Axonal growth and guidance during formation of the pyramidal tract in the rat

E.A.J. Joosten

Axonal growth and guidance during formation of the pyramidal tract in the rat

Axonal growth and guidance during formation of the pyramidal tract in the rat

Een wetenschappelijke proeve op het gebied
van de Natuurwetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Katholieke Universiteit te Nijmegen, volgens
besluit van het college van decanen in het
openbaar te verdedigen op
woensdag 11 oktober 1989 des namiddags om 3.30 uur

door

ENGELBERT ANTONIUS JOSEPH JOOSTEN

geboren te Horn (L)

Promotor: Prof.dr.R.Nieuwenhuys

Co-Referent: Dr.A.A.M.Gribnau

Dankwoord:

De medewerkers en oud-medewerkers van de afdeling Anatomie en Embryologie ben ik erkentelijk voor de leerzame samenwerking. Het contact met Theo, Jos, Alice, Jessie en wijlen dr.E.de Kort heb ik erg gewaardeerd. De prettige en vruchtbare samenwerking met betrokken medewerkers van de afdelingen Pathologische Anatomie, Celbiologie, Medische Fotografie, Medische Illustratie en het Centraal Dierenlaboratorium van de K.U.Nijmegen heb ik zeer op prijs gesteld.

aan vader en moeder

Bertine

CONTENTS

Chapter 1

General Introduction

CHAPTER 1 : GENERAL INTRODUCTION

1.1 Introduction

Man has been fascinated by the development of a multicellular organism from a single cell, the fertilized egg, formed by the union of an egg and a sperm. Up to the middle of the nineteenth century a generally accepted view on the development of an organism was that each sperm or egg contained a miniature of it, sitting inside.

Scientific discussions focussed mainly on the question whether the miniature organism was in the egg or in the sperm. Based on this preformationists concept, development involved only growth rather than the sequential acquisition of new structures. Largely as a result of the observations and arguments of C.F.Wolff and K.E.von Baer's "Entwicklungsgeschichte der Tiere" in 1828 a turning point was reached. From that time on it is well accepted that the development of an animal is the result of an initial plan operating in conjunction with external factors. The fertilized egg contains the genetic information and appropriate structure to program the development of an organism; yet the complexity of form is built up gradually.

The development of an animal as a whole includes the development of the central nervous system (CNS) which organizes the animal's behaviour. The development of the former, however, cannot be isolated from the generation of other organ systems.

The central nervous system (CNS) is formed by a sequence or complex of processes which is summarily designated neurogenesis. Among the many histogenetic events that occur during early CNS development is the generation of classes of cell types: among them nerve cells or neurons and glial cells. All nerve cells further differentiate to match their specific function within the CNS. A unique feature of developing neurons is the extension of processes or axons which are involved in the finding and subsequent formation of functional contacts between neurons.
Outgrowth and subsequent target finding of the axon are among the main events during the development of the CNS. Outgrowing axons must not only elongate but also have to find their targets by appropriate pathfinding. Our knowledge concerning the mechanisms underlying these events is still fragmentary. Elongation and subsequent target cell finding by an outgrowing axon is an astonishing phenomenon, a neuronal ability which may be 'guided' or influenced by chemical, mechanical, bio-electrical or other cues (Purves and Lichtman,1985; Edelman,1985; Dodd and Jessell, 1988). However, not all outgrowing axons will reach their appropriate target. CNS development is characterized by an overproduction of neurons, dendrites, outgrowing axons and synapses (see Purves and Lichtman,1985; Innocenti,1988). Hence, during CNS development cell death as well as the elimination of axon collaterals are very commonly occurring phenomena (Purves and Lichtman,1985; Stanfield et al.,1982; Innocenti,1988;

Provis and Penfold,1988).

This thesis is concerned with the development of the rat pyramidal tract (PT). The PT is a long descending central pathway, restricted to mammals, which is involved in both motor and sensory control. The rat PT is a very useful model in experimental research on the development of fibre systems in mammals because of its postnatal outgrowth throughout the spinal cord as well as its experimental accessibility. Hence, mechanisms underlying axon outgrowth and subsequent target cell finding as well as processes involved in developmental exuberance can be studied relatively easily in this fibre system.

1.2 Development of the rat pyramidal tract

The mammalian PT is defined as a set of fibres passing through the medullary pyramids. Its major components is the corticospinal tract (CST). The fibres of the CST originate from pyramidal cell bodies, which are situated in layer V of the cerebral cortex (Armand,1982; Hicks and D'Amato,1975; Wise et al.1979[a]; Kuypers,1981; Jones et al.,1982; Leong,1983; Hicks and D'Amato,1980; Miller,1987) and after decussating in the lower medulla oblongata continue in the ventralmost part of the dorsal funiculus and project to the contralateral spinal grey (Schreyer and Jones,1982). In rats the cells of origin of the CST are located primarily in sensorimotor cortex (Fig.1.1) (Woolsey,1958; Hicks and D'Amato,1975; D'Amato and Hicks,1978,1980; Wise et al.,1979[a]; Leong,1983; Miller,1987). Based on retrograde tract tracing experiments various authors demonstrated the somatotopic arrangement of the parent CST neurons in the rat cortex (Wise et al.,1979[a]; Leong,1983; Miller,1987). Those corticospinal (CS) neurons projecting to the cervical spinal cord segments are predominantly located in area 4 and rostral area 6/8 (motor-cortices), and medial area 3 and caudal area 2 (somatosensory cortices), whereas the CS neurons with their targets in the lumbar spinal grey are included in the caudal parts of areas 4 and 3 and area 18[b] (Krieg,1946; Miller,1987). Thus, rat CS neurons projecting to lumbar spinal cord segments are located more caudomedially as compared with those projecting to cervical spinal levels (Fig.1.1). These data are consistent with electrophysiological mapping studies (e.g. Hall and Lindholm,1974; Donoghue and Wise,1982; Neafsey et al.,1986). Besides, there are broad similarities between the distribution of CS neurons in the rat, cat and monkey (Miller,1987; Wise and Donoghue,1986).In early development, rodent layer V neurons, which give rise to CST axons, are also found in occipital parts of the cortex. This implies a reorganization of CS neurons in layer V of the cortex during development which is due to the elimination of axonal collaterals rather than the death of neurons (see Introductory note Chapter 2) (Stanfield et al.,1982;

O'Leary,1985[a]; O'Leary and Stanfield,1985,1986).

Although in man, after the decussation in the lower medulla,the extension of CST fibres into the spinal cord occurs during the fetal period (Humphrey,1960; Wozniak and O'Rahilly,1982) in rodents CST fibres enter the spinal cord at birth whereas their outgrowth comes about postnatally (DeMyer,1967; Kalil and Reh,1979; Reh and Kalil,1982; Jones et al.,1982; Schreyer and Jones,1982; Terashima et al.,1983; Gribnau et al.,1986; among others). With the use of varying anterograde tracing techniques, a timetable of the caudal extension of CST fibres at different postnatal ages was determined previously (Donatelle,1977; Schreyer and Jones,1982; Gribnau et al.,1986).

