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Astrocytic development, astrocytic scar formation and myelin formation as possible factors in loss of rubrospinal plasticity

Ghooray, Ganesh Terbenie, Ph.D.
The Ohio State University, 1992

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ASTROCYTIC DEVELOPMENT, ASTROCYTIC SCAR FORMATION AND MYELIN FORMATION AS POSSIBLE FACTORS IN LOSS OF RUBROSPINAL PLASTICITY.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Ganesh Terbenie Ghooray, B.S.

********

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1992

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Graduate Program in Anatomy
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To my Parents, my Wife and my Daughter
ACKNOWLEDGMENTS

I express sincere appreciation to Dr. George F. Martin for his guidance and insight throughout the research. Thanks go to the other members of my advisory committee, Drs. Michael Beattie and Richard Burry, for their suggestions and comments. Gratitude is expressed to Dr. James S. King and the Department of Cell Biology, Neurobiology and Anatomy for their unlimited support. The technical assistance and friendship of Mary Ann Jarrell is gratefully acknowledged. To my wife, Julie, I offer sincere thanks for understanding and supporting my endeavors. To my daughter, Kala, I thank you for making every day of my life interesting, refreshing and enjoyable.
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INTRODUCTION

Plasticity in the central nervous system is generally greater in developing animals than in adults (Weber and Stelzner, '77; Stelzner et al., '79; Goldberger and Murray, '85) and a well documented example of developmental plasticity is the ability of cortical axons to grow around a lesion of their pathway in newborn hamsters, rats and cats (Kalil and Reh, '79; Bernstein and Stelzner, '83; Bregman and Golberger, '82, '83; Tolbert and Der, '87), an ability which is lost with maturity. At birth in the same species, however, brainstem axons fail to grow around a comparable lesion (Prendergast and Stelzner, '76; Bregman and Golberger, '82, '83). Brainstem axons are relatively mature at birth in hamsters, rats and cats, however, at least in comparison to axons from the cerebral cortex (Bregman and Golberger, '82, '83; Schreyer and Jones, '83), and may have simply lost their potential for plasticity. It was suggested, therefore, that brainstem axons might be able to grow around a lesion if it was made at stages of development earlier than that present at birth in hamsters, rats and cats. It would be difficult to make lesions prenatally in the above species, however, so the North American opossum was chosen as the experimental animal. Opossums are born in a very immature state, 12-13 days after conception and
most of their brainstem-spinal development occurs postnatally (Cabana and Martin, '84, Martin and Cabana, '86). Rubrospinal axons were chosen for initial study since they are among the last from the brainstem to reach the spinal cord and their laterality is well documented (Cabana and Martin, '86, Xu and Martin, '91).

In a series of experiments employing retrograde and orthograde transport techniques it was shown that rubral axons can indeed grow around a lesion of their spinal pathway and that a critical period exists for that plasticity (Martin and Xu, '88). Such plasticity results primarily from the growth of late arriving axons around the lesion, but regeneration of cut axons also occurs (Xu and Martin, '91). Depending upon the size and geometry of the lesion, rubral axons either grow through the remaining part of the lateral funiculus, the dorsal horn or the dorsal funiculus to reach their destination (Martin and Xu, '88). The end of the critical period for rubrospinal plasticity has recently been determined to be between postnatal day (PD) 21 and 26 (unpublished results).

Since rubrospinal plasticity results primarily from growth of late arriving axons around the lesion, it is possible that the end of the critical period results from failure of new axons to reach the lesion site. Axons may be added to the rubrospinal tract after the end of the critical period, however, (unpublished data) suggesting that the environment traversed by late growing and regenerating axons
play a role (Aguayo, '85; Reier et al., '83; Schwab and Caroni, '88; McKeon et al., '91).

One factor in the environment that may impede rubrospinal plasticity is the development of an astrocytic response to lesioning, which may inhibit axonal elongation (Reier et al., '83). In order to study the development of an astrocytic response, however, we first needed to determine whether astrocytes are present in the spinal cord of the opossum by the end of the critical period. Therefore, we have studied the normal development of radial glial cells and astrocytes in the opossum's spinal cord and this study is described in Chapter I.

The results of the previous study enabled us to ask whether there is a temporal correlation between the development of an astrocytic response to injury and the end of the critical period for plasticity. Subsequent to injury of the spinal cord, astrocytes proliferate and hypertrophy as demonstrated by an increase in glial fibrillary acidic protein (reviewed in Reier and Houle, '88). The glial "scar" thus formed may be a physical barrier to growing axons (reviewed in Reier and Houle, '88). The development of an astrocytic scar to lesioning the spinal cord and the role it may play in the end of the critical period for plasticity is described in Chapter II.

The development of myelin may also influence axonal growth and thus the end of the critical period for rubrospinal
plasticity. Schwab and Caroni ('88) have isolated 2 proteins from central nervous system myelin which prevent neurite growth in vitro and it has been shown that corticospinal axons exhibit some degree of regeneration if myelin formation is suppressed by X-irradiation (Savio and Schwab, '90; Schwab and Schnell, '91). It is possible, therefore, that the development of myelin correlates temporally with the loss of rubrospinal plasticity. This possibility is discussed in Chapter III.

Our results indicate that the transition from radial glial cells to mature appearing astrocytes, the appearance of hypertrophied astrocytes at the grey/white matter junction and the formation of cystic cavities, and myelin formation correlate temporally with the end of the critical period for rubrospinal plasticity. It is likely, therefore, that factors other than the potential for axonal growth play a role in loss of plasticity.
CHAPTER I

THE DEVELOPMENT OF RADIAL GLIA AND ASTROCYTES IN THE SPINAL CORD OF THE NORTH AMERICAN OPOSSUM (DIDELPHIS VIRGINANA). AN IMMUNOHISTOCHEMICAL STUDY USING ANTI-VIMENTIN AND ANTI-GLIAL FIBRILLARY ACIDIC PROTEIN.

ABSTRACT

In previous studies, we have shown that rubrospinal axons are able to grow around a lesion of their pathway in developing opossums and that a critical period exists for that plasticity. Possible explanations for loss of plasticity includes the development of an astrocytic response to lesioning which might inhibit axonal growth. As a first step toward addressing that possibility, we have studied the normal development of radial glial cells and astrocytes in the spinal cord of the opossum to determine whether astrocytes are present by the end of the critical period. The normal development of radial glial cells and astrocytes was examined by immunostaining for vimentin (VIM) and glial fibrillary acidic protein (GFAP) using the Avidin-Biotin Complex (ABC) technique. Vimentin-like immunoreactivity (VIM-LI) is present in radial glia throughout the spinal cord at birth (12 days after conception), whereas GFAP-like immunoreactivity (GFAP-LI) is
limited to cells of comparable morphology in the ventral part of the cervical cord. The subsequent appearance of GFAP-LI follows ventral to dorsal and rostral to caudal gradients and by postnatal day (PD) 15, it is found in radial glia throughout the cord. At the same age, processes immunostained by either antibody have lost their radial orientation in the ventral part of the cervical cord. By PD 26, mature appearing astrocytes were immunostained by both antibodies at thoracic levels of the spinal cord, the levels lesioned in the plasticity experiments referred to above, and by PD 41, they were found at all levels of the cord. The transformation from radial glia to astrocytes also followed ventral to dorsal and rostral to caudal gradients. VIM-LI is still present in the adult opossum where it coexists with GFAP-LI in white matter astrocytes. Since rubral axons are able to grow around a lesion of the thoracic cord until sometime between PD 21 and 26, there is a rough temporal correlation between the transition from radial glia to mature appearing astrocytes and the end of the critical period for plasticity.
INTRODUCTION

In the North American opossum rubral axons can grow around a lesion of their spinal pathway during early stages of development and a critical period exists for that plasticity (Martin and Xu, '88; Xu and Martin, '89). The results of recent studies suggest that rubrospinal plasticity results primarily from growth of new, undamaged axons around the lesion, although regeneration of cut axons also occurs (Xu and Martin, '91). Possible explanations for loss of plasticity include the development of an astrocytic scar after lesioning which might impede growing or regenerating axons (Reier et al., '83, Reier and Houle, '88). In an initial attempt to address that possibility, we have studied the normal development of radial glia and astrocytes in the spinal cord of opossums to determine if astrocytes are present by the end of the critical period for plasticity. If so, it would be reasonable to pursue experiments designed to determine if there is a temporal correlation between the development of an astrocytic scar and the end of the critical period for rubrospinal plasticity.

