.2%, 3.5% and 1.5% as many labeled ventricular zone cells as had been counted on day 3 (Fig. 4-2).

Three days after the [^{3}H]-thymidine treatment many cells with the anatomy described in chapter 3 for the "new cell type" appeared labeled (Fig. 4-1 B-E and C-F). The number of these cells increased up to day 20 as the labeled ventricular zone cells decrease (Fig. 4-2). After day 20 there was a rapid decrease in the number of labeled cells of the new type that coincides with a steady increase in the number of labeled neurons (Fig 4-1D and 4-2). This result strongly suggested that ventricular zone cells give rise to the small elongated cells of the new type, which in turn convert into neurons. It remained now to prove that the new cell type migrates.

Figure 4-1. Photomicrographs of cell types involved in neurogenesis.
A. Exposed silver grains overlie the nuclei of many [^{3}H]-thymidine labeled ventricular zone cells in the lateral wall of the lateral ventricle (V). The dark staining at a different focal plane correspond to 40E-C positive radial glia cell bodies and processes. The bird survived 24 h after [^{3}H]-thymidine treatment.
B. Neuron appear labeled between 20 and 40 days after [^{3}H]-thymidine injection. The one on this photomicrograph was found in the hyperstriatum ventralis (HV in Fig. 4-3) after 30 days survival.
C. Labeled migrating cells (arrows) in HV, showing 40E-C positive fibers and exposed silver grains. Notice that the long axis of the clusters of exposed silver grains corresponds with the axis of the 40E-C positive fibers.
D. Same field as C but viewed under fluorescence; arrows point to four elongated nuclei of migrating cells that correspond to the four clusters of exposed silver grains in C. Stain is Hoechst 33258.
E. High magnification of migrating cell labeled with exposed silver grains (arrow) and apposed to a 40E-C positive fibers.
F. Hoechst 33258 fluorescently stained nuclei of the same cell shown in E (arrow).
Calibration marks 20 um.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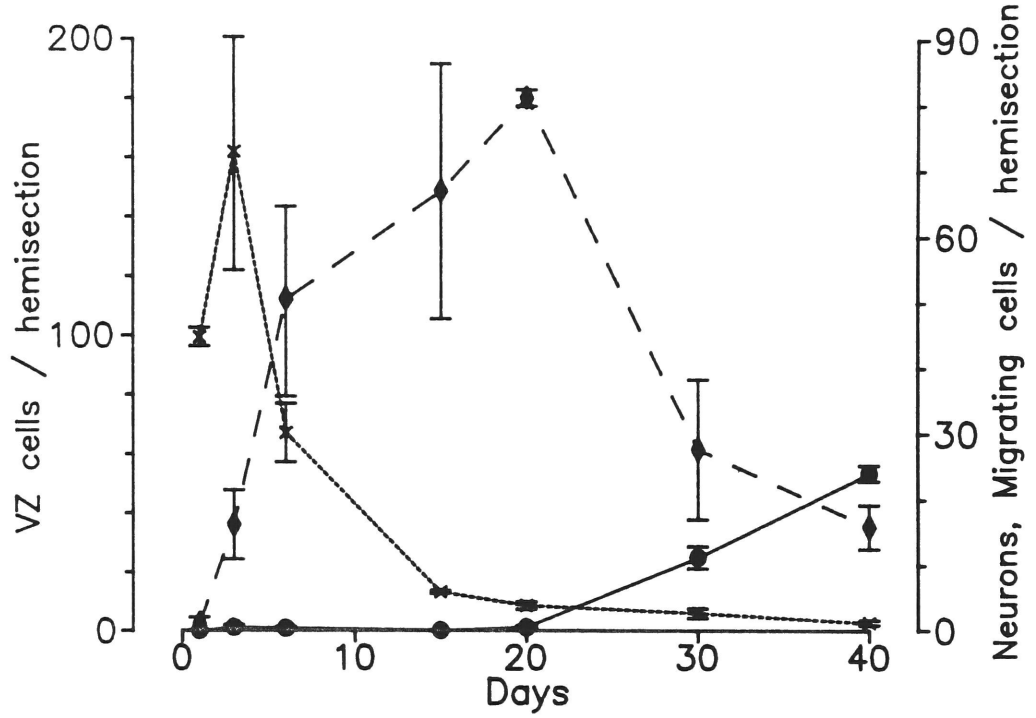


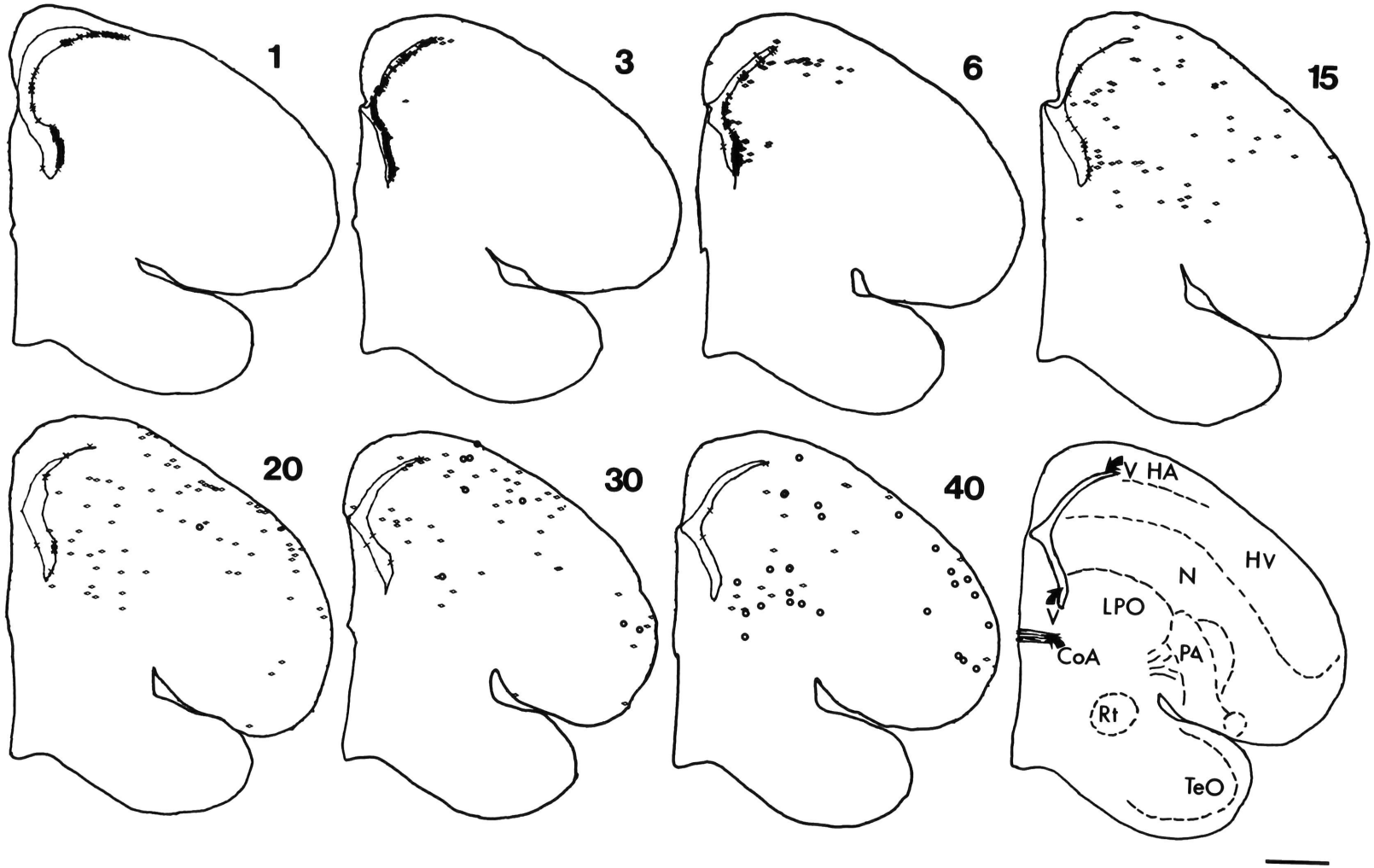
Figure 4-2. Mean number of labeled ventricular zone cells (X·····X), (left-side scale), labeled migrating cells (◆- - -◆), and labeled neurons (●—●), (right-side scale) as a function of time. The horizontal axis indicates number of days after [^{3}H]-thymidine treatment. Each point in a curve is the mean of 3 birds; for obtaining this mean, each bird was represented by the average number of labeled cells of a particular type per hemisection (4 hemisections sampled). Vertical bars correspond to standard error.

Dispersal of the new cell type.

The position of the different cell types at different survivals after [^{3}H]-thymidine treatment was mapped using the computer-microscope system described in appendix A. A few labeled cells of the new type were first seen one day after [^{3}H]-thymidine injection very close to the lateral ventricle (Fig. 4-4). By day 3 the number of these cells had increased and some were seen slightly lateral with respect to the lateral ventricle (Fig. 4-4) though at distances of less than 500 um from the nearest ventricular zone (Fig. 4-3). Three days later, two groups of the new cell type, one in the hyperstriatum and the other in lobus parolfactorius, appeared labeled (Fig. 4-3, 4-4). Some of these cells are now as far as 3mm from the ventricular zone, and concentrated in regions rich in radial glia (compare Fig. 3-2 B and Fig. 4-3 [6]). At this survival time many labeled elongated cells of the new type were found tightly apposed to radial glia fibers (Fig. 4-1 B-E, C-F).

By day 15 labeled cells of the new type -which will now be called "migrating cells"- had reached further laterally. The number of migrating cells was still considerably higher in the radial glia- rich regions but some cells were also present in areas where radial glia fibers are not common (Fig. 4-3). By day 20, some labeled migrating cells were found in the most lateral regions of the telencephalon, as far away the lateral ventricle as possible. Figure 4-3 also shows the position of the new neurons as they appeared between days 20-40. One might expect that labeled neurons would first appear close to the ventricle, since migrating cells will reach this point sooner than most distant points. However this was not the case, suggesting that the delay between birth and differentiation may not be dependent on migration distance alone.

FIGURE 4-3. Maps of transverse hemisections of 6 adult male canaries at the level of the anterior commissure (A.P. 1.6 of canary atlas, Stokes *et al.*, 1974), obtained from birds sacrificed at 1, 3, 6, 15, 20, 30 and 40 days after the second of two doses of [^{3}H]-thymidine. The position of labeled ventricular zone cells (X), migrating cells (<>) and neurons (O) is shown. The last section, was taken at the same level to show the anatomical regions present at this section level. Abbreviations: CoA, anterior commissure; HA, hyperstriatum accessorium; HV, hyperstriatum ventralis; LPO, lobus parolfactorius; PA, paleostriatum augmentatum; Rt, nucleus rotundus; TeO, optic tectum; V, lateral ventricle.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Migration time-table.