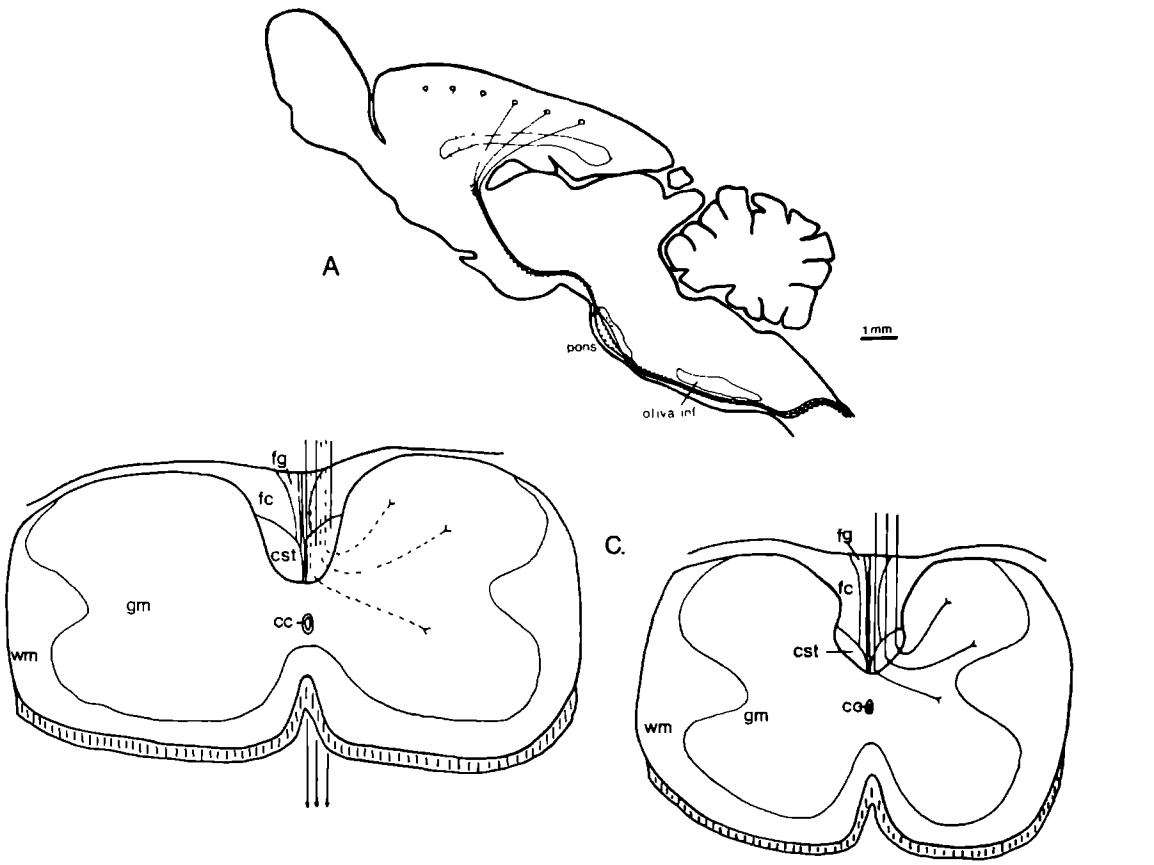


Fig.1.1 : The rat corticospinal tract originates in the cortex **(A)** and projects to the spinal gray via the medullary pyramids. After decussation it continues within the ventral part of the dorsal funiculus. The more anteriorly situated neurones project to the cervical spinal gray (- - - -) **(B)** ,whereas the lumbar spinal gray appears to be the termination area of the more posterior situated corticospinal cell bodies (———) **(C)** . cc=central canal; cst=corticospinal tract; fc=fasciculus cuneatus; fg=fasciculus gracilis; gm=gray matter; wm=white matter.

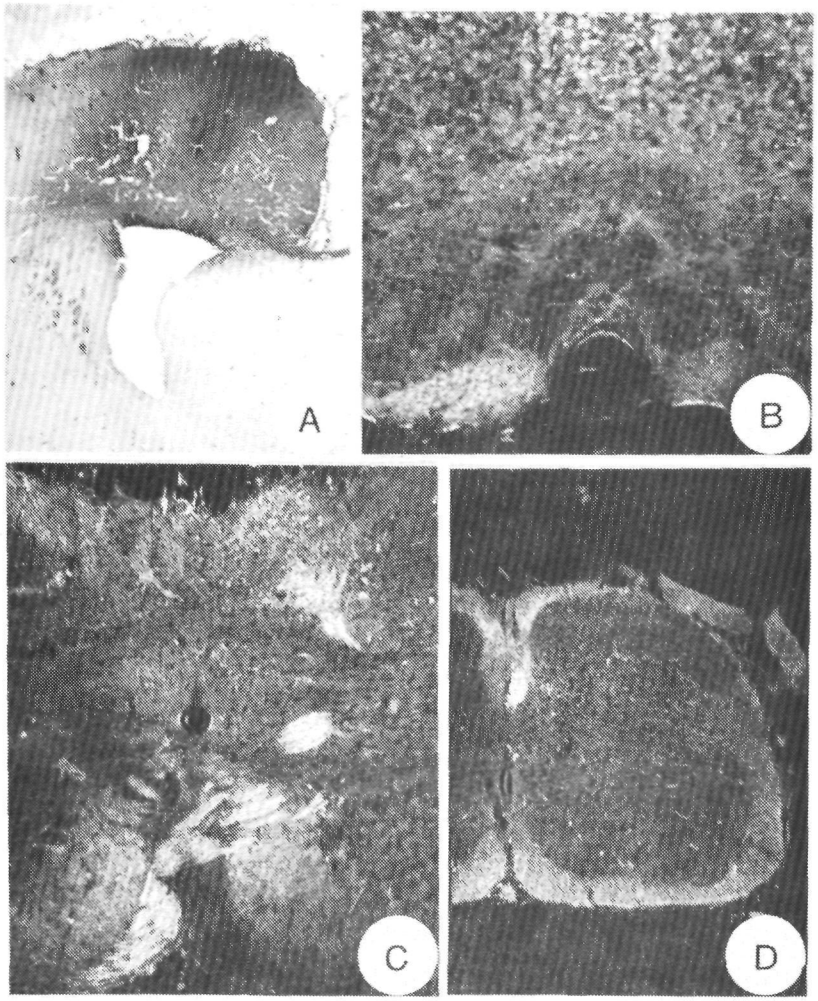


Fig.1.2 : With the use of the anterograde tracer horseradish-peroxidase (HRP) CST axons can be localized after labelling the CS neurons in the sensorimotor cortex. A :Photomicrograph of a representative example of HRP labelled injection area in the sensorimotor cortex of a ten day old rat. B-D :Dark-field illuminated photomicrographs of labelled CST axons of the same rat at various positions in the tract :medullary level **(B)** , decussation **(C)** and lumbar spinal cord **(D)** Bar=100 µm.

Such an experiment using horseradish-peroxidase (HRP) as a tracer is illustrated in Fig.1.2. The results show that CST outgrowth mainly occurs during the first postnatal week as is summarized in Fig.1.3.

A quantitative analysis on the amount of the anterograde labelling in

the developing rat CST at various ages revealed a characteristic pattern (Gribnau et al.,1986). From these results it was deduced that the developing rat CST is characterized by a staggered mode of outgrowth, as was also found in the hamster (O'Leary and Stanfield,1986). It became an intriguing question which guidance cues might underlie the outgrowth of the CST fibres (see Introductory note Chapter 5).

Between the arrival of the CST axons in the dorsal funiculus of a given spinal cord segment and the extension into the respective spinal gray matter in rodents a delay was noted of about 2-4 days (see Fig.1.3) (Wise et al.,1979[a]; Reh and Kalil,1981; Schreyer and Jones,1982; Gribnau et al.,1986).This waiting period might be attributed to the lagging behind of the developmental state of the target neurons in the spinal grey.