In order to study radial glial cells and astrocytes at different stages of development, we employed immunohistochemistry to identify the intermediate filament proteins vimentin
(VIM) and glial fibrillary acidic protein (GFAP). VIM is present in radial glia and immature astrocytes (Pixely and de Villis, '84; Voigt, '89; Yanes, '90; Tohyama, '91), whereas GFAP is found within radial glia and mature astrocytes (Eng, '71; Bignami and Dahl, '74; Levitt and Rakic, '80). In the mammals studied to date, VIM disappears and is replaced by GFAP as the animal matures (Pixely and de Villis, '84; Raff, '84). Our results show that: 1) vimentin like-immunoreactivity (VIM-LI) is present in radial glia of the opossum's spinal cord at birth and that it does not disappear completely with maturity, 2) GFAP-like immunoreactivity (GFAP-LI) is seen in radial glia of the ventral cervical cord at birth and its subsequent appearance follows ventral to dorsal as well as rostral to caudal gradients, and 3) the transition from radial glial cells to mature appearing astrocytes also follows ventral to dorsal and rostral to caudal gradients and a rough temporal correlation exists between that transition and the end of the critical period for rubrospinal plasticity.
MATERIALS AND METHODS

Pouch-young opossums were obtained from adults purchased from licensed collectors in Florida and from animals bred at the Ohio State University. The age of the pouch-young from captured opossums was estimated by comparing their snout-rump length (SRL) and external features with those of animals at known ages from timed litters (Cutts et al., '78, and data from our own collection).

Immature opossums were removed from the mother's pouch at various ages (Table 1) and anesthetized by inhalation of Metofane. Adult opossums were anesthetized by an intraperitoneal injection of sodium pentobarbitol (0.6ml/kg). The anesthetized animals were perfused intracardially with 0.9% saline followed by Zamboni's fixative (2% paraformaldehyde and 12% picric acid in 0.1M phosphate buffer at pH 7.2). The spinal cords of pouch-young were postfixed in situ for 2-4 hours and transferred into Sorenson's phosphate buffer with 30% glucose overnight at 4°C, whereas those of adult opossums were removed, postfixed for 2-4 hours, and placed into Sorenson's phosphate buffer with a 15% glucose solution overnight at 4°C. Sections of the spinal cord were cut at 60-80um on a freezing microtome and then processed immunohistochemically for vimentin and GFAP.
IMMUNOHISTOCHEMISTRY:

All incubations were performed at room temperature unless otherwise specified. Antibodies were diluted in 2-3% normal goat serum (NGS) in phosphate buffered saline (PBS) and after each step, free-floating sections were rinsed 4 times (5 minutes each) in PBS.

The tissue sections were collected in PBS, incubated in 0.3% Triton-X/PBS for 30 minutes, rinsed for 10 minutes and incubated in 2-3% NGS/PBS for 30 minutes on ice. The sections were then rinsed for 10 minutes and adjacent sections were incubated in either a monoclonal mouse anti-vimentin IgG or a monoclonal mouse anti-GFAP IgG for 48 hours at 4°C. Both antibodies were obtained from Boehringer-Mannheim and were diluted 1:3 in 2-3% NGS in PBS. The primary antibodies were removed by rinsing in PBS after which the sections were incubated in the secondary antibody (biotinylated goat anti-mouse IgG from the ABC kit, Vector Laboratories) for 1 hour, diluted as described in the ABC kit. After rinsing, the sections were incubated in the Avidin-Biotin HRP Complex (from the ABC kit, Vector Laboratories) for 1 hour, diluted as described in the ABC kit. For controls, the primary or secondary antibodies were replaced by 2-3% NBS in PBS and the primary antibodies were absorbed with the antigens obtained from Boehringer-Mannheim.
The sections were rinsed and reacted with a Diaminodenidine (DAB)-Glucose Oxidase (GOD) solution (25mg DAB in 25ml distilled water to which was added 25ml PBS, 20mg ammonium chloride, 100mg beta-D glucose, and 25ul GOD which was freshly prepared by dissolving 3mg GOD in 500ul distilled water) until a brown reaction product was observed. The reaction was stopped by rinsing with PBS. The sections were mounted onto gelatin coated slides and dried overnight. The slides were then dehydrated in graded alcohols, cleared in Xylene and coverslipped with piccolyte. Immunoreactive structures were viewed and photographed with a Leitz Orthoplan photomicroscope equipped with differential interference contrast optics.

COLOCALIZATION STUDIES:

To determine if vimentin and GFAP were present in the same processes in the adult opossum, we utilized fluroscent markers in a double labeling experiment. An adult animal was sacrificed and its spinal cord was removed and sectioned on a freezing microtome at 60um. The sections were incubated in NGS/PBS for 30 minutes, rinsed 3 times, and then placed in 0.3% Triton-X/PBS for 30 minutes. The sections were rinsed once for 10 minutes and then incubated in mouse anti-GFAP (Boehringer-Mannheim) and rabbit anti-vimentin (Chemicon) for 48 hours at 4°C. The primary antibodies were diluted in 3% NGS/PBS at 1:4 for the anti-GFAP and 1:20 for the anti-
vimentin. The sections were rinsed 4 times, 5 minutes each, and incubated in a solution containing goat anti-mouse IgG-FITC (Boehringer-Mannheim) and goat anti-rabbit IgG-L-Rhodamine (Boehringer-Mannheim) for 1 hour at room temperature. Both secondary antibodies were diluted 1:20. The sections were rinsed 3 times, 5 minutes each, and mounted onto slides using Entellan for viewing under a Leitz Orthoplan photomicroscope equipped for epifluorescence. Processes labeled with FITC (GFAP-positive) were viewed with cube 2 of the Ploem filter system and processes labelled with Rhodamine (vimentin-positive) were viewed with cube 4. To test for cross-reactivity of the secondary antibodies, one of the primary antibodies was omitted from a few sections that contained both secondary antibodies. In general, the appropriate filter blocked the inappropriate fluorochrome.
RESULTS

NORMAL DEVELOPMENT:

At PD 1 (15mm SRL), vimentin-immunostaining was present throughout the length of the spinal cord (Figs. 1A, C, and E) where it was found in processes which extended from the ventricular zone to the pial surface. It was our interpretation that such processes belonged to radial glial cells. In the intermediate zone, the immunostained processes were relatively straight, but in the marginal zone they became highly branched (Fig. 1C) and terminated at the pial surface as clubs or end-feet (Fig. 1C). VIM-LI was present dorsally and ventrally, although that in the dorsal part of the cord did not show up well in the focal plane photographed in Fig. 1A. At the same age, GFAP-LI was restricted to the ventral part of the cervical cord (Figs. 1B and D) where it was present in thin processes which also extended from the ventricular zone to the pial surface (arrows in Fig. D). At PD 3, VIM-LI was present in processes which appeared comparable to those present at birth, but there was a modest increase of GFAP-LI in the ventral part of the upper cervical cord (data not shown).

At PD 5 (20mm SRL), VIM-LI was comparable to that
observed at PD 1 (data not shown), but GFAP-LI increased dramatically in the ventral part of the cervical cord (Fig. 2A) where it was associated with radial processes extending from the ventricular zone to the pial surface (Fig. 2A and B). Individual processes became irregular and branched as they reached the periphery (arrows in Fig. 2B) and they were thinnest dorsally. Although GFAP immunostaining was restricted primarily to the ventral cord, a few immunoreactive processes veered dorsally (arrows, Fig. 2C) and some were found in the dorsal midline. Some GFAP-LI was present in the ventral part of the rostral thoracic cord, but none was present more caudally (Fig. 2D). By PD 10, GFAP-LI was found in radial glial cells throughout the length of the spinal cord although it was sparse to absent in the dorsal part of the caudal thoracic and lumbosacral cord (data not shown).

By PD 15 (30mm SRL), processes which immunostained for VIM and GFAP were identical in distribution and appearance and, for simplicity, only GFAP-immunostained structures will be described. GFAP-LI was present at all levels of the spinal cord and immunostained structures began to lose their radial orientation in ventral parts of the grey matter at cervical levels (Figs. 3A and B). Immunostaining within the latter area was associated with processes of varying size as well as with cells which were identified as astrocytes (arrows in Fig. 3B). The thicker processes appeared to be fragments of radial glial cells, whereas the thinner ones were probably associated with
maturing astrocytes. Immunostaining in the developing white matter was limited to tortuous and radially oriented processes at all levels (Fig. 3A, C and D). By PD 21, mature appearing astrocytes were immunostained in the ventral part of the thoracic cord whereas radial glia were immunostained dorsally. By PD 30, only mature appearing astrocytes were immunostained at thoracic levels (data not shown). The transformation from a radial configuration of immunostained processes to a more mature pattern which included astrocytes was complete at all spinal levels by PD 40 (71mm SRL).