Using the coordinates of each labeled migrating cell (stored into the computer while mapping, see appendix A) it was possible to calculate the distance from each labeled migrating cell to the nearest lateral ventricle. The pattern of dispersal is shown in figure 4-4 (upper panel). If we use the leading cells in the wave of migration to measure the maximal rate at which these cells moved away from the ventricle, then this rate peaked between days 3 and 6 (28 um/h), slowing down between days 6 and 20 (8 um/h) (Fig. 4-4). It may be inferred that during each of these two stages the migrating cells traveled at least that fast, since the calculation of distance migrated assumes a straight path between each migrating cell and the nearest ventricle. The apparent slowing down that occurs after day 6 does not necessarily imply that the pace at which each migrating cell is moving has diminished, but, that the absolute rate of separation from the ventricle has diminished. This would be the case if migrating cells follow now a more meandering path.

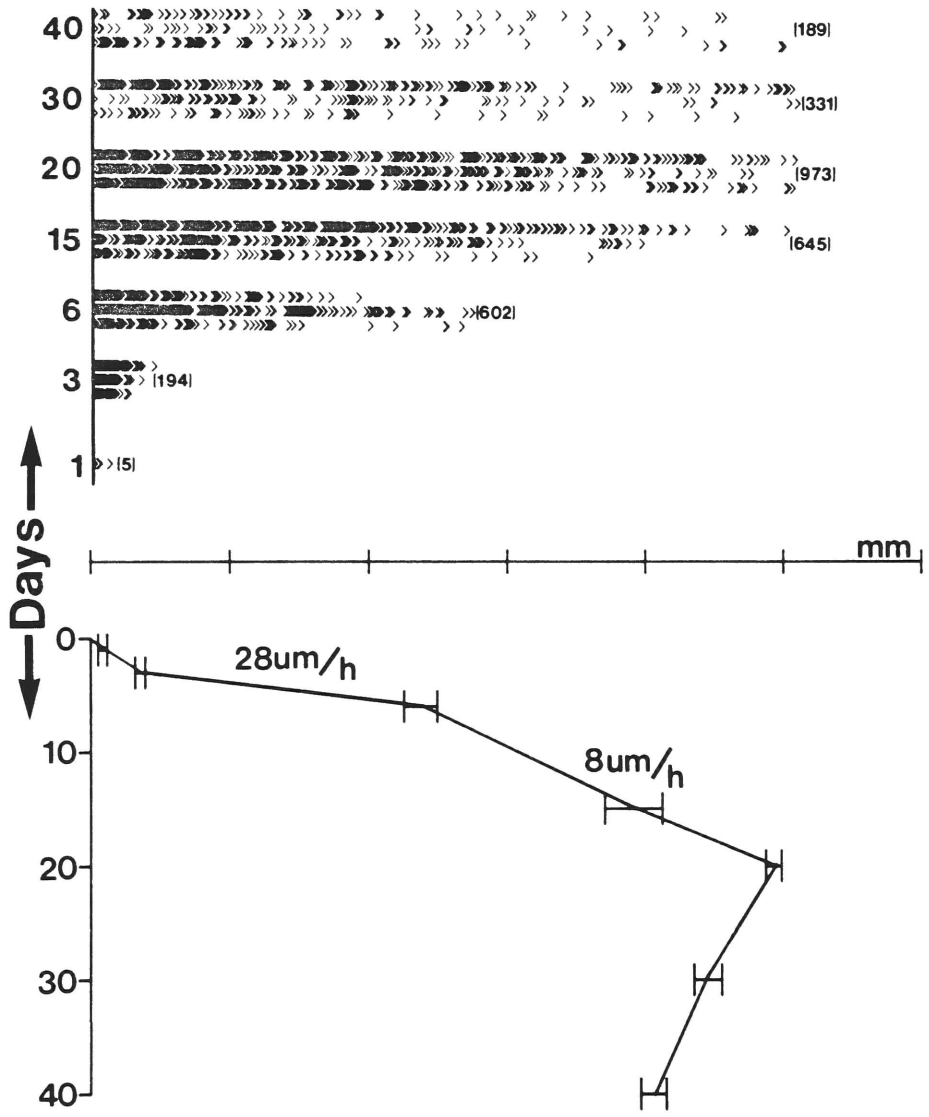


Figure 4-4. Upper panel: Dispersal of [^{3}H]-thymidine labeled migrating cells (>) as a function of time. The horizontal axis (shared with lower panel) indicated distance in mm from a labeled migrating cell to the closest ventricular wall. The vertical axis indicates survival time in days. Each survival time is represented by 3 birds (each row corresponds to all 4 hemisections from 1 bird). The number of labeled migrating cells per survival group is shown in brackets, to the right.
Lower panel. Mean distance from the nearest ventricular wall for the 5 migrating cells per bird (total 15 cells) which at each survival time were the farthest from the ventricular wall. The calculated slowest speed at which these cells could have traveled from 3 to 6 days and from 6 to 15 days is indicated. Mean +/- standard error.

DISCUSSION

Results presented in this chapter show, 1) that the elongated cells of the new type described in chapter 3 come from cells that are dividing in the ventricular zone. 2) That the new cell type is migratory; initially moving away from the ventricle at a fast rate and slowing down after 2-3mm of the journey. 3) That the appearance of new neurons coincides with the disappearance of migrating cells, suggesting that the migrating cells convert into neurons. Taken together, these results chart the process of neurogenesis in adult canary brain, from neuronal birth, through neuronal migration, to neuronal differentiation.

The site of birth.

The majority (72%) of the cells dividing in the adult canary brain are in the lateral walls of the lateral ventricle. The medial wall of the same ventricle facing the septum; and walls of the other ventricles of the canary brain are practically devoid of dividing cells (also see Chapter 5). The very few cells found to divide in these other ventricles probably indicate the low level of cell proliferation required to maintain ependymal function. The excess of dividing cells in the lateral walls of the lateral ventricle of the canary telencephalon are probably related to neurogenesis, since only the telencephalon receives new neurons (Nottebohm, 1985). Two other observations presented in this chapter support this interpretation. 1) The number of [^{3}H]-thymidine labeled cells in the ventricular zone decreases after the first three days following [^{3}H]-thymidine administration, suggesting that they are not dividing to generate cells that would perform local functions in the ventricular zone but rather for cell export, and 2) The first labeled migrating cells to appear do so very close to the ventricle suggesting that at least some daughter cell of cells that divide in the lateral ventricle convert into migrating cells. At

the level studied the dividing cells in the lateral walls are concentrated in ventral and dorsolateral domains in the lateral walls of the lateral ventricle. These region were also found to give rise to many radial glia fibers (Fig. 3-2 B), suggesting that ventricular zone cell proliferation and radial glia are functionally related.

Migration

Labeled migrating cells in their initial phase of dispersal appear to cluster near the dorsal and ventral tips of the lateral ventricle, in regions that have the highest density of radial glia fibers and many of the labeled migrating cells found there, are attached to these fibers (Fig. 3-1 C-DE and E-F). The maximum percent of labeled migrating cells attached to radial glia fibers occurred at 1, 3 and 6 day survivals; at those times 50%, 29% and 32% of the labeled migrating cells, respectively, were tightly apposed to radial glia fibers. However, even in the cases where a labeled migrating cell was not attached to a radial glia fiber, its orientation was usually very similar to that neighboring fibers (Fig. 3-1 B and E). By days 15 and 20 after [^{3}H]-thymidine treatment, the proportion of labeled migrating cells attached to radial glia fibers decreased to 16% and 9% respectively, and by then many of the labeled migrating cells had moved past the radial glia fiber-rich region (Fig. 3-3). The labeled migrating cells moved away from the ventricle at 20-30um/h for the first 2-3 mm and slowed down (8um/h) thereafter. Perhaps the slowing down of the migrating cells occurred as these cells detached themselves from the radial glia. The first 3mm away from the ventricle contain the highest concentration of radial glia fibers (Fig. 3-2 B). These observations suggest that during the first week of a migrating cell's journey, radial glia play an important role in the dispersal of these cells away from the ventricle; thereafter other cues take over. It has been suggested before that in embryos and fetal vertebrate brains neuronal processes as

well as radial glia play a role in guiding the migration of young neurons (Chapter 1, Zagon, *et al.*, 1985; Rakic, 1985).

The speed of migration reported here is considerably faster than the 2-5 um/h which is deemed to be typical for migrating neurons in developing vertebrate brain (Jacobson, 1978, pp.66 and 87). The migration of young neurons during development is difficult to measure because the brain is constantly changing size. In addition the many cells migrating during histogenesis might also create traffic jams, slowing down cells with the capacity to move faster. In vitro studies support this interpretation, since granular neurons migrating on glia processes in culture move at a speed of 33 um/h (Edmondson and Hatten, 1987). Alternatively the young neurons migrating in adult brain may have developed a fast way of locomotion. If they were to migrate at the 2-5um/h typical of development, it would take them 45-100 days to reach their furthest destinations in adult canary brain.

Migrating cells become neurons.

There are many reasons to believe that the migrating cells are young neurons. 1) If a significant fraction of those migrating cells were to become glia, then the number of labeled glia should increase at longer survival periods. This was not the case. A very similar number of labeled glia were seen at survival times of 1 day and 40 days (means of 20.4 vs. 21.9). Furthermore, the distribution of labeled glia with respect to the ventricular zone did not change with survival time, suggesting that all labeled glia became labeled and divided in situ. 2) No labeled neurons whose positions were mapped after achieving adult neuronal morphology occurred outside the forebrain or in the septum (this Chapter and Nottebohm, 1985). As discussed in Chapter 3, labeled migrating cells also occurred only in the telencephalon and were absent from the septum

or non-telencephalic regions. 3) None of the migrating cells were ever labeled with [^{3}H]-thymidine at short survival times, indicating that they are postmitotic cells committed to differentiation or death. This is also the case for neurons during development (Jacobson, 1978, p.44) that unlike glia do not divide after they start migration. 4) The migrating cells were negative to glial markers such as GFAP and vimentin (Chapter 3). To my knowledge there is no marker that would recognize young migrating neurons. Finally and most definitive is the evidence presented in this Chapter, i.e. 5) the natural history of the elongated cells of the new type. They were the only cells that originated from the ventricular zone, dispersed throughout the telencephalon and disappeared as neurons started to appear.

Overproduction of migrating cells.

The number of labeled cells in the VZ as well as in the migrating cell category exceeded considerably the number of labeled neurons. So, for example, a total 288 neurons appeared labeled in the l2 sections of the 3 birds killed after 40 days survival (Fig. 4-4); at that time those same sections also showed l89 migrating cells. The maximal number of migrating cells, 973, occurred at 20 days. If we subtract l89 from 973, we are left with 784 migrating cells that had disappeared by 40 days. The ratio between 784/288 = 2.72. Roughly one-third of the cells that migrated from the VZ became neurons. We do not know, of course, how many of the l89 labeled migrating cells still present at 40 days survival would have turned into neurons, and whether all labeled neurons present at that day were destined to survive much longer. Even if all 189 labeled migrating cells were to become neurons, more than half of the migrating cells would had disappeared. The above numbers suggest that many migrating cells died either before finishing their migration or after reaching their destination and while differentiating into adult neurons. This observation is of interest because it suggests a way by which "cell

selection" may be used by young migrating neurons. The ones that survived were those that reached sites for neuronal replacement. However, the apparent excess of neurons produced in adulthood may be similar to the overproduction seen in embryos (Hamburger, 1975).

Summary.