In the spinal gray most CST axons end on interneurons in the base of the dorsal horn and intermediate regions (Goodman et al.,1966; Brown,1971; Armand,1982; Kuypers,1981), although electrophysiological experiments demonstrated some monosynaptic contacts from CST axons on motoneurons (Janszen et al.,1977; Elger et al.,1977,1978). After ingrowth the formation of functional synapses in the spinal gray has been related to the appearance of fore- and hindlimb placing responses (Castro,1972; Kalil and Schneider,1975; Donatelle,1977). The placing responses are less

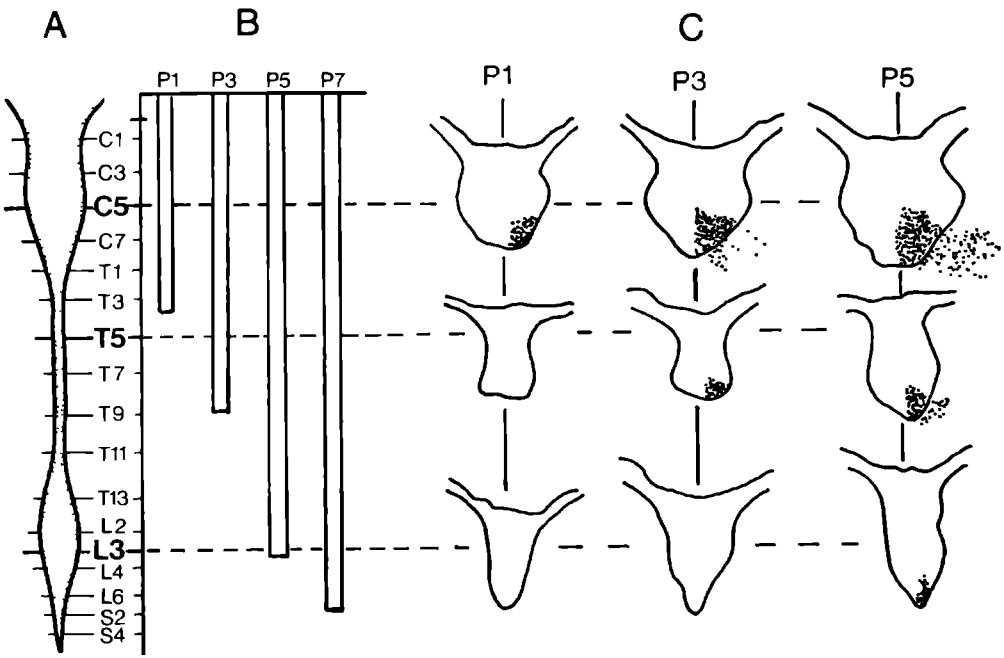


Fig.1.3 : A :Schematic representation of the rat spinal cord. B :CST extension diagram. C :camera lucida drawings of transverse sections of the dorsal funiculus with the labelled CST.

well coordinated in young postnatal animals as compared with adult rats (Donatelle,1977). This phenomenon may be correlated to the fact that myelination of PT fibres starts at about postnatal day 14 (Matthews and Duncan,1971).

Although in the adult rat many PT axons are myelinated, numerous unmyelinated PT fibres were encountered (Leenen et al.,1982,1985; Chung and Coggeshall,1987; Gorgels et al.,1989[a]).Unfortunately, the origin, destination as well as the function of the unmyelinated axons in the adult PT are unknown.

1.3 Scope of the present investigation

In the present study the postnatal development of the pyramidal tract and especially its corticospinal component has been investigated in the developing rat spinal cord using a variety of light- and electron-microscopic neuroanatomical techniques.

In essence this thesis can be subdivided into two main parts which, however, are closely related. First a detailed description of the development of the CST in the rat is given (Chapters 2-4). In the second part (Chapter 5) the role of several guidance cues on outgrowing CST axons in the rat is described. During the study on the development of the rat CST the following questions were of main interest:

- How does the corticospinal component of the rat pyramidal tract develop?

- Is collateral elimination confined to the occipital cortex or is it a general phenomenon in the development of the corticospinal tract?

- Which factors do play a role in the outgrowth and guidance of corticospinal axons in the rat?

In Chapter 2 first an Introductory Note is presented on the loss of axonal projections in the developing mammalian brain, with special attention to the rat CST. In Chapter 2B the development of the rat CST is described with respect to the relation between the site of WGA-HRP injection within the cortex and the pattern of labelling in the spinal cord from postnatal day 1 (P1) through postnatal day 10 (P10). With the use of iontophoretic WGA-HRP injections the development of transient CS projections from the medial prefrontal cortex is described in Chapter 2C. In Chapter 3 a description is given of the morphology of the distal ends of the CST pioneer axons in the lower spinal cord segments during white and grey matter development as analysed with the use of a new combination of HRP-staining techniques. After introduction of a method for the electron-microscopic (EM) visualization of anterogradely transported HRP in developing neural tissue the postnatally developing

CST as well as the mature CST were analysed (Chapter 4). Factors which might play an important role in the development of CNS fibre systems are discussed in the Introductory Note of Chapter 5. The possible role of precursor-astrocytes in the guidance of outgrowing CST axons is pointed out in Chapters 5B. The localization of the cell adhesion molecule L1 during the outgrowth of CST axons is based on LM (Chapter 5C1) and EM-observations (Chapter 5C2). Finally, in Chapter 6 the results of this study are discussed and summarized.

Chapter 2

Collateral elimination during postnatal corticospinal tract development in rat

CHAPTER 2A INTRODUCTORY NOTE: DEVELOPMENTAL EXUBERANCE IN CNS

CNS development is characterized by an overproduction and subsequent elimination of neurons, dendrites, spines, axons and synapses (Purves and Lichtman,1985; Innocenti,1988). These phenomena are often collectively referred to as developmental exuberance or regressive developmental events. It has been suggested that the developmental overproduction serves to ensure that each target structure eventually receives an adequate input. Furthermore, the subsequent elimination serves the matching in size of an innervating population of cells to the capacity of its target: "system matching" (Hamburger and Oppenheim,1982; Cowan et al.,1984). For instance, in rodents retinal axons initially project to all parts of the ipsilateral superior colliculus whereas in adults its projection is restricted to a region along the rostral and medial margins of the colliculus (Frost and Schneider,1979). The phenomenon of "system matching" is closely correlated with the plasticity of the young CNS. An important implication of this process is that developmental errors can effectively be eliminated (Cowan et al.,1984; O'Leary,1987). However, although it seems unlikely, some of the transient connections might play a temporary role in development (O'Leary and Thanos,1985; Innocenti, 1988).

Up till now most research on CNS developmental exuberance has been focussed on "neuronal death". During the development of virtually all cell groups in the vertebrate central nervous system, there is a period during which about half of the initial population of neurons dies. This phase of "naturally occurring cell death" was observed in various parts of the CNS (e.g.Hamburger and Oppenheim,1982; van Eden,1985; Provis and Penfold,1988) and coincides with the actual formation of connections between the neuronal population and its target. In normally occurring death some neurons are programmed to die by virtue of intrinsic instruction. This type of strategy is well suited to regulate cell numbers in relatively simple, in most cases invertebrate, systems (Goodman and Bate,1981; Shankland, 1984).

In vertebrate systems naturally occurring cell death is probably not preordained but depends on interactions with other cells at the level of the target. Even when axons follow their normal pathways and reach their correct target areas they might terminate in an inappropriate section of this target. This topographic targeting error may subsequently result in death of the cells of origin as has been demonstrated in e.g. the retinal projection to the contralateral superior colliculus in the rat (O'Leary et al.,1986).

It has been suggested that vertebrate neurons commonly die because of failure in competition for a factor or factors produced by the target. In the peripheral nervous system such a factor, namely nerve growth factor (NGF) has been isolated (Levi-Montalcini and Booker,1960). In vitro studies using dissociated sympathetic ganglion cells provided substantial evidence that a major effect of NGF is promotion of cell survival

(Levi-Montalcini and Angletti,1963; Berg,1982).

Although the process of "system matching" often can be explained by the death of neurons, research during the past decade demonstrated the overproduction and elimination of (long) axonal connections without death of the parent cells. This process of 'collateral elimination' presents itself in mammals during the development of corticocortical (Innocenti et al.,1977; Ivy et al.,1979), corticofugal (Reh and Kalil,1982; Stanfield et al.,1982), retinofugal (Williams and Chalupa,1982; Frost,1984) and other pathways (Stein et al.,1985).

The first evidence for the occurrence of a transient corticospinal projection from neurons localized in the occipital part of the cortex in the rat was provided in retrograde tracer studies with the fluorescent marker True blue (Stanfield et al.,1982). During the first week, injections of True blue into the pyramidal decussation resulted in the labelling of pyramidal tract neurons which are found virtually throughout the tangential extent of layer V of the neocortex. In contrast, after comparable injections during the fourth postnatal week the distribution of such cells appeared to be much more restricted; a restriction which is most obvious in the occipital part of the cortex. Double labelling retrograde experiments excluded the possibility of neuronal death in layer V of the occipital cortex. Based on these data it was concluded that occipital layer V neurons extend corticospinal axons well down into the spinal cord during the first postnatal week, but that all of the occipital corticospinal fibres are subsequently eliminated by collateral pruning even though many of their parent neurons remain intact projecting to the tectal region (Stanfield and O'Leary,1985b).