By PD 42 (75mm SRL), immunostaining for vimentin began to disappear in the grey matter and in adult animals, VIM-LI was present mainly in the white matter where it was found in tortuous, radially oriented processes (Fig. 4A). The only vimentin-positive immunostaining in the grey matter of the adult opossum was present around the central canal and around or within neuronal somata. In contrast, GFAP-LI was present throughout the grey and white matter (Fig. 4B). In the grey matter, immunostaining was associated with astrocytic cell bodies and short processes; whereas in the white matter, it was found within tortuous, radially oriented processes as well as profiles that resembled fibrous astrocytes. Some GFAP-LI was also present around blood vessels at the grey/white matter junction (arrows in Fig. 4B).

In the colocalization studies, most of the processes which immunostained for vimentin in the white matter (Fig. 4C)
also showed evidence for GFAP (Fig. 4D). Some structures were immunolabeled for vimentin (arrows in Fig. 4C) but not GFAP (arrows in Fig. 4D), however. In the grey matter, most processes were immunostained for GFAP but not vimentin.

Immunostaining was absent in the absorption controls except for a few VIM-positive (arrow in Fig. E) and GFAP-positive (arrow in Fig. F) processes at the periphery of the white matter. Immunostaining was not observed when either the primary or secondary antibodies were replaced by 2-3% NGS in PBS.
To the best of our knowledge, this is the first report of radial glia and astrocytic development in the spinal cord of any marsupial. In the following discussion we will compare our results with those obtained in other species and explore the possibility that some aspect of astrocytic development correlates temporally with the end of the critical period for rubrospinal plasticity.

Our results show that VIM-LI is present in radial glia throughout the spinal cord of the newborn opossum and that it can be demonstrated before immunoreactivity for GFAP. This is not surprising since vimentin is the predominant intermediate filament protein in radial glia of other species (Pixely and De Villis, '84; Engel and Muller, '89; Voigt, '89) and it can be demonstrated before GFAP. Vimentin is postulated to provide a structural framework in glial cells (Lazarides, '82).

Immature astrocytes which are immunoreactive for vimentin may provide an adhesive substrate for growth of pioneer axons (Joosten and Gribnau, '89). In the opossum, supraspinal and propriospinal axons have grown into the lumbar cord by PD 1 (Martin et al., '92) and it is possible that vimentin-positive radial glia support that growth. It would be interesting to see if cell adhesion molecules (CAM's) are present on vimen-
tin-positive glia during early development.

By PD 15, radial glial cells showed GFAP as well as vimentin-like immunoreactivity at all levels of the cord and at cervical levels they began to lose their radial orientation in the ventral grey matter. GFAP immunostained cells, identified as astrocytes, were also present in the latter area. This change from radially oriented glia to mature appearing astrocytes followed ventral to dorsal and rostral to caudal gradients. By PD 42, there was a gradual loss of VIM-LI in the grey matter that followed the same gradients. The loss of vimentin did not correlate with the expression of GFAP, which occurred earlier, or the onset of myelination (unpublished results) as has been described by others (Dahl, '81; Pixely and DeVillis, '84).

In the adult opossum, vimentin-positive structures are present in the peripheral white matter and around or within neuronal cell bodies in the grey matter. Double-labeling experiments indicated that vimentin is colocalized with GFAP in astrocytic processes of the white matter as reported for the adult rat brain (Schnitzer et al., '81) and optic nerve (Calvo et al., '90), the ferret's neocortex (Voigt, '89) and the rat's spinal cord (Joosten and Gribnau, '89). The presence of vimentin in the peripheral white matter may indicate a role in communication between the extracellular space and the internal environment of the spinal cord (Schnitzer, '81; Pixely and DeVillis, '84).
Our results indicate that GFAP-like immunoreactivity is present in radial glia of the ventral cervical cord shortly after birth in the opossum and that its development proceeds according to ventral to dorsal and rostral to caudal gradients. The ventral to dorsal gradient is similar to that described in monkeys (Levitt and Rakic, '80) and rodents (Gilmore and Sims, '89). Neither of the above studies mentioned a rostral to caudal gradient.

It has been suggested that GFAP-containing radial glia are important in guiding migrating neurons and axons to their targets (Levitt and Rakic, '80; Norris and Kalil, '91). Based on the relative timing of GFAP expression in radial glial cells in the opossum's spinal cord and the development of descending spinal pathways (Pindzola et al., '90; Martin et al., '91,'92), that may not always be the case. For example, axons from the pontine reticular formation, the locus coerulus and the brainstem raphe reach the lumbar cord before GFAP-like immunoreactivity can be demonstrated (compare Martin et al., '92 with the present study). It is possible, of course, that GFAP-positive radial glial cells help guide later growing axons, since the development of descending spinal pathways occurs over an extended period of time in the opossum (Cabana and Martin, '84).

The transition from radial glial cells to mature appearing astrocytes occurred primarily in the grey matter and followed ventral to dorsal and rostral to caudal gradients.
comparable to those reported in the monkey by Levitt and Rakic ('80). During the transition period, however, it was possible to find GFAP-positive structures resembling protoplasmic astrocytes as well as what appeared to be fragments of GFAP-positive radial glia. Eventually, radial glial cells were no longer demonstrable in the grey matter by either GFAP or vimentin immunostaining. In the white matter, however, GFAP-positive structures retained their radial orientation even in the adult animal and some of them contained vimentin. These results support the view that mature astrocytes originate from radial glia as described in the monkey brain (Schmechel and Rakic, '79; Levitt and Rakic, '80), the rat spinal cord (Hirano and Goldman, '88) and the ferret's cerebral cortex (Voigt, '89).

The classification of astrocytes used above is from early studies of glial cell morphology (Peters et al., '76). More recently, however, it has been suggested that there are two types of astrocytes in the white matter of the rat's spinal cord: one that has classically been referred to as the fibrous astrocyte and another that is radially oriented (Liuzzi and Miller, '87). The radial oriented astrocytes are more numerous and appear to extend from the grey/white matter junction to the pial surface as seen in this study. The radial and fibrous astrocytes may be analogous to the type I and type II astrocytes, respectively, of the rat's optic nerve (for review see Miller et al., '89).
Rubral axons are able to grow around a lesion of their pathway at thoracic levels of the cord until sometime between PD 21 and 26 and it is about that time that the transition from radial glial cells to mature appearing astrocytes occurs at the same level. It has been suggested that this transition occurs when axonal growth is declining (Engel and Muller, '89). It is possible, therefore, that immature, radially oriented glial cells help support growing and regenerating axons. Loss of these substrates could result in the loss of a preformed pathway or the loss of permissive substances which are needed by rubral axons in order to grow around a lesion of their pathway. Since laminin is synthesized by astrocytes during development (McLoon et al., '88), it would be interesting to see if it is transiently associated with radial glia during the critical period for rubrospinal plasticity. Glial maturation is probably not the only influence on the end of the critical period for rubrospinal plasticity, however. It is possible, for example, that the astrocytes present by the end of the critical period form a scar after lesioning which impedes axonal growth (Reier et al., '83). That possibility is considered in the next chapter.
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Age in postnatal days (PD), snout-rump length (SRL) in millimeters, and number of animals at each age (N) is provided.
Fig. 1  A, C and E are photomicrographs of the spinal cord from a PD 1 (15mm SRL) opossum showing Vim-LI in radial glia. Fig. C shows a higher magnification of the area enclosed within the rectangle in A. Adjacent sections were immuno-stained for GFAP and these are shown in Figs. B, D and F. Fig. D shows a higher magnification of the area enclosed within the rectangle in B.
Fig. 1
Fig. 2 A is a photomicrograph of the cervical cord from a PD 5 (20mm SRL) opossum showing GFAP-LI in radial glia. Fig. B shows a higher magnification of the area enclosed within rectangle 'a' of Fig. A and Fig. C shows a higher magnification of the area enclosed within rectangle 'b'. GFAP-LI processes are not present in the lumbar cord (Fig. D). cc = central canal.
Figs. 3 A-D are photomicrographs from a PD 15 (30mm SRL) opossum showing GFAP-LI at cervical and lumbar levels of the spinal cord. Fig. B is a higher magnification of the area enclosed within the rectangle in A and Fig. D shows a higher magnification of the area enclosed within the rectangle in C.
Fig. 3
Fig. 4 A is a photomicrograph of the lateral funiculus of the thoracic cord from an adult opossum immunostained for vimentin. Adjacent sections were processed for GFAP (Fig. B). Figs. C and D are immunofluorescence photomicrographs of vimentin-positive (Rhodamine, 580nm, cube 4) and GFAP-positive (FITC, 500nm, cube 2) structures, respectively, in the white matter of the spinal cord from an adult opossum. Figs. E and F are photomicrographs of the lateral funiculus of the thoracic cord in control sections when the antibodies to vimentin and GFAP, respectively, were preabsorbed with their respective antigens.
LIST OF REFERENCE