Neurons labeled in adult canary brain were born in selected areas of the VZ of the lateral ventricle. From there they dispersed, adopting the elongated shape described for the new cell type in chapter 3. Many of these cells followed radial glia as they dispersed at 20-30um/h away from the ventricle; migration then slowed down, as other cues probably were used to guide their migration. Differentiation into adult neuronal morphology was achieved by only one-third of the young migrating neurons after a period of at least 20 days.

CHAPTER 5

CONCLUSIONS AND FUTURE VISTAS

When neurogenesis in adult avian brain was first reported (Goldman and Nottebohm, 1983; Nottebohm, 1985), skeptics like myself argued that it seemed very unlikely that young neurons born in the ventricular wall could penetrate the adult brain's parenchyma, there to migrate and find their way over relatively long distances. Knowledge of neurogenesis during development provided three reasons for this disbelief: 1) Radial glial fibers were thought to be absent in adult avian brain. 2) Radial glia fibers were thought to be important to guide neuronal migration. 3) The slow migration speeds reported for young neurons during development made it impossible to conceive that only 1 month after final mitosis, differentiated neurons appeared as far as 5-6mm away from the nearest ventricular wall.

This thesis offers direct evidence that neuronal migration continues to occur in the adult avian brain and suggests ways in which this may be happening. The observations reported in chapter 2 indicate that radial glia persist in the adult avian brain, confirming the early observation of Nottebohm *et al.* (1985). In chapter 3 it is shown how radial glia fibers orient in a mediolateral plane to reach far lateral into the telencephalic parenchyma. Also in chapter 3 an elongated cell frequently attached to radial glia is shown to occur only in the telencephalon. These cells are identified as young migrating neurons in chapter 4; cells born in restricted regions of the lateral ventricle become elongated cells that move away from the ventricle at a much higher

speed than that described for development. A fraction of these migrating cells then differentiate into adult neurons.

The main conclusion from these findings is that **the adult avian brain has specific properties related to the production, migration and differentiation of neurons and thus the adult functioning brain can support the processes involved in neurogenesis.** There is no reason to believe that these properties are unique to the canary brain. Ventricular zone cells that send long processes into the brain parenchyma of adult cold-blooded vertebrates have been described for almost a century (e.g. Van Gehuchten, 1897; Studnicka, 1900; Ramón y Cajal, 1911; Horstmann, 1954). These cells may be very similar to radial glia in adult canaries. In addition many cold-blooded vertebrates continue to grow throughout life and in some fish, at least, persistent neurogenesis into adulthood has been demonstrated (Leonard *et al.*, 1978; Birse *et al.*, 1980; Raymond and Easter, 1983; Easter, 1983; Anderson and Waxman, 1985). In adult fish, as in embryonic mammals and adult birds, radial glia may guide the migration of young neurons. It will be interesting to see how many other groups of vertebrates have ventricular zone neurogenesis and radial glia-guided dispersal of neurons in adulthood. Do marsupials, reptiles or amphibians continue to generate neurons into adulthood?

The findings presented in this thesis may be representative of how neurogenesis and neuronal migration occur in an adult brain. If these findings are applicable to cold-blooded vertebrates, then mammals, from whom the dogma of neurogenesis being strictly a developmental process arises, could in fact be the exception; where mammalian adult neurogenesis has been studied, it is either not present or restricted to microneurons in a few regions of the brain (Altman, 1970; Kaplan and Hinds, 1977; Bayer, 1985; Rakic, 1985). Why is adult neurogenesis so poorly represented in mammals? Are mammalian

brains missing the precursor cells in the ventricular zone? are they lacking an inducer of proliferation or is there a repressor present? Does the absence of radial glia in adult mammals restrict the expression of neurogenesis? Proliferation in the ventricular walls of the mammalian brain is minimal (for review see Bruni *et al.* 1985), probably comparable to that of non-telencephalic ventricles in canaries (see below). It may be possible, eventually, to induce these cells to proliferate, migrate and differentiate into new neurons.

The model of neuronal migration in adulthood described here is unique in several respects. 1) The distances over which cells migrate are relatively long compared to the shorter distances observed in developing brain. 2) The size of the brain remains fixed from the onset to the termination of migration and the anatomical landmarks remain unchanged. 3) The number of migrating cells in any one part of the brain is considerably smaller than during development, making it easier to follow the fate of individual cells. 4) The speed of migration is faster than that reported in developing brain. 5) The presence of regions rich in radial glia fibers and of others poor in radial glia fibers permits comparisons of factors regulating migration under both these conditions.

Adult neurogenesis in canaries offers a new model to study what factors regulate the birth, migration and differentiation of new neurons. It also offers an opportunity to interfere in a piecemeal manner with the generation of specific types of neurons, and thus assess the behavioral relevance of those neurons. Three new approaches offer interesting areas for discovery; I would finish my doctoral dissertation by briefly describing these preliminary results.

Dynamics of the ventricular zone

One of the most interesting observations in this thesis is the apparently heterogeneous distribution of the labeled ventricular zone cells (Chapter 4). The walls of the lateral ventricle in the canary vary in anatomy from one region to another. In some walls, like those facing the lobus parolfactorius and its caudal pole, the ventricular zone is made up of 3-6 layers of cells. In this case it resembles the typical ventricular zone as it appears during development of the vertebrate brain (Boulder Committee). More lateral and dorsal, the ventricular zone gets thinner; being only one or two cells thick. In order to find where exactly cells are born in the adult canary brain ventricles, [^{3}H]-thymidine labeled cells in the ventricular zone were mapped in the brain of a 1 year old male canary. The bird was allowed 1 hour survival after the single [^{3}H]-thymidine injection and was processed for autoradiography as described in Chapter 6. The position of labeled ventricular zone cells in all ventricular walls (lateral ventricle as well as the third and fourth ventricles) was mapped. As shown in Figure 5-1, labeled ventricular zone cells appeared only in the lateral ventricle; of 429 cells labeled, only one was not in the lateral ventricle and was found in the ventricle of the tectum. This result further supports findings presented in previous chapters on the selective distribution of radial glia and migrating cells only in the telencephalon, strengthening the notion that adult neurogenesis happens only in the telencephalon (Nottebohm, 1985).

For scoring purposes the lateral ventricle was then divided into a latero-ventral wall facing most of the telencephalic mass, and a medio-dorsal wall facing the periphery, that includes the hippocampus and septum. Of the 428 labeled ventricular zone cells in the lateral ventricles, only 27 were in the medio-dorsal wall. Thus most of the cells proliferating in the lateral ventricle faced the striatal areas that in birds make up the

majority of the telencephalic mass. Otherwise, labeled ventricular zone cells on the walls of the lateral ventricle were found throughout the entire rostro-caudal domain of this cavity. In the most rostral tip, where the ventricle was found to become spherical (Fig. 5-1), cells were labeled also in its rostral walls. These cells probably give rise to young migrating neurons that service the rostral tip of the telencephalon. As shown in Chapter 3 (Fig. 3-3), a cohort of radial glia fibers travel in the sagittal plane to reach into rostral telencephalon.

Proliferative zones seemed to be segregated in the lateral-ventral wall of the lateral ventricle in two groups. One zone occurred ventrally, facing the lobus parolfactorius and the other zone occurred towards the lateral border of the lateral ventricle. Although these two regions had the highest density of dividing cells, labeled cells could also be found between these regions (Fig. 5-1). The very ventral tip of the lateral ventricle at the level of the stria terminal was always devoid of dividing cells.

The segregated distribution of labeled cells in the lateral-ventral walls of the lateral ventricle is of special interest. It could point to a site where a neuroblast population resides, and thus allow isolation by microdissection of small fragments of ventricular zone containing increased densities of neuroblasts. Such fragments could for example be used for in vitro bioassays to study the factors that regulate neuroblast proliferation.

There is another aspect of neurogenesis that is most intriguing and has to do with the origin and displacement of labeled cells within the ventricular zone. In order to study this problem, the distribution of ventricular zone cells was mapped at the level of the anterior commissure, 1, 3, and 6 days after [^{3}H]-thymidine treatment. At one day

survival, the two regions of the latero-ventral ventricular wall with a higher number of labeled cells were clearly detectable (Fig. 5-2). By day 3, the overall number of labeled cells had increased (Chapter 4, Fig. 4-2), presumably due to the division of cells already labeled. Unexpectedly, the ventral peak was now shifted to the left (i.e. dorsally, Fig. 5-2). Regions of the ventricular wall that had very few labeled cells at day 1, appeared to have gained 2-3 times more labeled cells by day 3 (Fig. 5-2). This finding has two possible explanations: 1) cells in different regions of the ventricular zone divide at different rates, and 2) cells may move within the ventricular zone. As shown in Chapter 4, very few cells had started migration at day 1 and the number of migrating cells at day 3 could not explain the proportional decrease in labeled ventricular cells in the day 1 ventral peak (Fig. 5-2).

The differential proliferation by regions of ventricular zone cells could be tested by counting the number of autoradiographic grains per labeled cell at different survival times. By using this technique the number of times cells divided in the different regions of the ventricular zone could be calculated. This approach together with others trying to label only certain regions of the ventricular zone with vital dyes are in progress in the laboratory and should resolve the interesting problem of the displacement and proliferation dynamics of the ventricular zone stem cells.

Many cells divided a fixed number of times after they became committed to terminal differentiation. The pattern of neuroblast division during the final stages of differentiation into young migrating neurons is not known. In the embryo the ventricular zone is thick and cluttered with dividing cells, making it difficult to follow the proliferation and movement of neuroblasts. The localized and sparse distribution of neuroblasts during adult neurogenesis may provide a unique opportunity to study this

problem. The most interesting possibility is that neuroblasts move in the plane of the ventricular zone as they differentiate while undergoing their final division. In this way, cells labeled with [^{3}H]-thymidine in different places of the lateral wall of the lateral ventricle at short survival times (Fig. 5-1), might correspond to unique stages of differentiation.

Figure 5-1. Map of [^{3}H]-thymidine labeled (dividing) ventricular zone cells (red crosses) in frontal section of 1 year old canary right brain hemisphere. This animal survived 1hr after receiving a single IM injection of [^{3}H]-thymidine. Left is medial and up is dorsal. The approximate anterior(A)-posterior(P) level in the stereotaxic atlas of the canary brain (Stokes, *et al.* 1974) is indicated. Major laminae are shown by green dashed lines, III V indicates the third ventricle while T V shows the ventricle of the tectum. The lateral ventricle is indicated by asterisks. Notice how labelled ventricular zone cells are almost unique to the latero-ventral wall of the lateral ventricle and how labeled cells on this wall are segregated in ventral and dorso-lateral groups.