The question remains why so many transitory structures are generated and what their functional role might be. Exuberance and subsequent regressive phenomena are correlated with "system matching", which is one example of the enormous flexibility for matching the phenotypic expressions of the various sets of genes provided during ontogenesis. These developmental strategies may be further clarified by a phylogenetic perspective (Innocenti,1988): The success of developmental exuberance in phylogenesis may be the plasticity provided in later stages of neural development. From this point of view axon elimination is more economical and gives more flexibility than neuronal death does.

CHAPTER 2B: AN ANTEROGRADE TRACER STUDY OF THE DEVELOPING CORTICOSPINAL TRACT IN THE RAT: THREE COMPONENTS

SUMMARY

Light microscopic analysis of anterogradely transported wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) has been used to study the developing corticospinal tract (CST) in the rat. This study was carried out to examine the relation between the site of injection within the cortex and the pattern of labeling of the developing CST in the spinal cord from postnatal day 1 (P1) through postnatal day 10 (P10). For this purpose the cortex was subdivided into three equal areas along the rostro-caudal axis: anterior, intermediate and posterior. After the operation the animals were allowed to survive for 24 hours.

The caudal extension of labeled CST axons originating in the anterior cortical area was restricted (L1 at P7 or P10) as compared with that of the CST fibres originating in the intermediate cortical area (S3 at P10). The axons of the posterior corticospinal (CS) neurons reach their most caudal extension in the spinal cord (Th5) at P7 but then gradually disappear up till P14.

Quantitative analysis of the amount of label along the length of the outgrowing CST fibres revealed the formation of a large stable peak at the level of the cervical enlargement after labeling of either the anterior or the intermediate cortical area. The formation of a second "running" peak which moves caudally from mid-thoracic levels at P5 to mid-lumbar levels at P10 was only accomplished by labeling the intermediate cortical area and is probably caused by the accumulation of label in the growth cones at the distal ends of the outgrowing CST fibres. After labeling the posterior cortical area no peaks could be detected neither at the cervical nor at the lumbar intumescence.

The major spinal gray termination field of the anterior CS neurons appeared to be the cervical intumescence, whereas the major spinal gray termination field of the intermediate CS neurons is the lumbar enlargement. By contrast, axons of posterior CS neurons never showed any outgrowth into the spinal gray matter at any level.

Concludingly the developing CST in the rat consists of three components the first, having its originating neurons in the anterior part of the cortex and its termination field in the cervical intumescence; the second with its originating neurons in the intermediate part of the cortex and its termination field predominantly in the lumbar enlargement and a third transient one originating in the posterior cortex and gradually disappearing from spinal cord levels.

Research using anterograde tracing techniques in combination with electron microscopy is necessary to further analyse these three different components.

Key words :corticospinal tract - development - termination field - anterograde tracing - rat.

INTRODUCTION

The pyramidal tract (PT) can be defined as a set of fibres passing through the medullary pyramids and its major component is the corticospinal tract (CST). The CST of the rat is a pathway which originates in layer V of the sensorimotor cortex and projects to the spinal gray matter via the medullary pyramids; after their decussation its fibres are located within the ventral part of the dorsal funiculus. In the rat the developing CST fibres reach the cervical spinal cord at birth and their outgrowth throughout the cord comes about postnatally (Donatelle,1977; Jones et al.,1982; Leong,1983; Leong et al.,1984^{b}; Gribnau et al.,1986). The CST in rodents is a very extensively studied pathway not only because of its postnatal outgrowth in the spinal cord but also because the CST provides a model in which the development of a long motor pathway can be studied. A close temporal relationship has been found between the growth of corticospinal axons into the spinal cord and the development of placing reactions in the rat (Hicks and D'Amato,1975; Donatelle ,1977; Hicks and D'Amato,1980).

Quantitative light microscopic analysis of anterogradely transported wheat-germ-agglutinin conjugated horseradish peroxidase (WGA-HRP) in the developing CST in the rat revealed a characteristic pattern of labeling within the outgrowing bundle varying with age (Gribnau et al.,1986). The analysis of the relation between the site of the originating neurons within the cortex and the pattern of labeling of the developing CST might provide interesting new information about the way in which this fibre tract arises.

Retrograde labeling studies in rodents revealed that during early postnatal developmental stages layer V neurons, which give rise to CST axons, are distributed over large parts of the cerebral cortex. Contrastingly, in adults these layer V neurons are concentrated in the sensorimotor cortex located in the rostral two-thirds of the cerebral hemispheres (Ivy and Killacky,1982; Adams et al.,1983^{a},1983^{b}; Leong,1983; Bates and Killacky,1984; Leong et al.,1984^{a},1984^{b}) showing a distinct somatotopical arrangement (Wise et al.,1979^{a},1979^{b}; Donoghue and Wise, 1982; Neafsey et al.,1986), neurons projecting to lumbar spinal cord segments are located more caudomedially than those which project to cervical spinal cord segments. This topographic reorganization of the layer V neurons during postnatal development is probably brought about by selective elimination of certain early-formed axon collaterals and not by degeneration of axons after neuronal death (O'Leary et al.,1981; Stanfield et al.,1982; Stanfield,1984; O'Leary and Stanfield,1985;

Stanfield and O'Leary,1985[a],1985[b]; O'Leary and Stanfield,1986; Schreyer and Jones,1988[b]). At the same time electron microscopic analysis of the developing PT revealed a notable loss of thin unmyelinated axons during the same period (Reh and Kalil,1982; De Kort and Van Aanholt,1983).

The present light microscopic study was undertaken to analyse the possible relationship between the developmental pattern of CST axons and the site of their parent neurons in the cortex on the one hand and the outgrowth of the terminal branches of these CST axons into the spinal gray matter on the other hand.

MATERIALS AND METHODS

In the present study postnatal Wistar rats (n=49), ranging in age from postnatal day 1 (P1) to postnatal day 10 (P10) were used; in which the day of birth was accounted for P0. At least three animals per injection area per age category were used. The ages of the animals given in the present paper are the ages at their respective days of injection.

After anaesthetizing the animals with sodium pentobarbital (18 mg. per kg. body weight, i.p.) and additional cooling in icewater, if necessary, the injections were placed after opening of the skin and making of a small hole in the skull using a fine needle.

The hemisphere of each animal was subdivided in three imaginary equal areas along the rostro-caudal axis: anterior, intermediate and posterior (Fig. 1). In the centre of each individual cortical area the injection of 5% wheat-germ-agglutinin horseradish peroxidase (WGA-HRP, sigma type VI) solution in distilled water was placed, using a 5 µl Hamilton syringe fitted with a glass micropipette. Each injection contained 0.05 ul WGA-HRP solution in order to label as many layer V neurons of the respective cortical area as possible. The postinjection survival times were kept constant at 24 hours. The animals were reanaesthetized and transcardially perfused with saline, followed by 1.25% glutaraldehyde and 1.0% formaldehyde in 0.1 M. phosphate buffer (PB) (pH 7.4).

After the perfusion the brains and spinal cords were immediately removed and postfixed by immersion overnight. Hereafter they were placed in phosphate buffer containing 30% sucrose (PBS) at 4°C during approximately 6 hours. Before cutting the brains and spinal cords into 30 µm transverse sections on a freezing microtome they were embedded in a gelatin-sucrose mixture (15% gelatin in PBS), stored overnight in 4% formaldehyde and washed in PBS for 1-2 hours. The material was then frozen in dry ice and cut into sections in the transverse plane on a freezing microtome. Every fifth (P1-P7) or sixth (P10) section was immediately incubated with tetramethylbenzidine (TMB) as a chromogen, according to Mesulam (1978). The sections were counterstained with neutral red and mounted in Depex. Control sections were processed identically but the incubation was carried out without TMB.