CHAPTER II

THE DEVELOPMENT OF AN ASTROCYTIC RESPONSE TO SPINAL CORD INJURY IN THE NORTH AMERICAN OPOSSUM AND ITS POSSIBLE CORRELATION WITH THE END OF THE CRITICAL PERIOD FOR RUBROSPINAL PLASTICITY. AN IMMUNOHISTOCHEMICAL STUDY USING ANTI-GLIAL FIBRILLARY ACIDIC PROTEIN.

ABSTRACT

The aim of this study was to examine the development of an astrocytic response to lesioning the spinal cord of opossums to determine if there is a temporal correlation between the development of a glial scar and the end of the critical period for rubrospinal plasticity. The astrocytic response was examined immunohistochemically, 2 and 4 weeks after lesioning, using an antibody to glial fibrillary acidic protein (GFAP). When the lesion was made at postnatal day (PD) 21, in the two weeks survival series, a relatively mild astrocytic response was observed in the white matter. When the lesion was made at PD 26, a few hypertrophied astrocytes were also present in the grey/white matter junction. The density of the astrocytic response in the white matter increased gradually and small cystic cavitations were observed when the lesion was made at PD 26. When the lesion was made at PD 41, the response had spread to include all of the grey matter and it
occupied a larger area rostral and caudal to the lesion site than at earlier ages. In adult animals, the astrocytic scar was even more pronounced and cavitations were large and numerous.

The animals allowed to survive 4 weeks after lesioning were subjected to a second operation 4-5 days before sacrifice so that Fast Blue could be injected bilaterally 2-3 segments caudal to the lesion. In such cases, a glial response was seen at an earlier age than in the cases that survived for 2 weeks. When the hemisection was made at PD 15, a response was present in the ventral funiculus and the ventral part of the lateral funiculus. As expected from previous studies, rubral neurons were labeled contralateral to the lesion suggesting that their axons had grown around it. When the lesion was made at PD 21, the glial response was more extensive. Although it was still limited to the white matter it now extended into the area normally occupied by rubral axons. In spite of the presence of a well developed glial response, rubral neurons were still labeled contralateral to the lesion. When the lesion was made at PD 26, astrocytes appeared to be hypertrophied at the grey/white matter junction and small cavities were present at the lesion site. In such cases, no evidence for rubrospinal plasticity was present. An astrocytic response was not observed in the grey matter of the dorsal horn, an area used by rubral axons to grow around a lesion of their pathway, until well after the end of the critical period. We conclude
that the initial development of a glial scar in the white matter of the lateral funiculus, the area occupied by rubral axons, does not correlate temporally with the end of the critical period for rubrospinal plasticity. However, there is a correlation between the appearance of hypertrophied astrocytes at the grey/white matter junction and the formation of cystic cavities with the end of the critical period.
INTRODUCTION

Injury to the central nervous system of adult mammals results in many histopathological changes. One of the more striking is the astrocytic response which includes increased synthesis of glial fibrillary acidic protein (GFAP), proliferation and hypertrophy (Bignami and Dahl, '76; Reier, '83; Janeczko, '88; Takamiya et al., '88). Such astrocytes are referred to as reactive. The initial effect of these changes is to fill the space left by degenerating neurons and to wall off the injured area by forming a new external glial limiting membrane (EGLM) (reviewed in Reier et al., '86). The result is a tightly knit meshwork of astrocytic processes often referred to as a glial scar. Although reactive astrocytes may initially participate in the healing phase following injury, they can also interfere with axonal elongation by forming a physical barrier (Windle, '56; Kao et al. '77; Reier, '83) or by producing inhibitory molecules (Rudge and Silver, '90; McKeon et al., '91).

We have shown previously that rubral axons are able to grow around a lesion of their spinal pathway in the North American opossum if it is made early enough during development (Martin and Xu, '88; Xu and Martin, '89). Such growth results primarily from the addition of new axons to the tract although
regeneration of cut axons also occurs (Xu and Martin, '91). Since reactive astrocytes restrict axonal regeneration in adult animals, we have asked whether the development of a glial scar after lesioning correlates temporally with the end of the critical period for rubrospinal plasticity. In experiments designed to address that question, we first studied the development of a glial response 2 weeks after hemisecting the thoracic cord at different ages and attempted to correlate the timing of that development with the end of the critical period (Martin and Xu, '88; Xu and Martin, '89). A two week survival was chosen because the response was reported to be maximal after that period of time in adult rat's (Barrett et al., '84). Individual variation in the time needed for a maximal response was observed in developing rat's (Barrett et al., '84), however, and the animals used previously for the rubrospinal plasticity studies, in opossums, had been allowed to survive for 4 weeks. For these reasons, we also studied the development of a glial scar 4 weeks after lesioning using a protocol which allowed us to determine whether rubral axons had in fact grown around the lesion. The second approach allowed us to study the development of a glial scar and the ability of rubral axons to grow around a lesion in the same animal. Our results suggest that the development of a glial scar in and of itself does not determine the end of the critical period for rubrospinal plasticity.
MATERIALS AND METHODS

In order to observe the development of a glial scar, pouch-young opossums at various ages (Table 2) were operated while still attached to the mother's nipple. The mother was anesthetized with an initial dose of Ketamine (im, 1.2ml) followed by inhalation of Metofane. The pouch-young were anesthetized individually by hypothermia and the spinal cord was exposed by laminectomy at mid to low thoracic levels so that it could be hemisected with a #11 scapel blade. Gelfoam was then placed over the lesion and the overlying connective tissue and skin were sutured. The operated animals were allowed to survive for 2 or 4 weeks.

Animals that survived for 2 weeks were removed from the pouch without further manipulation and anesthetized with Metofane. They were perfused intracardially with 0.9% saline followed by Zamboni's fixative. The spinal cord was remove and postfixied for 2-4 hours before being placed in Sorenson's buffer with 30% glucose overnight at 4°C. The lesion site was sectioned at 60um on a freezing microtome and the sections were collected in phosphate buffered saline (PBS) for immuno-histochemical processing.

All incubations were performed at room temperature unless otherwise specified. Antibodies were diluted in 2-3% normal
goat serum (NGS) in phosphate buffered saline (PBS) and after each step, free-floating sections were rinsed 4 times (5 minutes each) in PBS.

The tissue sections were collected in PBS, incubated in 0.3% Triton-X/PBS for 30 minutes, rinsed for 10 minutes and incubated in 2-3% NGS/PBS for 30 minutes on ice. The sections were rinsed for 10 minutes and incubated in a monoclonal mouse anti-GFAP IgG for 48 hours at 4°C. The antibody was obtained from Boehringer-Mannheim and was diluted 1:3 in 2-3% NGS in PBS. The primary antibody was removed by rinsing in PBS after which the sections were incubated in the secondary antibody (biotinylated goat anti-mouse IgG from the ABC kit, Vector Laboratories) for 1 hour, also diluted as described in the ABC kit. After rinsing, the sections were incubated in the Avidin-Biotin HRP Complex (from the ABC kit, Vector Laboratories) for 1 hour, diluted as described in the ABC kit. For controls, the primary or secondary antibodies were replaced by 2-3% NGS in PBS and the primary antibody was absorbed with the antigen which was obtained from Boehringer-Mannheim.