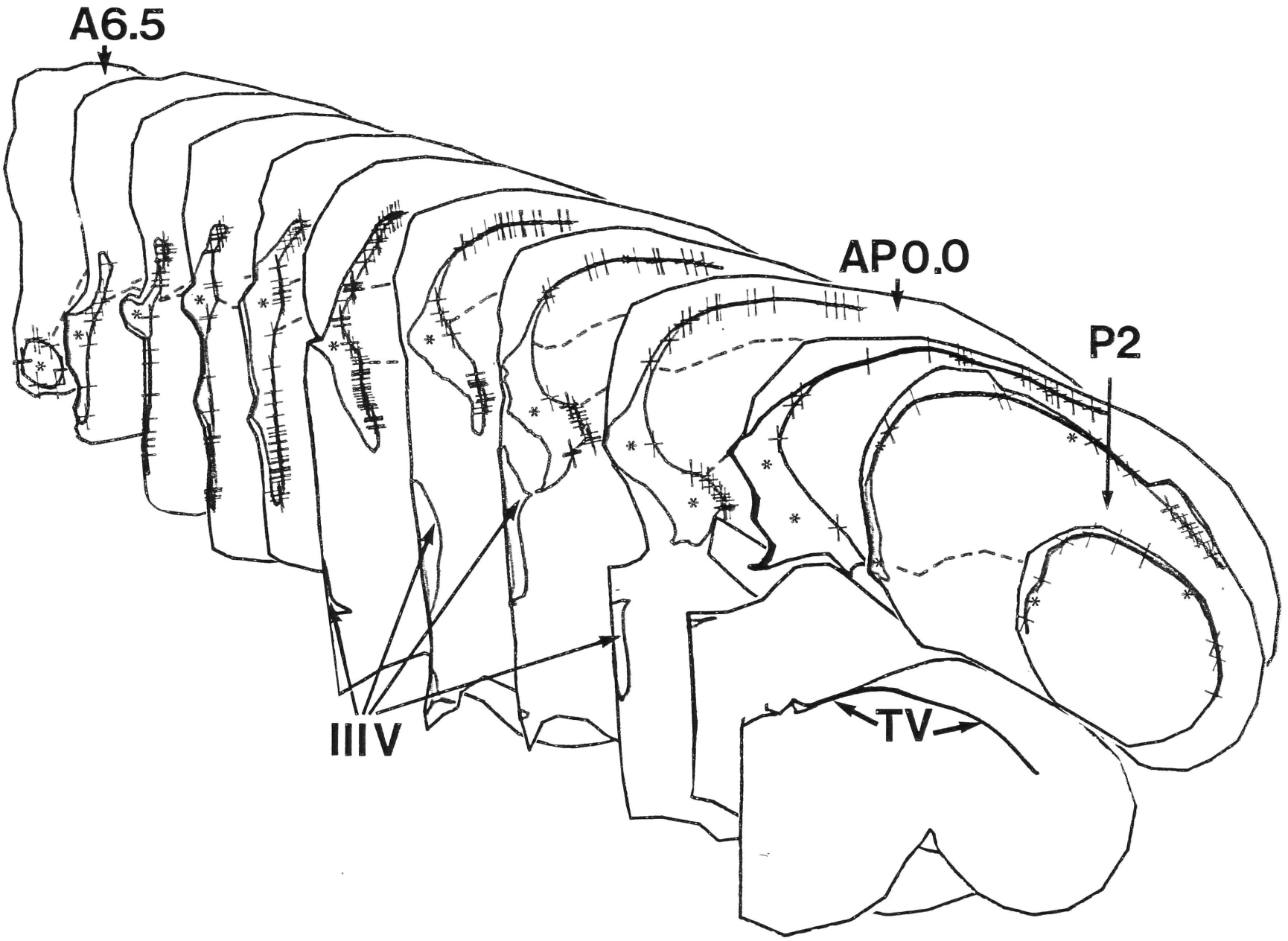
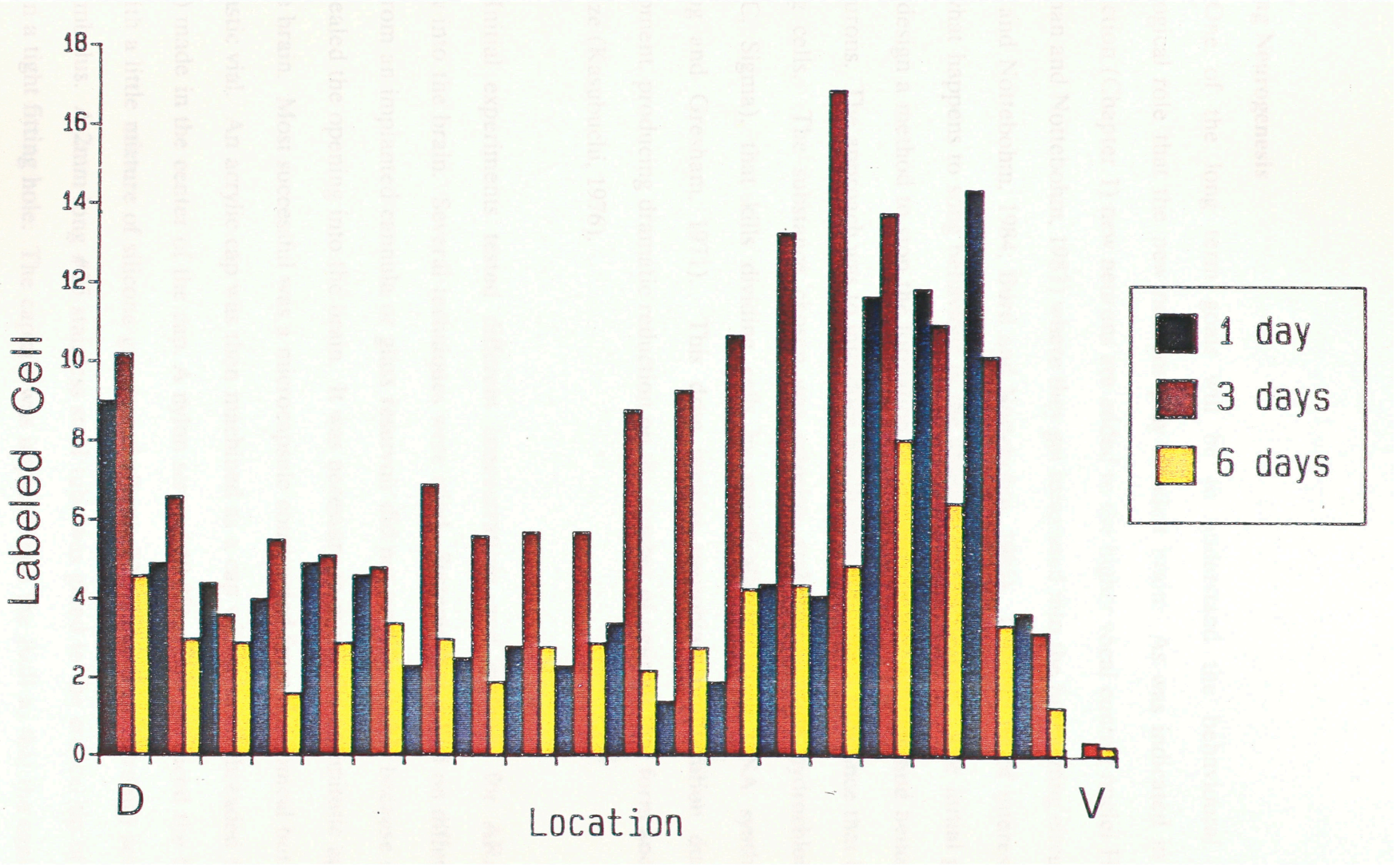

A6.5
AP0.0
P2
III V
TV

Figure 5-2. Distribution of labeled ventricular zone cells in the latero-ventral wall of the lateral ventricle at the level of the anterior commissure. This wall was divided in 20 equal segments from the ventral (v) to the dorsal (d) tip and the number of ventricular zone cells labeled per segment counted. The inset indicates the corresponding survivals after 2 injections of ^{3}H-thymidine (12 hours apart). Notice how the ventral peak of bars on day 1 (blue) becomes displaced dorsally by day 3 (red, see text for details). Each bar is the mean of 3 birds.


Labeled Cell
18
16
14
12
10
8
6
4
2
0
D
Location
V
1 day
3 days
6 days

Stopping Neurogenesis

One of the long term goals will be to understand the behavioural and physiological role that the new neurons play in adult brain. As was indicated in the introduction (Chapter 1) new neurons are added to the higher vocal control nuclei HVc, (Goldman and Nottebohm, 1983) where they get integrated into the functioning circuitry (Paton and Nottebohm, 1984; Burd and Nottebohm, 1985). It would be of interest to know what happens to song behaviour in the absence of new neurons. The initial goal was to design a method to stop the formation of new cells in the ventricle and hence of new neurons. The approach was to introduce locally into the brain a substance that kills dividing cells. The substance chosen was cytosine arabinofuronaside hydrochloride (ARA-C, Sigma), that kills dividing cells by specifically inhibiting DNA synthesis (Furlong and Gresham, 1971). This drug inhibits neuroblast proliferation during development, producing dramatic reduction on the number of neurons that form and on brain size (Kasubuchi, 1976).

Initial experiments tested different concentrations and methods for ARA-C delivery into the brain. Several techniques were tried. Systems that relied on diffusion alone from an implanted cannula or glass reservoir did not work, probably because scar tissue sealed the opening into the brain. It was necessary to pump the antimitotic agent into the brain. Most successful was a microcapsule constructed from the conical bottom of a plastic vial. An acrylic cap was then machined as a cap with a small threaded hole (#0-80) made in the center of the cap. A nylon screw (#0-80) which produced the final seal (with a little mixture of silicone grease and silastic 738 RTV, Dow Corning) served as an embolus. A 2mm long #25 stainless steel tube was glued to the conical tip of the vessel in a tight fitting hole. The capsule was implanted on the skull so that the cannula

on the conical tip went through a small slit on the dura and penetrated the telencephalon for 50-100 um. The capsule was then glued to the skull with dental acrylic.

After 24 hour surgical recovery, treatment was started: one quarter turn of the screw delivered 80 to 100 nl of ARA-C solution into the brain. A concentration of 10^{-2}M of ARA-C in the capsule is the minimal effective dose to inhibit cell proliferation. This dose did not seem to cause any generalized damage, or to be cytotoxic to neurons. Randomly chosen fields around the implantation site had the same number of neurons with saline filled (controls) or ARA-C capsules. The only region of damage was the implantation site, where the ARA-C produced a larger lesion than the control. This probably resulted from the proliferation inhibition of scar tissue. The first experiment was designed to test the effects of ARA-C filled capsules on ventricular zone cell proliferation.

Two capsules were implanted in the brain hemispheres of an adult canary, one with ARA-C on the left and a control with saline on the right. The screw was turned twice a day (at 12 hour intervals) for seven days. During the last 4 days of treatment, [^{3}H]-thymidine (see Chapter 6) was injected. The bird was sacrificed on the 8th day and its brain processed for autoradiography. The results are shown in Fig. 5-3 A, and show the virtual elimination of dividing cells on the ARA-C treated side while no effect on the control side. As one moves rostral from the site of implantation of the ARA-C, the number of [^{3}H]-thymidine labeled cells equals those of the control side. These results show that the effect of ARA-C is restricted, suggesting that the concentration of ARA-C drops below the effective dose at a certain distance. From Figure 5-3 A, a radius of approximately 3mm is affected and thus a volume of approximately 113ul. Hence the volume released by one screw turn is roughly diluted 1000 times, attaining a final

concentration of around 10^{-5} M ARA-C. This is the concentration normally used to kill rapidly proliferating cells in culture (Alvarez-Buylla and Valinsky, 1985).