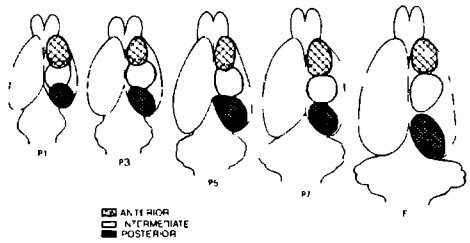


Fig.1 : Reconstructions showing the cortical injection areas in various stages studied.

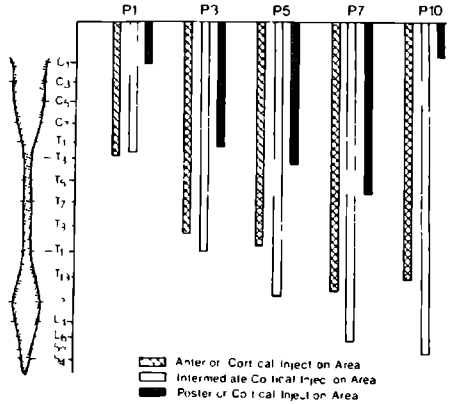


Fig.2 : Caudal extension of WGA-HRP labelled CST axons after injecting the cortical areas, as illustrated in Fig.1, at different postnatal ages.

In each experiment a graphical reconstruction was made of the cortical injection area, including both the heavily labeled injection site and its diffusely labeled surroundings, from the serial sections of the cortex as traced with a camera lucida. For that purpose the orthogonal projection of the cortex, with its labeled part as traced in the camera lucida drawings onto the midsaggital plane was used.

Drawings of the labeling in the spinal cord sections were made at higher magnification under dark field illumination using a Zeiss microscope eguipped with a drawing device (Figs. 4 and 5). For each spinal cord a labeling distribution chart of the CST was made according to the method as described by Gribnau et al.(1986). In this way a rostro-caudal labelingscurve of the CST could be deduced for each individual spinal cord (Fig. 3).

All photomicrographs were made with an automatic Zeiss-photomicroscope-II using dark field illumination using an Agfapan 25 film.

RESULTS

In the present paper attention was focused on the developmental pattern of the CST axons as related to the site of their parent neurons within the cortex on the one hand and to the outgrowth of the terminal branches of these CST axons into the spinal gray on the other hand. The results on both aspects of the developing CST axons during the postnatal period from P1 through P10 will be described for each of the three WGA-HRP injection areas (see Materials and methods) separately. Control sections were always negative.

Anterior cortical area

As is schematically shown in Figure 2 after labeling of the anterior cortical area (see Materials and methods) the labeled CST axons extend caudally into the second thoracic (Th2) segment at P1, into the ninth thoracic (Th9) segment at P3, into the eleventh thoracic (Th 11) segment at P5 and into the first lumbar (L1) segment at P7 and P10. Up to P10 the labeled CST fibres were not found at more caudal levels than the first lumbar segment.

The quantitative analysis of the WGA-HRP labeled CST axons provides some more interesting results. As can be seen in figure 4, which represents camera lucida drawings of the most salient spinal cord segments the amount of WGA-HRP label present in the CST at the spinal cord segment Th 5 is much less than that present at C5.

From the labeling distribution chart (Fig. 3) it can be concluded that after labeling of the anterior cortical area a large peak of WGA-HRP label arises at the cervical intumescence. The fact that anterior CST

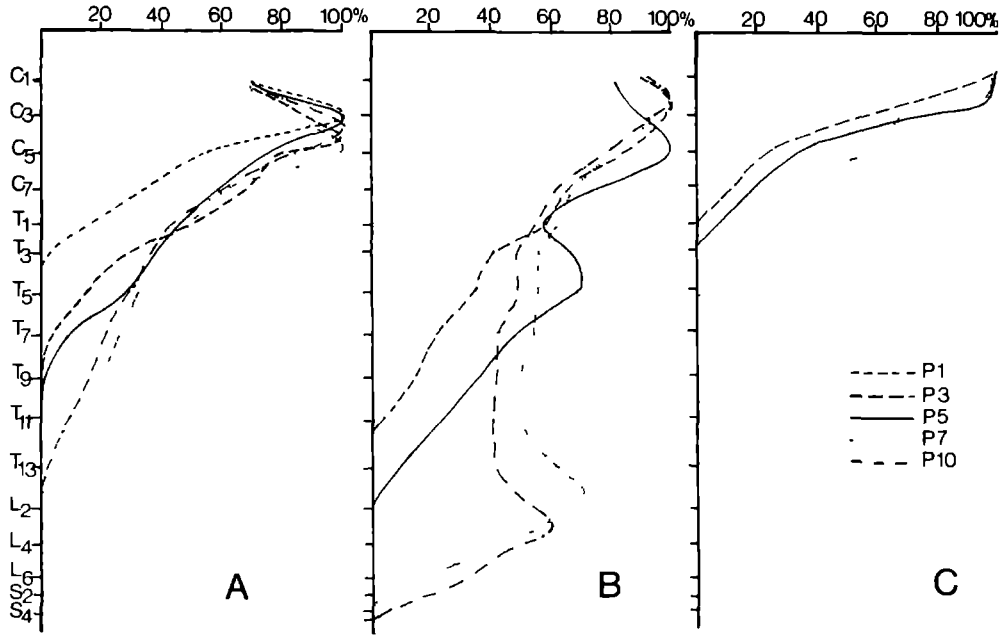
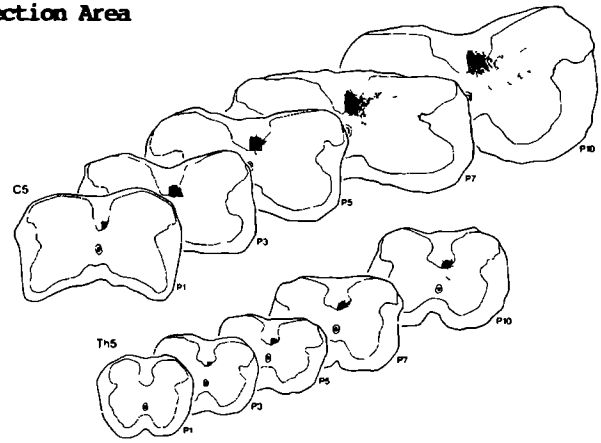


Fig.3 : Composition of labelling distribution charts at different postnatal ages resulting from the quantitative analysis of the amount of WGA-HRP within the fibre bundle. **A** :anterior cortical injection area. **B** :intermediate cortical injection area. C :posterior cortical injection area.

Anterior Cortical Injection Area



Intermediate Cortical Injection Area

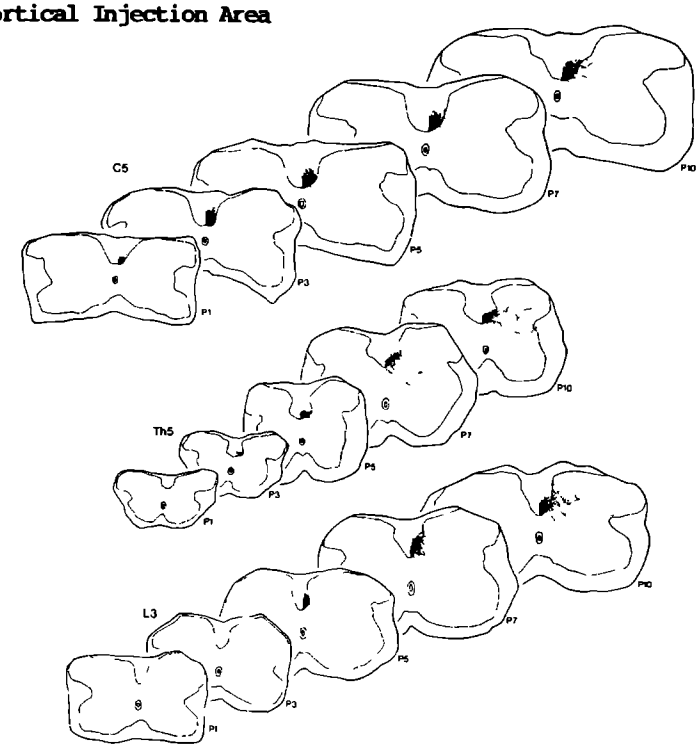


Fig.4 : Camera lucida drawings of transverse sections of the most salient spinal cord segments illustrating the labelled CST axons and their outgrowth into the adjacent spinal gray after injection of the anterior and intermediate cortical areas at different postnatal ages.

axons do not reach beyond L1 implies that a second peak of labeling which arises at the lumbar intumescence between the fifth and tenth postnatal day after labeling the entire left hemisphere (Gribnau et al.,1986), does

not originate from fibres originating in the anterior cortex.