The sections were rinsed and reacted with a Diaminodenzidine (DAB)-Glucose Oxidase (GOD) solution (25mg DAB in 25ml distilled water to which was added 25ml PBS, 20mg ammonium chloride, 100mg beta-D glucose, and 25ul GOD which was freshly prepared by dissolving 3mg GOD in 500ul distilled water) until a brown reaction product was observed. The reaction was stopped by rinsing with PBS. The sections were mounted on
gelatin coated slides and dried overnight. The slides were then dehydrated in graded alcohols, cleared in xylene and coverslipped using piccolyte. Immunoreactive structures were viewed and photographed with a Leitz Orthoplan photomicroscope equipped with differential interference contrast optics.

The animals that survived for 4 weeks were subjected to a second operation 4-5 days before sacrifice. The spinal cord was exposed as described above and 0.2-0.25ul of 4% Fast Blue (FB) diluted in distilled water was injected bilaterally 2-3 segments caudal to the lesion. After a 4-5 day survival, the animals were removed from the pouch and perfused as described above so that the lesion site could be dissected out, sectioned at 80um, and processed immunohistochemically for GFAP (see above). Sections from the midbrain and injection site were cut at 30um, mounted onto gelatin coated slides and coverslipped with Entellan (Merck) for viewing with a Leitz Orthoplan photomicroscope equipped for fluorescence. Neurons labeled with FB in the red nucleus contralateral to the lesion were viewed at an excitation wavelength of 340-380nm and were interpreted as supporting axons which grew around the lesion during the 4 week survival. Their presence was correlated with the presence or absence of a glial scar in the immunohistochemically processed sections. In the opossum, as in other species, rubrospinal axons are entirely crossed (Xu and Martin, '91).
RESULTS

Results after a 2 week survival:

In the animals allowed to survive for 2 weeks, the first astrocytic response was seen when the lesion was made at PD 21. At that age the response was relatively mild and localized very close to the lesion site (data not shown). When the lesion was made at PD 26, however, the astrocytic response was extensive and a large cavity was present at the lesion site (Fig. 5A). Rostral to the lesion site there was an obvious increase in density of immunostained processes ipsilateral to the lesion. This increase in density was limited to the white matter and the grey/white matter junction (Figs. 5B – D). In addition, immunostained processes on the lesion side had lost their radial orientation (compare Figs. 5C and D) and at the grey/white matter junction, they were larger than on the control side (compare Figs. 5C and D). A few small cystic cavities were also present on the lesion side.

When the lesion was made at PD 40, an astrocytic response was found in both the grey and white matter at the lesion site, as well as rostral and caudal to it, and the density of immunostaining in the grey matter appeared greater than that seen at PD 26. In sections distant to the lesion site, the response was confined mainly to the white matter (data not
When the lesion was made at PD 54, there was a new EGLM at the lesion site and the astrocytic response extended into the unlesioned side (Fig. 6A). The EGLM was identified by the presence of immunostained end feet of astrocytic processes. Adjacent to the lesion site, the response had spread throughout the grey matter on the lesion side (Fig. 6B and D) and the white matter was much more densely immunostained and more compact than on the control side (compare Fig. 6C and D). Cystic cavities were numerous ipsilateral to the lesion (Figs. 6B and D).

In the adult animals, only the white matter was damaged by the lesion (Fig. 7A and B). On the lesion side, the white matter was densely immunostained and Swiss cheese-like in appearance due to the presence of numerous cavities and hypertrophied astrocytes (compare Figs. 7C and D). An astrocytic response was found in the grey matter, however, when the grey matter was included in the lesion (data not shown). No immunostaining was present in the controls.

Results after a 4 week survival:

When pups were allowed to survive for 4 weeks, the first indication of an astrocytic response was found when the lesion was made at PD 15. The response was relatively mild and characterized by immunostained processes which were thicker and more abundant than normal. In addition, the immunostained
processes had lost their radial orientation. Although the cord had been hemisected, the glial reaction was restricted to the ventral funiculus and the ventral part of the lateral funiculus. At this age, immature or transitional glia were still present in the dorsal horn and the dorsal funiculus. The glial reaction did not spread very far rostrally or caudally. As expected, rubral neurons were labeled in the red nucleus contralateral to the lesion suggesting that their axons had grown around it (data not shown).

In the PD 21 case illustrated, there was extensive expansion of the central canal and reconstitution of the external glial limiting membrane (EGLM) at the lesion site (Fig. 8A). The white matter on the lesion side appeared to be more heavily immunostained than at PD 15 and the reaction spread to the dorsal part of the lateral funiculus, the area normally occupied by rubral axons. Rostral to the lesion site, the central canal was still enlarged (Fig. 8B) and the white matter on the lesion side (Figs. 8B and D) was more compact and heavily immunostained than on the control side (Figs. 8B and C). The reaction did not extend into the grey matter, however. Fragments of immature radial glia were still present in the dorsal horn. Although the glial response was well developed in the area of white matter which contains the rubrospinal tract, evidence for rubrospinal plasticity was still present. Neurons were labeled in the red nucleus contralateral to the lesion (Fig. 8E), although they were
fewer in number than those labeled on the ipsilateral side (Fig. 8F). It will be recalled that the rubrospinal tract is almost entirely crossed (Xu and Martin, '92).

When the lesion was made at PD 26, the lesion site showed an enlarged central canal and a new EGLM around the lesion (Fig. 9A). Although a glial response was well developed in the white matter rostral to the lesion site (Figs. 9B and D), it still did not extend throughout the grey matter. However, astrocytes at the grey/white matter junction appeared larger on the lesion side than on the control side (compare Figs. 9D and C). In the white matter, the astrocytic response appeared denser and more compact than at PD 21 (compare Figs. 9D and 8D) and small cystic cavities were found on the lesion side (Fig. 9D). These observations were similar to those obtained at the same age in the 2 week series. At this age, rubrospinal plasticity was not present. In the case illustrated, many neurons were labeled in the red nucleus ipsilateral to the lesion (Fig. 9F), but none were labeled contralaterally (Fig. 9E). In our series, the end of the critical period for rubrospinal plasticity was between PD 21 and 26. By PD 41, the astrocytic response had spread to include the entire grey matter.
DISCUSSION

Our results suggest that an astrocytic response to lesioning the spinal cord occurs more slowly during development than in the adult animal. Comparable results have been reported in the rat (Barrett et al., '84). In young rats, the glial response to a spinal lesion takes 7-60 days to develop, whereas in the adult animal it was maximally developed after only 2 weeks. In the rat optic nerve, however, the situation was reversed. It took 1-2 weeks for a compact scar to form in neonates and 3-5 months in adults (Trimmer and Wunderlich, '90). This discrepancy was probably due to differences in the area injured and/or the type of injury (Trimmer and Wunderlich, '90). The greater response to spinal hemisection 4 weeks after lesioning than after 2 weeks in our experiments suggests that late maturing astrocytes respond to signals which remain for some time after lesioning. The specific factor or factors that stimulate astrocytes to become reactive is(are) unknown.

In our experiments, the astrocytic response to lesioning the spinal cord never became as well developed in pups as it was in the adult animal. Immature astrocytes may simply lack the metabolic machinery needed to mount a large response (Barrett, et al., '84), but it is also possible that fewer axons were injured in the developing animal and that the
relatively mild astrocytic response reflects that fact (Murray et al., '90). In any case, the response increased in intensity and spread farther from the lesion site as the animal matured.

The earliest astrocytic response was present in the ventral white matter 4 weeks after the spinal cord was hemisected at PD 15 and, with age, it progressed dorsally following the ventral to dorsal gradient observed in astrocytic maturation (chapter 1). Since radially oriented astrocytes were the only type observed in the white matter of the thoracic cord at PD 15 (chapter 1), they may have been the ones that responded to the lesion. It has been suggested that radial astrocytes in the white matter of the spinal cord are analogous to the type 1 astrocytes of the optic nerve (Liuzzi and Miller, '87) and it is the type 1 astrocytes that respond to enucleation (Miller et al., '86). It is possible, however, that late maturing astrocytes respond to the lesion during the 4 week survival.

Our results suggest that a glial response to lesioning the spinal cord of developing opossums occurs in the white matter before it is seen in the grey matter. It is possible that the grey matter was not included in our lesions, but that is unlikely. To our knowledge, such a gradient has not been described previously.