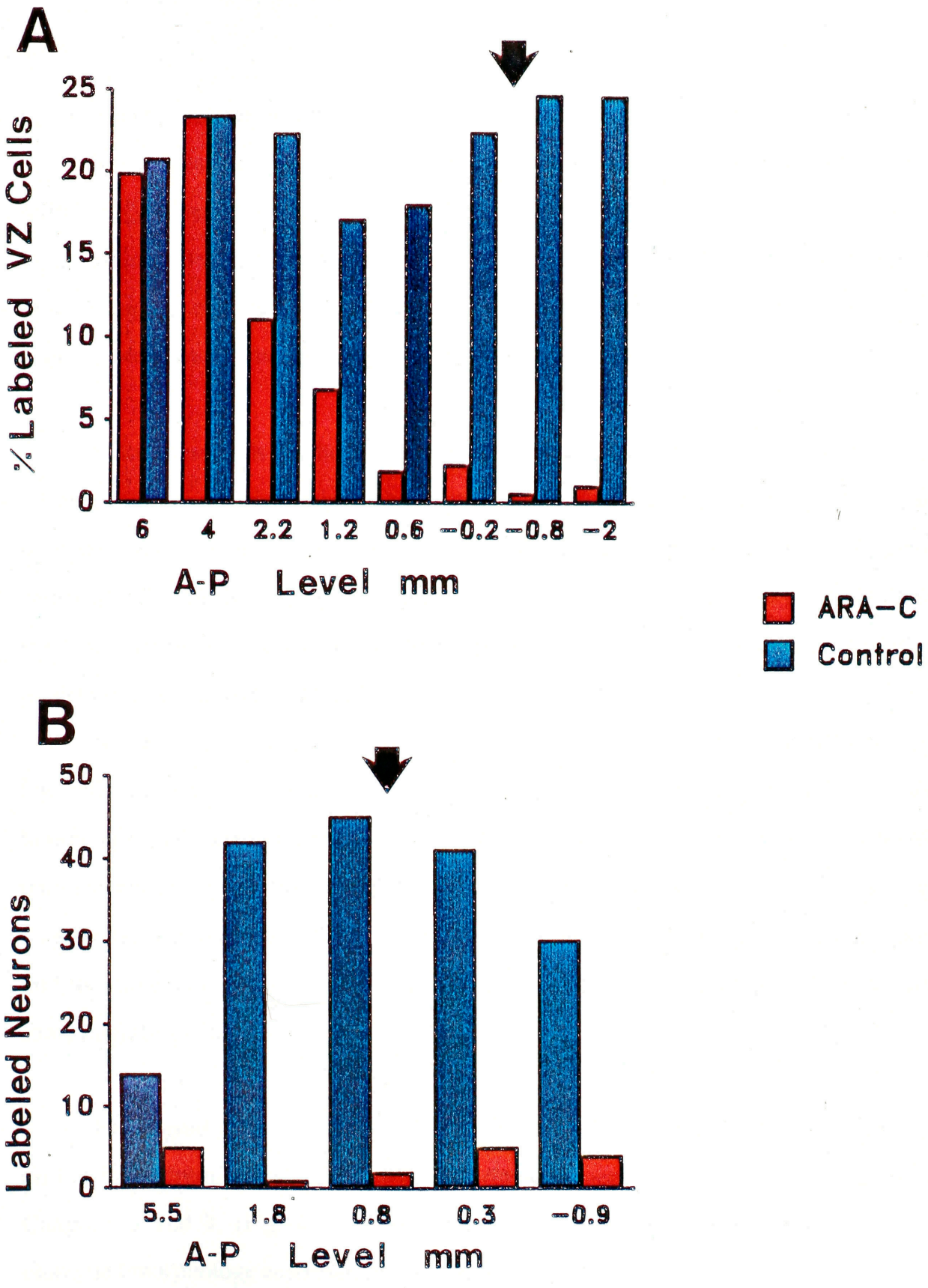
From the findings described in Chapter 4 one could predict that preventing cell division in the ventricular zone should abolish neuron formation. This was tested by repeating the procedure shown above but sacrificing the bird 60 days after the last [^{3}H]-thymidine injection. The results confirmed the prediction (Fig. 5-3 B). The number of [^{3}H]-thymidine labeled neurons in the treated side was greatly reduced, being on average 10% of the control. Again the site of maximal effect was close to the point of implantation. As mentioned above the average number of neurons counted in randomly selected fields is the same in control and ARA-C treated sides, discarding the possibility of generalized neuronal death.

The effect of ARA-C was reversible; if the bird was allowed to survive 60 days after ARA-C treatment and then administered [^{3}H]-thymidine, the treated side had as many labeled cells in the walls of the lateral ventricle as the control. Even after 2 weeks of screw turning, labeled ventricular zone cell division and new neurons reappeared, suggesting that after the ARA-C treatment, non-dividing stem cells could start dividing and re-establish the neuroblast population in the ventricular zone.

In summary, antimitotic delivery into the brain by capsules implanted onto the skull eliminated dividing cells in the ventricular walls and in this way stopped neurogenesis. The behavioural effects of this treatment are under study. This technique could also help in the study of ventricular zone dynamics. It would be interesting to kill dividing cells for a short period and then follow the location of reappearing [^{3}H]-thymidine labeled cells. If as suggested in the previous section, there is a primary source

of mitotic cells in the ventricle, this protocol could point to the location of the primary source.

Figure 5-3. Effect of ARA-C on ventricular zone cell proliferation (A) and on the formation of new neurons (B). The brain of 2 adult canaries was implanted with two microcapsules (see text), on the left hemisphere with ARA-C (red) and on the right with saline (control, blue).
A: The bird received 7 days of antimitotic and during the last 4, [^{3}H]-thymidine (see text and Chapter 6). On day 8 the bird was sacrificed. The number of [^{3}H]-thymidine labeled and unlabeled ventricular zone (VZ) cells was counted at different Anterior-Posterior (A-P) levels in both sides of the brain (Stokes *et al.* 1974).
B: The same procedure was used as for A, but the bird survived for 60 days after ARA-C and [^{3}H]-thymidine treatment, allowing neurons to migrate and differentiate. Total labelled neurons present in control (blue) and ARA-C treated (red) hemisection were counted at different A-P levels. The arrows in A and B indicate the approximate level of the capsule implantation.


A
% Labeled VZ Cells
25
20
15
10
5
0
6
4
2.2
1.2
0.6
−0.2
−0.8
−2
A-P Level mm
ARA−C
Control
B
Labeled Neurons
50
40
30
20
10
0
5.5
1.8
0.8
0.3
−0.9
A-P Level mm

Cytoplasmic processes of young migrating neurons.

The migrating cells have so far been identified by their characteristic nuclear morphology (Chapter 3). The size and shape of the cytoplasm is not known since under the conditions used so far it is not visible. Preliminary EM studies suggest that these cells have a highly contoured cytoplasm, that wedges between other processes. This complicated anatomy makes these cells difficult to follow in sections thinner than 5 um.

Since these cells are born in the ventricular zone, it was reasoned that vital dyes injected into the ventricle might label neuroblasts and in this way migrating cells would later be found labeled. A number of tracer substances were injected into the lateral ventricle. The birds were then allowed to survive for 6-7 days and 30 um thick brain vibratome sections were cut in the frontal plane. The sections were then processed to detect the tracer used (see table 5-1).

Rhodamine beads (Katz *et al.*, 1984) injected into the lateral ventricle labeled a few cells lining the ventricular walls; many macrophages were seen in the ventricular lumen with phagocytosed beads. Three other tracers tried labeled the periventricular parenchyma as if the substance injected had partially diffused into the brain. The other three were negative (Table 5-1). Of the three tracers that labeled the ventricular zone and its immediate surroundings, only Fluorogold (Schmued and Fallon, 1986) labeled some migrating cells.

Fluorogold-filled migrating cells were found at less than 2mm from the ventricle on the hyperstriatum and lobus paraolfactorius as expected from findings presented in Chapters 3 and 4, (Fig. 4-3). These fluorogold-filled cells had nuclei of the same elongated morphology described in Chapter 3. The fluorogold cytoplasm allowed for the

first time to visualize the processes of these cells (Fig. 5-3). Clusters of irregularly shaped fluorescent material were frequently seen in the proximal segment of the leading process (Fig. 5-3), while lighter fluorescence defined the rest of the processes. The leading process was usually longer than the trailing process as revealed by the fluorogold. However, it was difficult to ascertain how long these processes were since they may have come out of the plane of the section or simply not have been completely labeled by fluorogold. Most of these cells were in radial glia-rich regions and had their processes oriented as radial glia in the medio-lateral plane.

Fluorogold has also been injected into the brain parenchyma, just lateral to the ventricular zone in the region where one finds many proliferating cells (see above). In this case too, some migrating cells become labeled. However, in both intraventricular or intraparenchymal injections, the number of cells labeled is small when compared to that expected (Chapter 4). I do not know if this is because fluorogold is available for only a short period of time to a small number of migrating cells or whether the fluorogold is killing migrating cells. Experiments addressing this problem are underway in the laboratory.

The anatomy of the migrating cell, as defined by fluorogold, is very similar to that of a typical migrating young neuron (Rakic, 1972; Jacobson, 1978). The fluorogold-filled migrating cell also greatly resembles the bipolar cells of Cajal (see for example Ramón y Cajal, 1929, p. 79). One would like to know what is the function of the migrating young neuron's processes. Are they exploratory appendages of the migrating cell extending the reach of the cell to potential differentiation nitches? Are they part of locomotor machinery that helps in the translocation? or are they -as suggested by Ramón y Cajal- the axonal primordium, maybe transiently trying to find potential target cells?. All these