In all cases the most caudal tip of the labeled outgrowing CST only contains a very small amount of WGA-HRP labeling randomly distributed in the ventral part of the dorsal funiculus (Fig. 4).

Up to the tenth postnatal day the outgrowth of the CST fibres into the spinal gray matter is restricted to cervical and thoracic levels (Figs. 4 and 6). The extension of the labeled fibres into the spinal gray matter can be found at an average delay of two days after the arrival of the CST axons at their respective spinal cord segments (Fig. 4). Labeling of the entire left hemisphere (Gribnau et al.,1986) resulted in a similar pattern of outgrowth of labeled CST fibres into cervical and thoracic spinal gray matter.

Intermediate cortical area

Figure 2 shows the caudal extension in the spinal cord of the CST axons after labeling of the intermediate cortical area (see Materials and methods) from P1 through P10. The labeled CST axons extend caudally into the first thoracic (Th1) segment at P1, into the eleventh thoracic (Th11) segment at P3, into the first lumbar (L1) segment at P5, into the first sacral (S1) segment at P7 and into the third sacral (S3) segment at P10.

Comparison of these data with those obtained after labeling of the anterior cortical area clearly shows the much more caudal extension of the CST axons after labeling of the intermediate cortical area during the period analysed (P3-P10) (Fig. 2). The caudal extension of the CST axons after labeling of the intermediate cortical area closely resembles that observed by Gribnau et al.(1986), after labeling of the main part of the cortex of the left hemisphere.

Quantitative analysis of the labeled CST axons after intermediate cortex injections yields following results (Fig. 3), firstly: two peaks of WGA-HRP labeling arise during the development of the CST: one stable peak at the cervical intumescence and one "running" peak (which moves caudally from mid-thoracic levels at P5 to mid-lumbar levels at P10); secondly: the most caudal tip of the outgrowing CST bundle only contains a small amount of WGA-HRP labeling.

At least within the age P1-P10 a "running" peak can only be achieved by labeling the intermediate cortical area and, contrastingly, not by labeling of the anterior cortical area (Fig. 3).

Special attention was focussed on the outgrowth of the CST fibres into the spinal gray matter after labeling the intermediate cortical area. Up to the seventh postnatal day the outgrowth of the CST fibres into the spinal gray matter appeared to be restricted to thoracic and lumbar levels (Figs. 4 and 6).

An optimal separation of the outgrowth fields of the labeled CST fibres into the spinal gray matter after labeling the intermediate and the anterior cortical area exists at P7 (Figs. 4 and 6). At this stage the major spinal gray outgrowth field of the CS neurons of the anterior

cortical area appears to be the cervical intumescence whereas the major spinal gray outgrowth field of the CS neurons of the intermediate cortical area is the lumbar intumescence. However, it is conceivable that, due to a further expansion of the cortex beyond P7, the chosen experimental plan using three cortical injection areas (see Materials and methods) is not suitable to an optimal analysis of the spinal gray outgrowth or projection fields. This aspect will be discussed later.

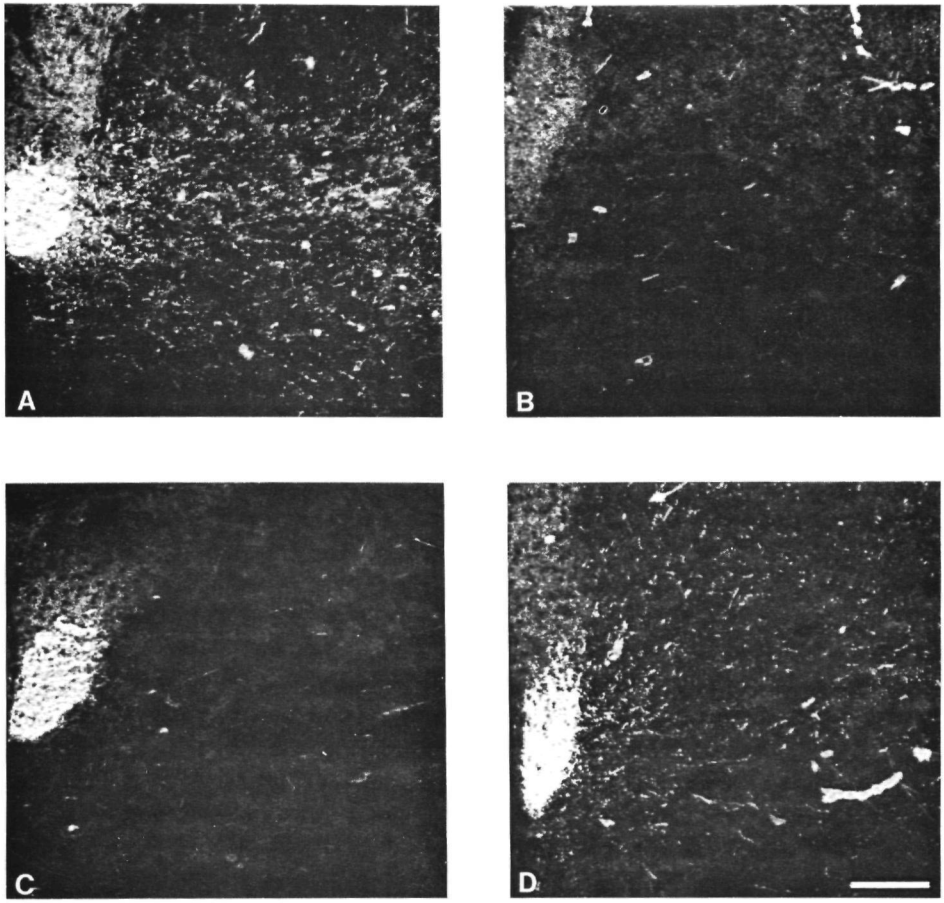


Fig.5 : WGA-HRP labelled corticospinal tracts in transversely sectioned spinal cord segments (dark-field illumination), of P7 animals. After labelling the anterior cortical area **(A,B)** ,extensive outgrowth at the fifth cervical segment (A) and no outgrowth at the second lumbar segment **(B)** was found. On the contrary, after labelling the intermediate cortical area **(C,D)** ,no labelling could be detected in cervical (C_5) spinal cord gray matter **(C)** ,whereas the lumbar (L_3) spinal cord grey matter was extensively labelled **(D)** .Bar= 75 µm.

Posterior injection area

The caudal extension of the labeled CST axons after injection into the posterior part of the cortex is much more limited as compared with

the outgrowth after labelling of the anterior or the intermediate cortical area (Fig. 2). After labelling of the posterior cortical area the labeled CST axons extend caudally into the first cervical segment (C1) at P1; into the eighth cervical (C8) segment at P3; into the fifth thoracic (Th5) segment at P7 and into the first cervical (C1) segment at P10. No labeled CST axons were found at any spinal cord level analysed at P14 (not shown). The transient nature of the corticospinal projection from the occipital cortex during the postnatal development of the rat, as described by Stanfield and O'Leary (1982,1985a,1985b) might be a possible explanation of this phenomenon (see discussion).