The lateral white matter on the lesion side always appeared smaller than on the control side, presumably due to axonal degeneration and phagocytosis. It is possible, there-
fore, that axonal loss resulted in greater concentration of the astrocytic processes already present prior to the lesion. If so, the response observed may not have been due to increased synthesis of GFAP or proliferation. Previous quantitative studies have demonstrated shrinkage of the dorsal horn in response to deafferentation in neonatal and adult rats, but astrocytic hypertrophy was also present (Murray et al., '90). In our study, the hypertrophied astrocytes at the grey/white matter junction resembled reactive astrocytes.

When the lesion was made at PD 21, an astrocytic response was present in the lateral funiculus, but rubral axons were still able to grow around the lesion. We conclude, therefore, that rubral axons are able to circumvent an immature glial scar. Previous studies have shown that rubral axons can use the dorsal horn to grow around a lesion during the critical period for plasticity and, if the hemisection is complete, the edge of the contralateral dorsal funiculus provides a suitable substrate (Xu and Martin, '88).

In most of the cases lesioned at PD 26, rubral axons no longer grew around the lesion. Since rubral axons may still be growing into the spinal cord at PD 26 (unpublished data), it is likely that a permissive substrate is no longer produced by normal astrocytes and/or that inhibitory factors have been produced by reactive ones. If inhibitory factors are produced by reactive astrocytes (McKeeon et al., '91), they must be produced sometime after astrocytes first respond to lesioning.
Although the astrocytic response did not extend throughout the grey matter at PD 26, hypertrophied astrocytes were present at the grey/white matter junction. Theoretically, rubral axons could use the rest of the dorsal grey horn, which was devoid of reactive astrocytes, but for some reason(s) they did not. It is possible that the reactive astrocytes referred to above and/or products produced by them are inhibiting or are non-permissive to growing axons.

Reactive astrocytes are not the only component of the so-called "glial scar", however; microglia, macrophages, oligodendrocytes and myelin debris are also present (Reier, '86; Giulian et al., '89; David et al., '90). In fact, the development of cystic cavitation at PD 26 indicates that non-CNS elements such as macrophages had invaded the lesion and that the cavity left after phagocytosis was walled off by astrocytic processes. It is possible, therefore, that the macrophages, oligodendrocytes and/or myelin debris also contribute to the end of the critical period for plasticity. It has been shown that inhibitory factors on oligodendrocytes impede neurite growth (Schwab and Caroni, '88; Pesheva et al., '89) and that possibility is explored in chapter 3.
Table 2

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Ages in postnatal days (PD), snout-rump length (SRL) in millimeters, and number of animals studied (N) at 2 weeks and 4 weeks post-lesion.
Fig. 5 A is a photomicrograph of the lesion site from the spinal cord of an opossum lesioned at PD 26 which survived for 2 weeks before sacrifice. Fig. B is a section rostral to the lesion site. Fig. C is a higher magnification of the area enclosed by rectangle 'a' in B and Fig. D is a higher magnification of the area enclosed by rectangle 'b'.

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Fig. 6 A is a photomicrograph of the lesion site from the spinal cord of an opossum lesioned at PD 54 which survived for 2 weeks before sacrifice. Fig. B is a section adjacent to the lesion site. Fig. C is a higher magnification of the area enclosed by rectangle 'a' in B and Fig. D is a higher magnification of the area enclosed by rectangle 'b'. 
Fig. 7 A is a photomicrograph of a section directly adjacent to the lesion site from the spinal cord of an adult opossum which survives for 2 weeks before sacrifice. Fig. B is a section adjacent to the lesion site. Fig. C is a higher magnification of the area enclosed by rectangle 'a' in B and Fig. D is a higher magnification of the area enclosed by rectangle 'b'.
Fig. 8  A is a photomicrograph of the lesion site from the spinal cord of an opossum lesioned at PD 21 which survived for 4-5 weeks before sacrifice. Fig. B shows a section rostral to the lesion site. Fig. C is a higher magnification of the area enclosed in rectangle 'a' in B and Fig. D is a higher magnification of the area enclosed by rectangle 'b'. Fig. E is a photomicrograph of the contralateral red nucleus (RN) showing Fast Blue labeled neurons and Fig. F is from the ipsilateral RN.
Fig. 9  A is a photomicrograph of the lesion site from the spinal cord of an opossum lesioned at PD 26 which survived for 4-5 weeks before sacrifice. Fig. B shows a section rostral to the lesion site. Fig. C is a higher magnification of the area enclosed in rectangle 'a' in B and Fig. D is a higher magnification of the area enclosed by rectangle 'b'. Fig. E is a photomicrograph from the contralateral red nucleus (RN) showing the lack of Fast Blue labeling and Fig. F is from the ipsilateral RN.
LIST OF REFERENCE


McKeon, R.J., Schreiber, R.C., Rudge, J.S., Silver, J. (1991). Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astro-


CHAPTER III

THE DEVELOPMENT OF MYELIN IN THE SPINAL CORD OF THE NORTH AMERICAN OPOSSUM, AS DEMONSTRATED BY MYELIN BASIC PROTEIN IMMUNOHISTOCHEMISTRY, AND ITS POSSIBLE ROLE IN LOSS OF RUBROSPINAL PLASTICITY.

ABSTRACT

The aims of this study were to observe the timing and sequence of myelin formation in the opossum's spinal cord by using myelin basic protein (MBP) immunohistochemistry and to determine whether the onset of myelination, as demonstrated by this technique, correlates temporally with the end of the critical period for rubrospinal plasticity (Martin and Xu, '88; Xu and Martin, '89). In selected cases, alternate sections of the spinal cord were immunostained for a myelin specific glycolipid (galactocerebroside, GalC) which has been reported to appear prior to MBP (Schwab and Schnell, '89). In this communication, rubrospinal plasticity refers to the ability of rubral axons to grow around a lesion of their spinal pathway.

MBP-like immunoreactivity (LI) was first observed in the opossum's spinal cord at postnatal day (PD) 15 where it was present in the ventral and dorsal funiculi at cervical levels.
and within the ventral funiculus at thoracic levels. By PD 21, it was also found in the ventral part of the lateral funiculus at cervical and thoracic levels and in the ventral funiculus at lumbar levels. Except for the dorsal part of the lateral funiculus, the area which contains rubrospinal axons, MBP-LI was present throughout the length of the spinal cord by PD 26. The dorsal part of the lateral funiculus did not become immunostained at cervical levels until PD 30 or at thoracic and lumbar levels until PD 33. By PD 26, some immunostaining was found in the ventral horn of the cervical cord and by PD 30, it was also found in the dorsal horn. MBP-LI was not present in the ventral horn of the thoracic cord until PD 30 nor in the dorsal horn until PD 33. By PD 41, immunostaining had reached adult-like levels except for the corticospinal tract which did not become immunoreactive until PD 54. Between PD 21 and 26, GalC immunostaining appeared at the same time as that for MBP.

Rubral axons are able to grow around a lesion of their pathway in the thoracic cord until approximately PD 26; but evidence for MBP and GalC-LI was not found where myelin proteins would be expected to inhibit such growth, i.e. in the dorsal part of the lateral funiculus or the dorsal horn, until PD 33. It is clear, however, that rubral axons do not grow around a lesion when myelin is present in their pathway.
INTRODUCTION

Previous studies have shown that rubral axons can grow around a lesion of their spinal pathway in developing opossums and that a critical period exists for that plasticity (Martin and Xu, '88; Xu and Martin, '89). The plasticity in question results primarily from growth of undamaged axons around the lesion, although regeneration of cut axons also occurs (Xu and Martin, '91). The marsupial opossum is particularly useful for such studies because it is born in a very immature state, 12-13 days after conception (McCready, '38; Hartman, '52; Martin, '78), and rubrospinal development occurs postnatally (Cabana and Martin, '84) rather than prenatally as in placental mammals (Prendergast and Stelzner, '76; Bregman and Goldberg, '82, '83). Because the rubrospinal tract develops postnatally, it is possible to manipulate it at early stages of development without intrauterine surgery. It is now appropriate to ask why rubral axons no longer grow around a lesion after the end of the critical period.