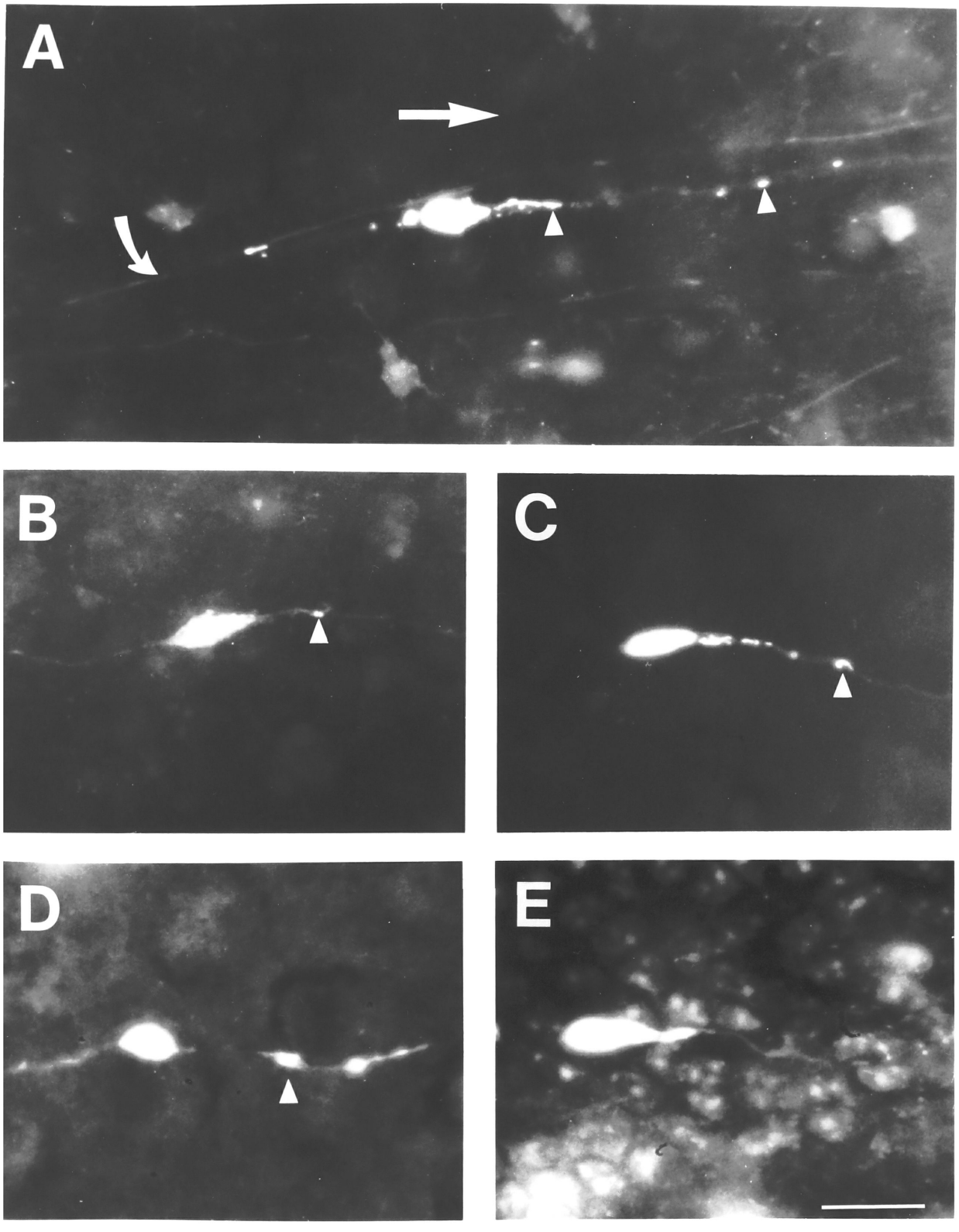
are exciting problems for which adult neurogenesis and the labeling technique described here might provide a new experimental model. For example besides fluorogold revealing for the first time a fuller picture of the migrating cells, this technique could be very useful to follow migration in sections of living brain. It would be interesting to study the behaviour of these cells in living tissue slices and follow their conversion into neurons.

			Labeling	
	Concentration	Volume	Peri-Ventricular	Migrating cells
Rhodamine beads	--	2ul	+/-@	-
Fluorogold	2%	1/2ul	+	+*
FITC-Peanut-agglutinin	0.1%	3ul	-	-
FITC-Dextran	1%	1.3ul	-	-
WGA	1%	1ul	-	-
Peroxidase	25%	2ul	+	-
PhA-L	1%	3ul	+	-

@ some ventricular wall cells appear labelled.
* only a few migrating cells become labelled.

Table 5-1. Birds were injected into the right lateral ventricle with the indicated volumes and concentrations of the different tracers. The following relative stereotaxic coordinates were used to reach the ventricle: anterior 0.6mm, lateral 0.39mm, deep 3mm (Stokes *et al.* 1974). Birds were sacrificed 7 days later and frontal vibratome sections were obtained as described in Chapter 6. WGA; (Wheat germ agglutinin, Miles lab.) and Peroxidase (grad 1, Boehringer Mannheim) where detected with diaminobenzidine (Chapter 6). Phal; phaseolus vulgaris leucoagglutinin was detected by immunocytochemistry (ABC Vector Laboratories). Fluorescent tracers; rhodamine beads (Katz *et al.*, 1984), Fluorogold (Schmued and Fallon, 1986), FITC-Peanut-agglutinin (Fluorescinated peanut agglutinin, Vector lab.) and FITC-Dextran (D1820, Molecular probes Inc.) were directly visualized through a fluorescent microscope.

Figure 5-4. Fluorogold filled migrating cells seven days after an intraventricular or intraparenchymal injection of fluorogold (see text). In all examples shown here the ventricular wall from where these cells presumably originate is to the left. The expected direction of migration is then from left to right (Straight arrow). The perinuclear cytoplasm is brightly fluorescent while the processes are partially filled with the dye. Notice how the fluorogold accumulates in small clumps (arrow heads), and how these clumps are more frequent in the leading process of the cell.
A: Cell found in lobus parolfactorius. Notice other fibers running parallel to the axis of this cell and how one of these fibers contact the trailing process of the migrating cell (curved arrow).
B,C and D: Show three other examples of filled migrating cells.
E: Brightly fluorescent cell showing the migratory morphology. It is of interest that this cell was found in the ventricular zone that is partially visible by the dim staining of the cells in the background. Calibration mark for all panels, 10um.


A
B
C
D
E

Summary.

This dissertation describes for the first time neuronal migration in an adult brain. It is shown, 1) that radial glia persist in the adult canary brain. 2) That a new cell type in adult bird brain correspond to migrating young neurons. 3) That initial migration paths are rich in radial glia to which the migrating cells are frequently attached. 4) That the dispersal speed is first fast and then slower as cells migrate past the radial glia rich region. The uniqueness of the adult model system to study neurogenesis is presented in this chapter. In addition three new experimental approaches show 1) that the sites of birth of most new neurons are restricted to certain regions of the latero-ventral walls of the telencephalic ventricles; 2) novel dynamics for neuroblast displacement within the ventricle are suggested. 3) A technique to stop neurogenesis is presented and 4) the cytoplasmic processes of migrating cells are revealed by a vital dye. Adult neurogenesis might be a place to look for the rules that underlie the steps in the formation of neurons in general. Furthermore the question of what might by the behavioural relevance of new neurons in adulthood is now open for experimentation.

CHAPTER 6

MATERIAL AND METHODS

Animals and Tissue Processing.

Birds were from the closebred colony of Waterslager canaries of the Nottebohm laboratory. One year old males except when indicated were used. Birds were fed ad libitum and maintained in circanual light-dark conditions so as to match New York state day-night cycle. Birds were sacrificed under deep anesthesia by an overdose of Chloropent (Fort Dodge). The tissue was processed for histochemical techniques in one of two ways.

1) Frozen sections: The brain, kidney, testis, liver and a sample of pectoral muscle were rapidly dissected, embedded in OCT (Tissue-Tek), frozen and cryostat sectioned at 8 um. Sections were thawed onto slides and air dried for 2-3 minutes followed by fixation in acetone at 4°C for 1-2 minutes. The mounted sections were then dried and stored in air tight boxes with desiccant at -30°C. Tissue stored in this manner showed no decrease in staining for up to 6 months.

2) Fixed sections: In order to improve preservation of brain tissue, canaries were perfused intracardially with 10-20 ml of saline followed by 50 ml of 0.15% picric acid and 2% paraformaldehyde in 0.1M phosphate buffer. The brain was then removed from the cranium and immersed overnight in fixative at 4°C. Vibratome sections 25-30 um were used when longer segments of glial fibers were to be preserved. The brain was blocked in the transverse plane with the aid of a canary brain matrix (Activational Systems) and attached to the tissue stage of the vibratome with cyanoacrylate. Sections were collected

in small plastic cylinders (cut from plastic tubes) that were slightly melted into a nylon mesh to form mini-strainers. Sections were passed through the different staining solutions with the aid of these strainers.

Polyethylene glycol (PEG) sections: This impregnation technique was used to obtain thinner sections. It was preferred over paraffin because sectioning is simpler and tissue processing is not as harsh, conserving antigenicity and better overall morphology. The brain was blocked as before, dehydrated by placing it sequentially in 50%, 70%, 95% and 100% ethanol dilutions in water for one hour each. It was then impregnated in polyethylene glycol in a small vacuum desiccator installed inside an oven at 46°C. Incubation in PEG was as follows: for one hour in PEG MW 1000, 3 hours in a 1 to 1 mixture of PEG MW 1000 and PEG MW 1500, an additional hour in a new volume of the same mixture, then for 10 minutes in PEG MW 1500. The block was cooled in the freezer (-20°C) for 2 minutes and stored at 4°C in an air tight bag with desiccant. Tissue embedded in this manner was cut at 2-10 um on a rotary microtome. Sections were collected in mini-strainers in 0.1 M phosphate buffer pH 7.4 (PB) for subsequent immunocytochemical staining. Alternatively sections were mounted directly onto to gelatin-coated slides with the aid of a slide holder designed in the laboratory. This device holds the slide at an angle, immersed in the mounting medium (0.01% BSA, one drop of Photoflo (Kodak) in 100ml of PBS). The section is floated on the mounting liquid and carefully manouvered onto the slide with a small brush. The slide holder greatly facilitates the mounting of PEG sections considered one of the most problematic aspects of the PEG technique (Humason, 1972, pp. 88).

Immunocytochemical staining.

Monoclonal antibody 40E-C is an IgM, generated by immunizing mice with homogenized adult canary brain. The 40E-C hybridoma has been subcloned seven times. Other antibodies used: i) Monoclonal antibody to vimentin was purchased from Labsystems. ii) Polyclonal rabbit antibodies to vimentin were kindly provided by Dr. Eugenia Wang (Rockefeller University). iii) Antibody 3A7 was the gift of Dr. V. Lemmon (University of Pittsburg). iv) Polyclonal antibodies prepared in rabbits against glial fibrillary acidic protein (GFAP) were generously given to us by Dr. L. Eng (Stanford University School of Medicine).

Immunocytochemical staining was done on frozen, vibratome or PEG sections. Frozen sections were used for immunofluorescence, they provide a fast and sensitive method for antigen detection, however the fine cellular detail is destroyed by the freezing and subsequent thawing of the section onto the slide. For better morphological preservation PEG or vibratome sections were preferred. Vibratome sections provide thicker sections (25-50um), useful for following longer segments of radial glia (see Chapter 3). The thinner PEG sections allow non overlapping structures to be clearly resolved, studies combining [^{3}H]-thymidine and used PEG sections as well as did the mapping of radial glia. Detail immunocytochemistry procedures follow:

Staining of frozen sections. Sections on slides were washed several times in phosphate-buffered saline (PBS) and pre-incubated for 10 minutes with PBS containing 0.4% triton X-100 and 3% serum from the species in which the second antibody was made (or skim milk) in order to block non-specific binding. Sections were then incubated in a humid chamber with a small drop of diluted antibody. Dilutions were as follows: 40E-C, 1:1;

anti-vimentin and 3A7, 1:10; anti-GFAP, 1:100. After incubation for 2 hours in the first antibody, sections were washed in PBS 4 times for a total of 30 minutes. Fluorescinated anti-mouse or anti-rabbit IgG's (Cappel) were used at 1:50 dilution for 1 hour, washed in PBS 4 times for a total of 30 minutes. For double label staining, anti-GFAP was mixed with 40E-C to the same final dilution as above; the second antibodies were swine anti-mouse coupled to rhodamine and fluorescinated goat anti-rabbit both at a 1:50 dilution. Sections were mounted in PBS-glycerol containing 1 mg/ml of P-phenylenediamine (Sigma), the pH adjusted to 9 with 0.2 M sodium carbonate. Substitution of the first antibody in the case of a monoclonal for a nonspecific supernatant or in the case of anti-GFAP for non-immune rabbit serum gave no staining. For double staining, special attention was exercised to control for cross-contamination: the substitution of one of the antibodies completely eliminated the staining for that antibody but did not affect the staining of the other.