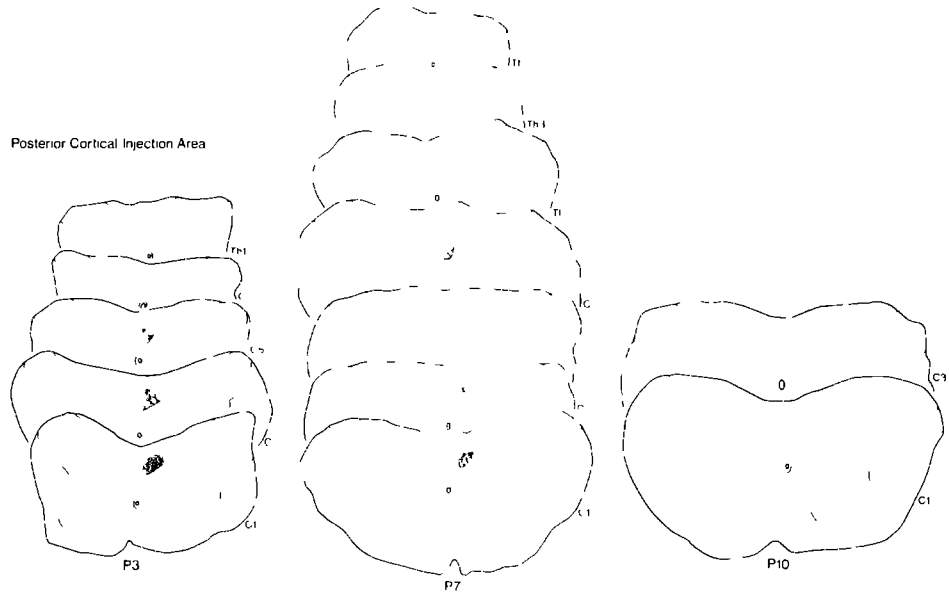


Fig.6 : Camera lucida drawings of transverse sections of spinal cord segments illustrating the caudal extension of the occipital CST axons into the spinal cord white matter. No outgrowth of these axons into the adjacent spinal grey was noted.

The amounts of WGA-HRP label present in the CST at different spinal cord levels at varying developmental ages (Figs. 6 and 7) after labeling the posterior cortical area differ dramatically from those observed after injections into the anterior and intermediate cortical areas, respectively. Firstly, the amount of WGA-HRP label is approximately ten times lower at all spinal cord segments analysed. Secondly, no peaks of labelling could be detected either at the cervical or at the lumbar intumescence. The decrease of the amount of WGA-HRP label from cervical to thoracic levels appears to be nearly linear (Fig. 3C).

As can be seen very clearly in Figs. 6 and 7 no outgrowth of labeled CST fibres into the spinal gray matter could be detected at any spinal cord level at any age after injections into the posterior cortex. This

phenomenon, in combination with the afore mentioned disappearance of all labeled CST axons of any spinal cord level between P7 and P14, possibly implies degeneration of these corticospinal projections (see Discussion).

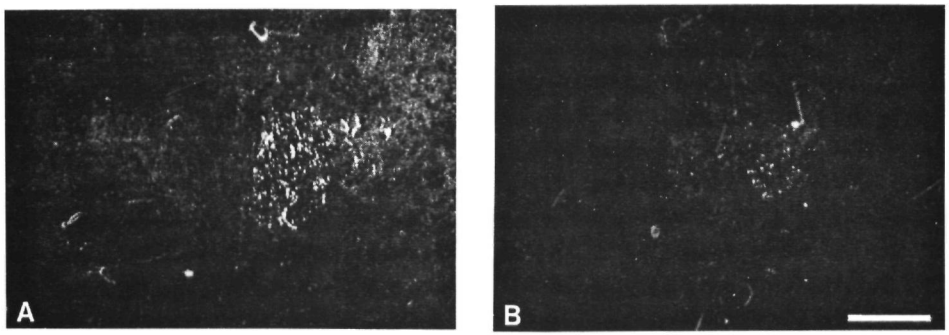


Fig.7 : Transverse sections of WGA-HRP labelled corticospinal axons at P7 (dark-field illumination) after labelling the posterior cortical area. A : second cervical segment. B :eighth cervical segment. Comparision of the quantity of label present with that after labelling the anterior or intermediate cortical area (Fig.5A,5C) illustrates the small amount of WGA-HRP. No outgrowth of labelled CST axons into the adjacent spinal grey could be detected. Bar= 75 µm.

DISCUSSION

The present anterograde tracer study of the developing CST in the rat, using WGA-HRP, demonstrates an obvious relationship between the site of injection in the cortex and the developmental pattern of the labelled CST axons in the spinal cord on the one hand and the outgrowth of their terminal branches into the spinal grey matter on the other hand. Before discussing our results it should be emphasized that we have made the assumption that the cerebral hemispheres expand in an isometrical way during the postnatal period studied (P1-P10), i.e. the rostral injection sites correspond to each other in all stages studied, and the same holds true for the intermediate and caudal injection sites, respectively.

After injecting the anterior cortical area, no labelled CST axons were found at more caudal levels than in spinal cord segment L_1 during the developmental period analysed (Fig.2). Quantitative analysis of the amounts of WGA-HRP within the fibre bundle, after labelling the anterior cortical area revealed the presence of a large stable peak at the cervical intumescence ar all stages analysed (Fig.3). In accordance with this development in the spinal cord white matter, the major spinal grey termination field of these outgrowing CST fibres appears to be the cervical intumescence (Figs.4 and 5). Labelling of the intermediate cortical area during the first postnatal week revealed marked differences as compared with the labelling of the anterior cortical area. The caudal extension of the CST axons after labelling the intermediate cortical area proceeds up to even sacral spinal cord segments S1 at P7 and S3-4 at P10 (Fig.2). Quantitative analysis of the amount of label within the CST at several spinal cord levels after labelling the intermediate cortical area

revealed two peaks: one large stable peak at the cervical intumescence, comparable with that found after labelling the anterior cortical area, and a running peak, which moves caudally from P5 till P10 and then stabilizes at the lumbar intumescence (Fig. 3). The occurrence of a large stable peak at the cervical intumescence after labelling the anterior or the intermediate cortical injection area and a stable peak at the lumbar enlargement after labelling the intermediate cortical injection area may possibly be explained by the branching of CST axons at these levels. The transient presence of a running peak which moves caudally with the front of the outgrowing bundle after labelling the intermediate cortical injection area probably arises by accumulation of tracer within the growth cones at the tip of the outgrowing CST. These statements, however, are based upon a number of presuppositions about different factors (such as the transport rate of the tracer, the varying axon diameter, the branching collaterals, the presence of varicosities, the number of axons and the uptake of tracer at the injection site) which possibly may affect the amount of label present in both the entire bundle and the individual axons, as was discussed upon earlier (Gribnau et al.,1986). Only a combined anterograde HRP tracing and electron microscopic analysis of the developing CST in the rat can provide additional information on these underlying factors.

Up till the seventh postnatal day the chosen experimental plan using three different cortical injection areas was highly suitable since a marked separation was obtained of the respective spinal gray outgrowth patterns (Figs. 4 and 6). However, as a result of differential expansion or rearrangement of the cortex beyond P7 the chosen experimental plan using three cortical injection areas may not be very usefull in getting further segregation of spinal gray termination fields. As can be seen in Figure 4 the spinal gray outgrowth of labeled CST fibres of a P10 animal after injecting the intermediate cortical area occurs, in addition to lumbar and thoracic parts, also in cervical regions. Extensive experiments using a great number of smaller injections especially within the intermediate cortical area might yield additional information about this matter.