Based on the literature, it is possible that the development of myelin contributes to the end of the critical period for plasticity. Schwab and Caroni ('88a) have demonstrated that myelin-forming oligodendrocytes and CNS myelin are non-permissive substrates for neuronal adhesion and neurite
growth. Two proteins, NI-35 and NI-250, were isolated from CNS myelin and when they were neutralized by an antibody specific to them, the inhibitory function of myelin on axonal elongation was diminished (Caroni and Schwab, '88a and b; Schnell and Schwab, '90). It has also been shown that if myelin formation is suppressed by x-irradiation, corticospinal axons exhibit some degree of regeneration (Savio and Schwab, '90; Schwab and Schnell, '91). Since CNS myelin inhibits axonal growth and regeneration, we have entertained the possibility that some aspect of myelin formation correlates temporally with the end of the critical period for rubrospinal plasticity. The proteins in myelin which inhibit axonal growth may be associated with the development of myelin basic protein (Schwab and Caroni, '88), so we have studied myelin formation immunohistochemically using antibodies against the latter protein. Monoclonal antibodies against the inhibitory proteins referred to above may be too species specific to be of use in the opossum (Schwab, personal communication).

It has also been suggested that inhibitory proteins in myelin may be present on oligodendrocytes which are galactocerebroside (GalC)-positive (Schwab and Caroni, '88). Since GalC may appear in oligodendrocytes before MBP (Schwab and Schnell, '89), it is possible that the end of the critical period for rubrospinal plasticity correlates temporally with its expression. We have, therefore, used immunohistochemistry to study the development of GalC.
Comparison between the end of the critical period for rubrospinal plasticity and the results reported herein suggest that rubral axons have lost the ability to grow around a lesion of their spinal pathway before either MBP or GalC can be identified immunohistochemically in areas where myelin might be expected to impede axonal growth. It is clear, however, that rubral axons do not grow around a lesion in the presence of myelin. Possible interpretations of our results are discussed.
MATERIALS AND METHODS

Pouch-young opossums were obtained from adults purchased from licensed collectors in Florida. The ages of the pouch-young from captured opossums were estimated by comparing their snout-rump length (SRL) and external features with those of animals at known ages from timed litters (Cutts et al., '78, and data from our own collection).

Opossum pups were removed from the mother's pouch at various ages (Table 3) and anesthetized by inhalation of Metofane before being perfused intracardially with 0.9% saline followed by Zamboni's fixative (2% paraformaldehyde and 12% picric acid in 0.1M phosphate buffer at pH 7.2). The adult opossums were anesthetized with sodium pentobarbital (1.2ml/Kg) and perfused intracardially with 0.9% saline followed by the same fixative. The spinal cords of the pouch-young were postfixed in situ for 2-4 hours and then transferred into Sorenson's phosphate buffer with 30% glucose overnight. The spinal cords of the adult animals were removed and then postfixed as described above. All spinal cords were sectioned at 60um on a freezing microtome and processed immunohistochemically for myelin basic protein (MBP). In selected cases, adjacent sections were immunostained for GalC (Table 3).
In all of the above cases, sections were collected in PBS on ice, incubated in 0.3% Triton-X in PBS for 30 minutes, rinsed once for 10 minutes and then incubated in 2-3% normal goat serum (NGS) in phosphate buffered saline (PBS) for 30 minutes. The sections were rinsed once for 10 minutes and then incubated in either a monoclonal antibody against MBP or one raised against GalC (Boehringer-Mannheim) at 4°C for 48 hours. The anti-MBP was diluted 1:100 and the anti-GalC was diluted 1:20 in 2-3% NGS/PBS. The primary antibodies were removed by rinsing in PBS (3 times, 5 minutes each) after which the sections were incubated in the secondary antibody (biotinylated goat anti-mouse IgG from the ABC kit, Vector Laboratories) for 1 hour at room temperature. The secondary antibody was diluted as described in the ABC kit. After rinsing, the sections were incubated in the Avidin-Biotin HRP Complex (ABC kit, Vector Laboratories) for 1 hour at room temperature, also diluted as described in the ABC kit. For controls, the primary or the secondary antibodies were replaced by 2-3% NGS in PBS. Absorption controls were also run for MBP using a primary antibody that had been incubated with an excess of the antigen (Chemicon).

All incubations were performed at room temperature unless otherwise specified and the antibodies were diluted in 2-3% NGS in PBS. After each step, free-floating sections were rinsed 4 times (5 minutes each) in PBS. The sections were rinsed and reacted with a Diaminobenzidine (DAB)-Glucose
Oxidase (GOD) solution (25mg DAB in 25ml distilled water, add 25ml PBS, 20mg ammonium chloride, 100mg beta-D glucose, and 25ul GOD which was freshly prepared by dissolving 3mg GOD in 500ul distilled water) until a brown reaction product was observed. The reaction was stopped by rinsing with PBS. The sections were mounted on gelatin coated slides and dried overnight. The slides were then dehydrated in graded alcohols (85%, 90%, 100%), cleared in xylene, and coverslipped with piccolyte. Immunoreactive structures were viewed and photographed with a Lietz Orthoplan microscope equipped with differential interference contrast optics (Nomarski).
RESULTS

Prior to PD 15, myelin basic protein-like immunoreactivity (MBP-LI) was not found in either the brainstem or the spinal cord. At PD 15, however, it was present in the medial longitudinal fasiculus at medullary (Fig. 10A) and rostral cervical levels. Additional immunoreactivity was present in the ventral funiculus of the cervical (Figs. 10B and arrows in C) and thoracic spinal cord and, although light, within the dorsal funiculus at cervical levels (Figs. 10B and D). That within the dorsal funiculus was limited to the fasiculus cuneatus. MBP-LI was not present at lumbosacral levels.

By PD 21, the amount of MBP-LI had increased throughout the length of the spinal cord. At cervical levels, immunostaining was present within the ventral part of the lateral funiculus as well as within the ventral and dorsal funiculi (Figs. 11A and B), and at thoracic levels (Fig. 11C), it was found in all of the areas immunostained at cervical levels, except for the dorsal funiculus. Immunostaining in the dorsal funiculus was still limited to the fasiculus cuneatus. In the lumbar cord, immunostaining was present only within the ventral funiculus (arrows in Fig. 11D). At this age, MBP-LI was not observed in the grey matter or the dorsal part of the lateral funiculus at any spinal level.
At PD 26, the end of the critical period for rubrospinal plasticity, MBP immunostaining was relatively heavy at cervical levels in the fasiculus cuneatus, the ventral funiculus, and the ventral part of the lateral funiculus (Fig. 12A). A small amount was also found in the fasiculus gracilis, the ventral white commissure, the dorsal part of the lateral funiculus, and the ventral grey matter (Figs. 12A). Little evidence for MBP was found in the dorsal horn, although an occasional oligodendrocyte and presumptive myelin sheath was immunostained. At progressively more caudal levels, the amount of immunostaining decreased (compare Figs. 12A, B and D). In the thoracic cord, where the lesions were made in the plasticity studies, MBP-LI was present in the fasiculus cuneatus (rostral thoracic levels), the fasiculus gracilis, the ventral funiculus and the ventral part of the lateral funiculus (Fig. 12B); but none was found in that part of the lateral funiculus which contains the rubrospinal tract (Figs. 12B and C), or within the dorsal horn, an area through which rubral axons were forced to grow in the plasticity experiments. MBP-LI was not found in the ventral horn at thoracic levels until PD 30 nor in the dorsal horn until PD 33. In the lumbar cord, MBP-LI was present in the same areas as in the thoracic cord, but its density was generally less (Fig. 12D). In the lateral part of the fasiculus gracilis, however, immunoreactive fibers appeared more numerous than at cervical levels. By PD 33, the fasiculus gracilis and the dorsal part of the lateral funicu-
lus were well immunostained throughout the length of the spinal cord.

By PD 41, MBP-LI was well developed in the white and gray matter at all spinal levels (Fig. 13A-D) except for areas occupied by the corticospinal tracts in the cervical cord (Figs. 13A and B). The corticospinal tracts became immunoreactive by PD 54 making the pattern of immunostaining similar to that seen at later stages of development and in the adult animal.

The immunostaining pattern seen with the GalC antibody (Fig. 14A and B) was almost identical to that obtained using the antibody to MBP in the same animal (Fig. 14C and D). In some instances, GalC immunostaining was associated with round profiles that looked like oligodendrocytes.