Staining of vibratome or PEG sections in suspension. Sections were washed for 1-2 hours in PB (phosphate buffer, 0.1M, pH 7.4) followed by incubation in PB with 0.4% triton X-100 and 3% skim milk for 1 hour. 40E-C was diluted 1:1 in PB, 2% skim milk and 0.1% triton X-100. (In some cases the skim milk was replaced with the same percent of normal rabbit serum; in this case the results were identical however the background was slightly increased). After overnight incubation with 40E-C, the sections were washed for 1 hour with at least 3 changes of PB, incubated in a 1:200 dilution of biotinylated horse anti-mouse antibodies for 2 hours at room temperature, washed as before and incubated for 1 hour in avidin-peroxidase (biotin-avidin peroxidase technique from Vectostain, Vector Laboratories) followed by PB wash as above. Sections were then incubated for 7 minutes in 30 mls of PB containing 2 mg/ml of diaminobenzidine and 7 drops of 0.3% hydrogen peroxide and subsequently washed and mounted onto 2.5 x 5cm slides. Control

experiments without first or second antibodies gave no staining. Some sections were counter-stained with 0.5% cresyl violet in water or with the fluorescent DNA stain Hoechst 33258 at a concentration of 20ug/ml in water.

Photography was on Tech-Pan 35 mm film (Kodak). This film was either used in high contrast (ASA100 developed in HC110 diluting 1:9, 7min at 20°C) for fluorescence or for lower contrast but higher resolution generally for light microscopy (ASA50 developed in HC110 diluting 1:19, 6 min at 20°C). Labophot compound or inverted Diaphot (Nikon) microscopes equipped for epi-fluorescence were used.

Biochemical characterization of antibody 40E-C.

An enriched intermediate filament protein fraction from adult canary brain was extracted as described by Eng and DeArmond (1983). The brains were homogenized in 20 volumes of a 50mM Tris HCl buffer, pH 6.8, containing 0.5% triton X-100, 2mM phenylmethylsulfonyl fluoride, and 2 mM EDTA. The pellet obtained after centrifugation at 12,000xg was re-extracted in the same buffer containing 30% sucrose. After centrifugation as above, the pellet was delipidized in 20 volumes of ether:ethanol (3:2 v/v), washed with acetone and dried overnight in a desiccator at 4°C.

The extract was weighed and dissolved at 10mg/ml in sample buffer containing 1% SDS, 1.5% Cleland's reagent, 8% sucrose and 0.5% bromophenol blue. Two to 10ul samples of the dissolved protein were run on a 10% polyacrylamide gel, 7cm long with a 4% stack (Laemmli, 1970). The separated proteins were transferred to nitrocellulose paper (Towbin *et al.*, 1979) and fixed for 15 minutes in acetic acid-isopropanol-water (10/25/65 V/V/V).

Strips of the nitrocellulose paper corresponding to the lanes of the gel were blocked, first overnight in Denhardt's solution (0.1% Ficoll, .1% polyvinylpirrolidone, 0.1% bovine serum albumin). Blocking was continued the next morning, for one to two hours in PBS containing 4% skim milk, and 0.4% triton X-100 (PBS-M-T) containing 10% horse serum. The strips were reacted for 24hr with antibody 40E-C, anti-vimentin or anti-GFA antibodies (described above). Dilutions in PBS-M-T and 10% horse serum were as follows: anti-vimentin monoclonal (Labsystems), 1:50; anti-vimentin polyclonal, 1:1000; anti 3A7, 1:10; anti-GFA, 1:1000. After washing for 2hr in PBS-M-T changed every 5 to 10 minutes, the strips were incubated for 2hr with a 1:500 dilution of biotinylated antibody (Vector Labs), washed as above and positive bands visualized using the avidin-biotin-peroxidase system and diamino-benzidine as described for immunocytochemistry. Control experiments included the substitution of the first antibodies with non-immune serum. Molecular weight standards were run in parallel and stained with amido black.

Mapping

Fifteen 1-2 year-old male and female canaries were used to study the distribution of radial glia. However, based on the preservation and the quality of the immunocytochemical staining, only the brains of 1 male and 1 female one-year-old birds were used for detailed mapping.

Mapping was done by reconstructing individual microscopic fields of the tissue section onto the computer screen with the aid of camera lucida. These fields were pieced together using software developed in our laboratory. The final output was a plot of cells and fibers, the outlines of the section, ventricles and laminae. Systematic scanning of the entire section was done with a 40X oil immersion objective. The microscope (Nikon,

Labophot) was equipped with X-Y digital encoders (Mitutoyo) mounted onto the stage. These encoders provide positional information to the computer. This mapping technique is described in detail in appendix A.

Labeling of dividing cells.

Each bird received two injections per day of methyl-[^{3}H]thymidine, 6.7 Ci/mmol (New England Nuclear) at 12h intervals, at 9-11PM and 9-11AM in the pectoral muscle. Birds received from 1/2 to 4 days treatments and were allowed different survival as indicated in the text. Slides with PEG mounted sections were washed of potential chemographic contamination by dehydration in a series of increasing concentrations of ethanol. The sections were then delipidized in xylene for 5 minutes and brought back to water through a decreasing series of ethanol. Sections were dried and dipped into nuclear tract emulsion NTB-2 (Kodak) and incubated for 30-35 days. The slides were then developed in D19 (Kodak) at 17°C for three minutes, wash in water and fix (Kodak fixer) for 10 min at room temperature. The emulsion on the back of the slide was scraped off and the sections were stained and coversliped. In cases when autoradiography was combined with immunocytochemistry the immunoperoxidase staining method was used before coating sections with the emulsion. A cell was recognized as labeled if the number of exposed silver grains overlying its nucleus was 20 or more times above background. This criterion was based on the mean diameter of neuronal nuclei; since the nuclei of ventricular zone cells and migrating cells are smaller than the nuclei of neurons, the labeling criterion used would seem to be more stringent for the smaller cell types. However, a previous study (Nottebohm, 1985) also using adult canary forebrain showed that the numbers of exposed silver grains after [^{3}H]-thymidine treatment are very similar in labeled neurons and glia, despite the differences in nuclear size. DNA may be more concentrated in the smaller cell nuclei, so that the amount of

DNA sampled per nuclear section may not depend on nuclear size. For this reason it seem reasonable to use the same criteria for the different cell types.

APPENDIX A.

Computer-Microscope system for mapping tissue sections.

Examination of tissue sections in the light microscope frequently requires the use of high power objectives to visualize details of interest (e.g. cell types, autoradiographic grains, immunohistochemical stains or degenerating fibers). The spatial relationship of such details to each other and to the rest of the section is often lost due to the small field size. Since several of the questions approach by this thesis involved mapping fine details (referred to here as 'landmarks') in relation to the whole section, we developed a simplified system that would help in this task.

Common but expensive and time-consuming methods for mapping include the construction of photographic montages or the positioning of landmarks by hand on a low magnification picture or drawing of the region to be mapped. It is easier and more efficient to use the position of the microscope stage to drive the X and Y axes of a plotter. Several such systems have been developed, such as the electronic plotters (Boivie *et al.*, 1968; Grant and Boivie, 1970; Eidelberg and Davis, 1977) or the light pantograph (Patterson *et al.*, 1976). Recent computer-assisted mapping systems (Curcio and Sloan, 1981; reviewed in Mize 1984, 1985) often make use of specialized hardware and frequently require that each feature of interest be aligned under a cross-hair in the microscope eyepiece (Forbes and Petry, 1979; Williams and Elde, 1982; Mize, 1985). Landmarks may be visualized throughout the microscope field, but, because they are only recorded by the computer when they fall under the cross hair, an excessive amount of stage movement is necessary.

In an ideal system, the operator would position a pointer visible through the microscope on a feature of interest anywhere in the field, and enter its position in the computer. While this can be accomplished with digitized video images displayed on a graphics terminal (DuVarney and DuVarney, 1985), such systems are expensive and do not approach the resolution of direct visualization through the microscope. The system described here makes use of common microcomputer hardware and a drawing tube to view a mouse-driven cursor on the computer screen, while two digital indicators relay the microscope stage position to the computer. Software was then written for this system allowing rapid entry, display and plotting of the positions of cells and fibers (or any landmark) throughout the section while looking through the microscope. This work was done in collaboration with Dr. David Vicario. (Alvarez-Buylla and Vicario, submitted)

Hardware

The standard hardware consisted of an IBM PC-XT with two serial ports, 1 parallel port, Hercules graphics card, monochrome monitor, mouse (Microsoft) and plotter (Hewlett-Packard models 7470A or ColorPro). The software has recently been adapted to run on the IBM PS/2 model 30 with similar peripherals. A related system is under development for Macintosh microcomputers (C. Ghez, personal communication). Specialized equipment consisted of two digital linear position sensors (Mitutoyo Digimatic) with a serial interface (Mitutoyo Multiplexer 10). These provided positional coordinates of the microscope stage to the computer. The indicators were mounted on the arm of a Nikon Labophot Microscope at right angles to one another with a bracket made at the University's instrument shop. The bracket was constructed of aluminum and adapts to any microscope with a 7cm square arm. The X indicator has a range of 2cm and rests directly against the back of the stage, thus reading the short axis of a 2.5 x 7.5cm slide. The Y indicator has a 5cm range (sufficient to cover the non-frosted portion of the

slide) and is oriented parallel to the long axis. On a Zeiss microscope, the indicators were mounted directly onto the sides of the stage at right angles to each other.

In order to enter all the landmarks within a field without having to position each of them in the center of the field, a camera lucida (drawing tube; Nikon) was modified to visualize the computer screen superimposed on the image of the tissue section (Fig. A1). The three screws just distal to the focusing ring were replaced with a single compression screw. This allows the end mirror to be oriented horizontally instead of in the manufacturer's fixed vertical position (looking down at the table). The axes of the reticle were aligned with the X-Y motions of the stage by rotating the eyepiece. The reticle of the microscope eyepiece was then superimposed on the image of the screen by adjusting the drawing tube zoom and moving the computer monitor. The screen and mouse cursor were clearly visible on the specimen image, enabling interactive data entry while scanning sections. This technique could not be used for mapping fluorescently-labelled landmarks, because the camera lucida optics attenuated too much of the light emitted from the tissue. In this case, positioning was accomplished by matching the location of landmarks in a reticle grid to corresponding positions in an identical grid displayed on the terminal. This method is effective, but more time-consuming.

Software

Computer programs were written in Basica (IBM) for graphics equipped computers. One version takes advantage of text cursor positioning commands to provide a pseudo-graphics display and can run on computers without graphics hardware. The programs were designed to aid in the systematic screening of tissue sections. Up to 20 different landmark types or line drawing can be selected from a screen menu by means of the mouse. The landmarks or lines are then displayed on the screen, superimposed on

the image of the section. In this way, the data entered on the image of a single field can be edited before being stored and plotted. The X-Y coordinates of the center of the field are transmitted to the computer by pressing the "load" bottom of the multiplexer interface. This also signals the program to store and plot the information in the current field at the appropriate X-Y location. To insure that fields or landmarks are not counted twice several check points are built into the program.