In controls, immunostaining was absent with either MBP-absorbed antisera or with the omission of the primary antisera. Absorption controls were not run with the GalC immunostaining because of the lack of a commercially available antigen.
DISCUSSION

In the present study, the sequence of myelination in the spinal cord of developing opossums was studied using MBP-immunohistochemistry. The only previous study of myelin formation in this species was reported by Langeworthy ('29) who utilized the Weigert technique. When our results are compared with his and with material in our own laboratory, it is clear that MBP is present well before myelin can be identified by the Weigert technique. A similar conclusion was reported for the rat (Rozeik and von Keyserlingk, '87; Schwab and Schnell, '89). The sequence of myelination revealed by the two methods appears comparable, however. For example, the first tract in the opossum's nervous system to acquire myelin, as demonstrated by either technique, was the medial longitudinal fasciculus and the last one was the corticospinal tract. Based on the above comparisons, it appears that there is a considerable temporal disparity between the growth of descending axons into the spinal cord (Cabana and Martin, '84) and their subsequent myelination in the opossum.

When our results were compared with those reported for the rat (Rozeik and von Keyserlingk, '87; Schwab and Schnell, '89), it appears that the sequence of myelination, as demonstrated by MBP-immunohistochemistry, is similar. The develop-
ment of myelination appears more protracted in the opossum than in the rat, however.

Myelin basic protein (mol. wt. 18,500) accounts for 30% of the total protein in adult CNS myelin (Sternberger et al., '78) and it is thought to be most pronounced during early stages of myelination (Sternberger et al., '78; Schwob et al., '85; Sternberger et al., '85). MBP-LI is diffusely distributed in the cytoplasm of oligodendrocytes before it is found in association with axons (Sternberger et al., '78; Schwob et al., '85). When axons become myelinated, immunostained oligodendrocytes are either obscured from view or lose their antigenicity (Sternberger et al., '85). As compact myelin increases during development, immunostaining becomes homogeneous making the outline of the myelinated tracts difficult to distinguish (Sternberger et al., '78). This occurs even when detergents are used in the processing.

Previous studies have shown that differentiated oligodendrocytes and/or proteins from central myelin can inhibit axonal growth (Schwab and Caroni, '88; Caroni and Schwab, '88a). For that reason, we have asked whether the development of myelin in the spinal cord, as judged by MBP-LI, correlates temporally with loss of rubrospinal plasticity.

If the rubrospinal tract is severed at thoracic levels prior to PD 26, rubral axons can grow around the lesion to innervate appropriate areas caudal to it (Martin and Xu, '88; Xu and Martin, '89). Depending upon the size and geometry of
the lesion, rubral axons either grow through the remaining part of the lateral funiculus, the dorsal horn or the dorsal funiculus to reach their destination (Martin and Xu, '88).

By the end of the critical period for rubrospinal plasticity (approximately PD 26), MBP is not present in either the dorsal part of the lateral funiculus or the dorsal horn at thoracic levels, areas where myelin proteins would reasonably have a negative influence on growing axons in the plasticity experiments. It was not until approximately one week later that MBP-LI was first seen in such areas. The same pattern of immunostaining was observed in the GalC experiments. Although it is obvious that rubral axons do not grow around a lesion after myelin is present, a time gap appears to exist between the end of the critical period for rubrospinal plasticity and the expression of MBP and GalC. That gap could be explained, however, if late arriving and regenerating axons do not grow around the lesion during the first week or if the myelin associated proteins which inhibit axonal elongation are present before MBP or GalC. Opossums have a protracted development (Cutts et al., '78) and it is possible that individual variation in the appearance of myelin and/or the end of the critical period contribute to the temporal disparity described above. If so, it may be more apparent than real.
| Ages in estimated postnatal day (PD), snout-rump length (SRL) in millimeters and the number of animals in each cases (N) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PD  | 15 | 21 | 26 | 30 | 33 | 37 | 41 | 43 | 54 | 62 | 70 | 76 | 78 | 85 | 89 | adult |
| SRL | 32 | 40 | 50 | 55 | 60 | 65 | 72 | 77 | 90 | 102 | 120 | 140 | 149 | 180 | 195 | - |
| N(MBP) | 3 | 2 | 3 | 2 | 3 | 2 | 2 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| N(GalC) | 2 | 2 | 4 | 1 |
Fig. 10 A is a photomicrograph from a PD 15 opossum showing MBP-LI in the medial longitudinal fasciculus (MLF) of the medulla and Fig. B is a photomicrograph of the cervical spinal cord from the same animal. Fig. C is a higher magnification of the area enclosed by rectangle 'a' in B and Fig. D is a higher magnification of the area enclosed by rectangle 'b'. The arrows in Figs. C and D point to MBP-LI structures in the ventral and dorsal funiculi, respectively. Hg = hypoglossal nucleus; Rv = nucleus reticularis medullae oblongatae ventralis; iv = fourth ventricle; df = dorsal funiculus; vf = ventral funiculus.
Figs. 11 A, C, and D are photomicrographs from a PD 20 opossum showing the distribution of MBP-LI at cervical, thoracic and lumbar levels of the spinal cord. Fig. B is a higher magnification of the area enclosed by rectangle in A. The dashed lines in Fig. A, C and D demarcate the approximate boundaries between white and grey matter. The arrows in Fig. D point to MBP-LI structures in the ventral funiculus.
Fig. 11
Figs. 12 A, B and D are photomicrographs from a PD 26 opossum showing the distribution of MBP-LI at cervical, thoracic and lumbar levels of the spinal cord. Fig. C is a higher magnification of the area enclosed by rectangle in B. The dashed lines in Figs. B and C demarcates the approximate boundaries between white and grey matter. fc = fasciculus cuneatus; fg = fasciculus gracilis.
Figs. 13 A, C and D are photomicrographs from a PD 41 opossum showing the distribution of MBP-LI at cervical, thoracic and lumbar levels of the cord. Fig. B is a higher magnification of the area enclosed by rectangle in A.
Fig. 13
Fig. 14 A is a photomicrograph from a PD 26 opossum showing the distribution of GalC in the thoracic spinal cord. Fig. B is a higher magnification of the area enclosed by rectangle in A. Fig. C is a photomicrograph of an adjacent section showing the distribution of MBP and Fig. D is a higher magnification of the area enclosed by rectangle in C.
Fig. 14
LIST OF REFERENCE


DISCUSSION

The results in Chapter 1 suggest that a rough temporal correlation exists between the transformation from radial glia and mature appearing astrocytes and the end of the critical period for rubrospinal plasticity. It is possible, therefore, that rubral axons need radial glial cells as a scaffold to maneuver around a lesion of their pathway. The link between growing axons and the surface of radial glial cells may be laminin since it is transiently synthesized by astrocytes during development (McLoon, et al., '88). It would be appropriate, therefore, to identify proteins that are transiently expressed on radial glial cells during the critical period. If such proteins could be isolated, it might be possible to block them with appropriate antibodies and study the effect on rubrospinal plasticity.

In Chapter II, we report that the first glial response to lesioning the spinal cord occurs before the end of the critical period for rubrospinal plasticity. By the end of the critical period, however, the glial response is still limited to the white matter and it is surprising that rubral axons cannot avoid it by growing through the dorsal horn. Hypertrophied astrocytes are present at the grey/white matter junction.
at the end of the critical period, however, and it is possible that they produce inhibitory proteins (McKeon et al., '91). This possibility should be explored.

During the critical period for rubrospinal plasticity, myelin was not present in the dorsal part of the lateral funiculus or the dorsal horn, areas occupied by normal or rerouted rubrospinal axons, and it was not found in these regions until approximately a week after the end of the critical period (Chapter III). Inhibitory proteins associated with myelin (Schwab and Caroni, '88) might influence rubrospinal plasticity, however, if late growing axons do not reach the lesion site until approximately 7 days after the lesion is made and/or if the proteins in myelin which inhibit axonal growth are expressed before myelin basic protein (MBP) can be detected. The latter viewpoint was suggested by Schwab and Schnell ('89) who found the inhibitory proteins on galactocerebroside (GalC)-positive oligodendrocytes. In the opossum, however, GalC and MBP appeared at the same time. It is possible to conclude, however, that rubrospinal plasticity does not occur once MBP-LI can be demonstrated in those areas of the spinal cord which contain rubrospinal axons. A logical next step would be to identify these proteins in the opossum so that they can be blocked using appropriate antibodies to see if it extends the critical period for rubrospinal plasticity. Alternatively, it may be feasible to demyelinate the spinal cord using X-irradiation.
The above results are purely correlative and do not establish cause and effect relationships. The experiments were necessary, however, to determine which, if any of the phenomena studied were likely candidates for more definitive experiments. It is important to identify the factors which limit spinal cord plasticity with maturity in order to ask whether they can be blocked or modified to recapitulate plasticity in the injured spinal cord of adult animals, including man.
LIST OF REFERENCE