The final output is a plot of the mapped histological section, showing all tracings and landmarks entered. The program also stores all the information on the hard disk. Additional features include erasing landmarks on the screen, erasing the previous field, finding the center of the scanning row, tracing by moving the stage, calculating distances, perimeters and areas, counting landmarks, concentricity compensation for different objectives and replotting stored information.

With a separate program, data can be recalled from the disk and the map redrawn on the computer screen. Plots of selected landmarks can be made, and statistical analyses of frequencies and distributions for features of interest can be conducted. The mouse can also be used to select screen positions, allowing distances, areas and perimeters to be calculated.

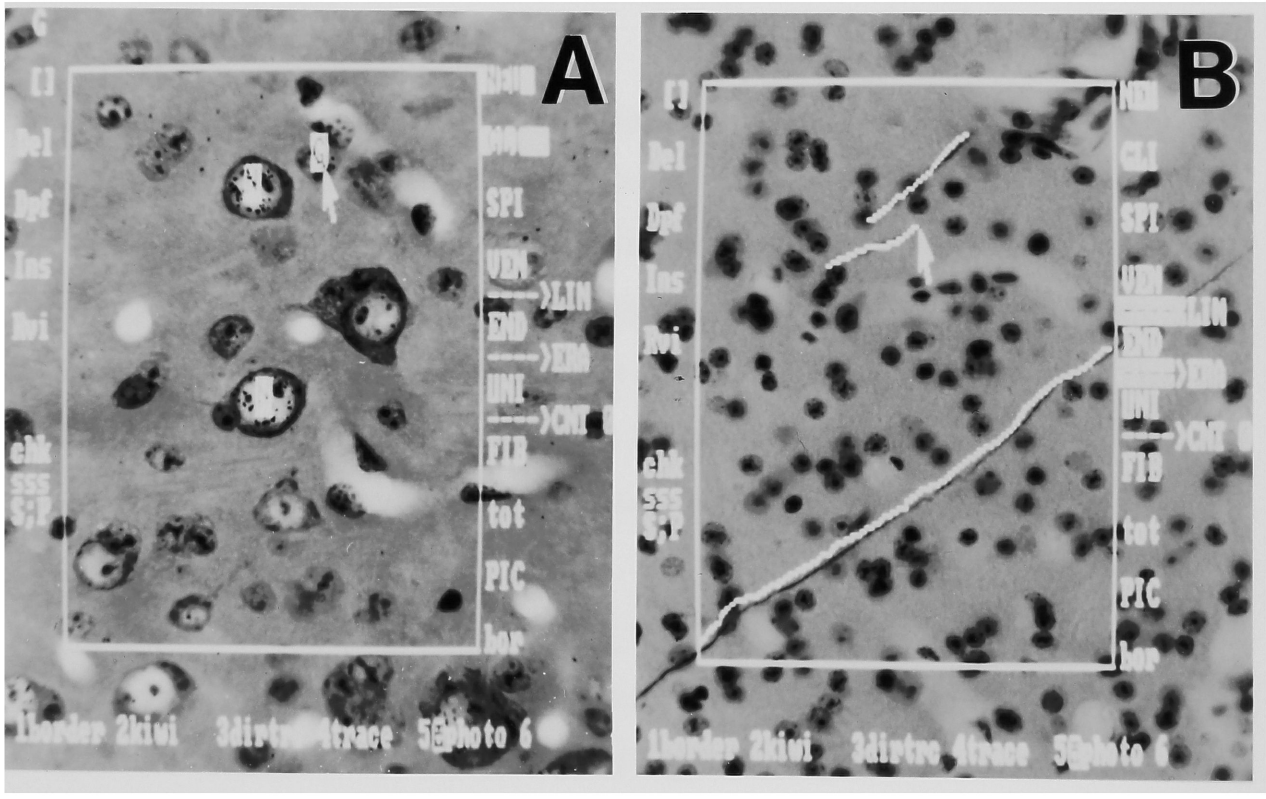
Procedure

The mouse is used to enter the location of landmarks or other features in the correct position while viewing the section at high magnification (Fig. A1). Position within each field is determined as the mouse's cursor screen position. The absolute location of each field is determined from the positional indicators which record

movement of the stage and slide. In order to map an entire section or region, successive fields need to be mapped according to a scanning strategy.

The program allows a choice between scanning parallel to the X axis, the Y axis or a random trail. The best choice depends on the size, shape and orientation of the specimen as well as on the preference of the user.

Figure A1. Superimposed computer screen (screen field) and specimen image as seen through the microscope.
A: The positions of two neurons and a glial cell with autoradiographic grains overlaying their nuclei are marked by mnemonic screen characters (faintly seen in this photograph). The mouse-driven cursor (arrow) serves as a pointer to designate the position of cells to be marked by pressing one of the two mouse buttons. In this case, each mouse button is loaded with neuron (NEU) or glia (GLI) as indicated in the highlighted landmark menu to the right of the field. The menu to the left contains different editing functions. Del: clears the field; Dpf: deletes previous field; Ins: inserts into previous field; Rvi: recovers previous field; chk: activates double landmark checking subroutines; sss: silences computer codes; S;P: skip plotting for this field. The menu at the bottom contains: 1: enters border in center of the field; 2: activates subroutine to find previous field; 3 and 4: pantograph trace functions; 5: records position where photo was taken.
B: Line drawing of radial glia fibers (see Chapter 3). In this case the mouse is loaded with the "LIN" function (menu to the right) and the cursor is used to trace the fibers on the field. The second button on the mouse was loaded with "ERA", used to erase individual marked landmarks from the screen.


A
B
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bor
1Border 2Kivi 3Diptre 4Trace 5Photo 6

Initialization. The digital indicators are first zeroed at the upper right corner of the slide and set to read in millimeters. The area of the section to be mapped is selected by entering the maximum and minimum values for the X and Y coordinates. This automatically sets the best scale to maximize the size of the plot. A reference point at a known location in the section is entered. This can be used later, if superimposed mapping is desired.

The display then shows a list of program variables that are automatically set but can be modified by the user. These include: scale, field size, relative symbol size, landmark names and plotting status. This menu also allows a perimeter to be entered that limits the area mapped; this is especially useful when mapping certain details within a nucleus for which the borders are not distinct at high magnification. Thus the perimeter-limiter is first drawn around the nucleus with a lower power objective, while actual mapping is done at higher magnification; the computer will signal if a landmark maps outside this perimeter.

The mapping screen. A square or a rectangle (screen-field) set to match the size and shape of the eyepiece reticle (eyepiece-field) appears on the screen. A menu of function codes appears to the left of the screen field and a menu of landmark names to the right (Fig. A1).

By adjusting the relative illumination of the microscope and the screen intensity, one can visualize the microscope field superimposed on the computer screen image (Fig. A1). A 90° rotation of the screen image is needed to match the optics of the drawing tube when oriented horizontally to view the computer screen. The cursor is moved by

the mouse and serves as a pointer to the desired function or landmark. To compensate for the 90° rotation of the field on the computer screen the mouse is used sideways with the buttons to the left. This allows the movement of the mouse, as seen through the microscope, to correlate with that of the cursor.

Mapping. In order to begin entering landmarks, each button of the mouse is loaded with a landmark name (e.g. neuron or glia) selected from the menu (Fig. A1). The cursor is then moved inside the screen-field to point to the desired feature of the superimposed microscope image. The appropriate landmark symbol is placed at this position on the screen by pressing the correct mouse button. In case of error, one can chose the erase function to remove one landmark or to clear the whole field. When all landmarks have been entered, the completed field is loaded, signaling the computer to calculate the absolute coordinates of all the landmarks. plot them and store them on the disk.

The program signals if a field overlaps a previously loaded field or if the perimeter limiter (see above) has been violated. The program is usually used in the plotting mode, which allows plotting during data entry and verification of the output. In case of an error the previous field can be erased and entered again correctly.

The stage is then moved to view the adjacent field. The scanning strategy depends on the density of landmarks to be mapped. If very dense it may require stopping and completing every contiguous field. In this case the stage is moved one field at a time in the Y or X direction depending on the chosen scanning mode. If the landmarks to be mapped are more scattered the stage can be moved until the region of interest is reached and only those fields that contain such landmarks mapped.

Built-in functions help the operator keep track of his position during systematic scanning. When scanning a row of fields along the X or Y axis, a row border can be established; the computer signals when a subsequent field falls outside the established row. Also, in case of accidental or intentional movement of the stage, a subroutine can be called that helps to move the stage back to the center of the scanning row.

In addition to entering tissue landmarks which map to a point, more complex structures (e.g. radial glia) can be entered as line drawings. This can be done in two ways: 1. Within a field, the mouse can be used to trace the structure of interest, using the "Lin" function (Fig A1, also see Fig. 3-2); 2. For larger structures, extending beyond a single field, the tracing mode can be used to draw lines. In this mode the microscope and computer work like a pantograph: the plotter follows the movement of the stage, using the color pen and line type selected. This is useful to draw the contour of the section (Fig. 3-2 and 4-3), laminae, long fibers, large blood vessels or the ventricles.

Termination. All plots receive an identifying legend, including the filename, date and time, a scale bar, counts of landmarks, scanned area and, when using the perimeter limiter, the area and perimeter. All plotting data are stored in a random access file enabling the plot to be repeated, modified or completed.

The enormous advantage of computer-based plotting methods is the ability to edit and analyze this map during and after data collection (Mize, 1985). This includes calculating distribution statistics of certain landmarks, calculation of distances, replotting only part of the file or modifying the plot. The scale, symbol size or pen colors can also be selected as desired during replotting.

The mapping resolution in our system is determined by two parameters: 1. The stage position indicators have a 1 um resolution, and 2. the resolution of the screen. The effective size of the pixel image seen through the microscope depends on the objective used. This means that at high magnification, the effective screen resolution is equal to or better than the stage resolution of 1 um, which then sets the limit. At lower magnification, the screen resolution is the limiting factor, reducing resolution to about 2 or 4 um at 40 or 20X, respectively. The map resolution is limited by the number of addressable points in the screen-field of the computer monitor and the plotter. In our experience, the graphics mode of the screen exceeds what is required for most applications. A slight error is introduced because of the curvature of the screen, making the sides of the screen field appear slightly curved when observed through the microscope (Fig. A1). The calculated maximum deviation is 0.8% of the size of the field. This could only be of significance when working with a very low magnification objective. Flat-screen monitors which would solve this problem have recently become available and affordable for personal computers.

A unique feature of this system is that points are mapped while directly viewing the tissue through the microscope at the optimum magnification, using a camera lucida. Other systems, designed mainly for neuronal reconstruction, have used a similar principle (Capowski and Sedivec, 1981; Glaser *et al.*, 1983; Freire, 1986; Capowski, 1985). By assembling a complete map from individual fields, the absolute locations of cellular features can be stored at high resolution (1 um) for tissue sections up to 2.5 cm in size. These are important advantages when compared to the digitized video images with a superimposed cursor that are commercially available. While recently developed high resolution video systems (4096x4096 pixels), using expensive specialized hardware and

software, may provide improved resolution (Hillman *et al.*, 1987), the images they generate, howeve, require large amounts of mass storage space.

Mapping of radial glia or autoradiographically labelled cells (Chapters 3 and 4) was done by using this system. Other features of the system, like mapping retrograde labeled cells, fiber tracts, muscle fibers and counting cells or measuring areas are currently being exploited in the laboratory.

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