Previous studies revealed that reorganizing or focusing of cortical neurons during the first postnatal weeks is a common feature (Stanfield et al.,1982; Adams et al.,1983[a]; Mihailoff et al.,1984; Stanfield and O'Leary,1985[a]). The normal development of the nervous system also encompasses a number of regressive phenomena. In some regions of the central nervous system naturally occuring neuronal death has been well documented (Heumann et al.,1978; Oppenheim,1986). On the other hand the focussing of specific populations of neurons can be explained by the elimination of early formed axon-collaterals. This has not only been demonstrated for neurons whose axons project via the corpus callosum (Innocenti,1981; Ivy and Killacky,1981; O'Leary et al.,1981; Ivy and Killacky,1982; Killacky and Chalupa,1986) but also for corticospinal neurons in the rat and the hamster (Stanfield et al.,1982; Stanfield,

1984; O'Leary and Stanfield,1985; Stanfield and O'Leary,1985[a],1985[b]; O'Leary and Stanfield,1986). The elimination of early-formed corticospinal axon-collaterals was demonstrated for layer V neurons in the occipital part of the cortex (Stanfield et al.,1982; Stanfield,1984; O'Leary and Stanfield,1985; Stanfield and O'Leary,1985[a],1985[b]; O'Leary and Stanfield,1986; Schreyer and Jones,1988[b]).

Our results after labeling the posterior injection area (occipital cortical area) clearly demonstrate a maximal caudal extension of the labeled CST fibres up to midthoracic levels at P7 (Fig. 2). After the first postnatal week these corticospinal projections gradually disappear from spinal cord levels (Fig. 2). Besides, no outgrowth of these transient occipital corticospinal projections into the spinal gray matter could be detected at any developmental stage (Figs. 6 and 7). Both phenomena point to a possible degeneration of these corticospinal axons. The presence of a transient corticospinal projection from the occipital cortex during the postnatal development of the rat is in line with previous results of Mihailoff et al.(1984) after anterograde or Stanfield et al.(1982),Adams et al. (1983[a],1983[b]), Stanfield (1984), Stanfield and O'Leary (1985[a],1985[b]) and O'Leary and Stanfield (1985,1986) after retrograde or combined labeling studies. O'Leary and Stanfield (1985) previously demonstrated that many of the occipital neurons with early corticospinal projections stabilize and maintain permanent axonal connections to the superior colliculus or to the pons. The stabilized projections most likely result from a selective elimination of a transient component represented by the occipital corticospinal collaterals (O'Leary and Stanfield,1985; Stanfield and O'Leary,1985[b]). In addition, the time course of the disappearance of transient occipital corticospinal projections corresponds with an electron microscopically demonstrated reduction of the total number of axons present during the development of the PT in the rat at the third cervical segment (Th. Gorgels, pers. communication). In the hamster the number of PT axons in the medulla oblongata peaks at P7 and declines dramatically up till P14 to about 70% of the number at P7 (Reh and Kalil,1982). The elimination of transient occipital corticospinal projections certainly partially accounts for this decline but a great deal of the reduction might be explained by the axon pruning of neurons that maintain a pyramidal projection (Crandall et al.,1985). We intend to study the possible degeneration of the transient occipital corticospinal projections during the postnatal development in the rat at the ultrastructural level using a modified anterograde HRP-EM technique (Chapter 4). The occipital cortical projections did not show any outgrowth in the spinal cord gray matter. This implies that these projections do not make contact with target cells which may lead to the consecutive degeneration of the axons in question.

In the present study we noted a delay of about two days between the arrival of the bulk of the CST axons at a given spinal cord segment and their outgrowth into the respective spinal gray after labeling the

anterior cortical area. After labeling the intermediate cortical area this waiting period of approximately two days was found only at thoracic and lumbar levels; at cervical spinal cord levels no outgrowth was noted at all even after a waiting period of six days.
The presence of CST fibres within the spinal cord white matter about two days before their entry into the adjacent spinal gray was previously noted in rodents (Wise et al.,1979[a],1979[b]; Martin et al.,1980; Schreyer and Jones,1982,1983; Gribnau et al.,1986). Whether this waiting period can be attributed to the lagging behind of the developmental state of the neurons in the dorsal horn remains to be established.
Besides, the maturation of the layer V cortical neurons projecting to spinal cord levels and especially their positioning in tangential and/or radial dimensions along the cortex (Stanfield,1984), may play a role in the waiting period between the arrival and the outgrowth of the axons at a given spinal cord segment.

The principal findings in this chapter can be summarized as follows: 1. The CST fibres originating in the intermediate cortical area extend much more caudally than the CST fibres originating in the anterior cortical area; 2. The major spinal gray termination field of the anterior CS neurons appears to be the cervical intumesence, whereas the major spinal gray termination field of the intermediate CS neurons is the lumbar intumescence; 3. The axons of the posterior CS neurons attain their most caudal extension in the spinal cord at P7 and then gradually disappear up till P14; these axons do not show any outgrowth into the spinal gray matter at any level or any age.

Acknowledgements

We like to thank Prof.Dr. R. Nieuwenhuys, Dr. E. de Kort, Dr. P. van Mier and Dr. P. Jap for valuable discussion on the data and critical reading of the manuscript. The authors are grateful to Mr. J. de Bekker for preparing some of the drawings and to Miss M. Rieck for her secretarial assistance.

CHAPTER 2C: AN ANTEROGRADE TRACER STUDY ON THE DEVELOPMENT OF CORTICOSPINAL PROJECTIONS FROM THE MEDIAL PREFRONTAL CORTEX IN THE RAT.

SUMMARY

The aim of the present study is to investigate, both qualitatively and quantitatively, the development of corticospinal (CS) projections from the medial prefrontal cortex of the rat. This study was carried out with the use of anterogradely transported wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) after iontophoretic injections in the medial prefrontal cortex. For comparison similar injections were made in the sensorimotor cortex.

The CS axons of neurons situated in the medial prefrontal cortex have reached the first thoracic segment (T1) at postnatal day 3 (P3) and reach their most caudal extension in the spinal cord sixth thoracic segment (T6) at postnatal day 7 (P7) and then gradually disappear during the second postnatal week. Quantitative results revealed that after labelling of the medial prefrontal cortex no peaks in labelling density neither at the cervical nor at the lumbar intumescence were present. Furthermore, the CS axons of medial prefrontal neurons never showed any outgrowth into the spinal grey matter at any age studied. Concludingly, the extension and subsequent elimination of CS axons originating in the medial prefrontal cortex follow a similar time course as those from the occipital cortex (Chapter 2B).

Key words :corticospinal tract - collateral elimination - medial prefrontal cortex - anterograde tracing - rat.

INTRODUCTION

The mammalian corticospinal tract (CST) represents a major descending motor pathway (Armand,1982), with its cell bodies located mainly in the fifth layer of the sensorimotor cortex (Hicks and D'Amato,1975; Wise et al.,1979[a]; Murray and Coulter,1981; Leong,1983; Neafsey et al.,1986).

Using retrogradely transported horseradish peroxidase (HRP) Miller (1987) was able to describe the exact localization of corticospinal (CS) neurons in the adult rat: besides retrogradely labelled CS neurons in areas 4 and 6/8 (motor cortices), medial area 3 and caudal area 2 (somatosensory cortices), less dense concentrations also were observed in occipital, cingulate and prefrontal cortices.

The CS neurons in the visual cortex of the adult rat may represent a remnant of the transient occipitospinal projection that is evident in young postnatal rats (Stanfield et al.,1982;Stanfield and O'Leary,1985[b]). Occipital neurons with early CS projections stabilize and maintain

permanent axonal connections to the superior colliculus or to the pons (O'Leary and Stanfield,1985). These transient occipitospinal projections demonstrate a maximal caudal extension up to mid-thoracic levels at postnatal day 7 (P7), whereas they gradually disappear from spinal cord levels during the second postnatal week (see Chapter 2B).

In the adult rat besides the visual cortex also the medial prefrontal cortex makes a minor contribution to the CST. Within the prefrontal cortex two parts can be distinguished: the medial- and the orbital prefrontal cortex (Krettek and Price,1977; van Eden and Uylings,1985).The medial prefrontal cortex forms the greatest part of the cortex on the medial wall of the frontal pole and caudally extends up to 300 um dorsal to the genu of the corpus callosum. The orbital prefrontal cortex is situated on the dorsal bank of the rostral part of the rhinal sulcus (Krettek and Price,1977; van Eden and Uylings,1985). The prefrontal cortex has its main projections to the caudatoputamen (McGeorge and Faull,1989) complex and the mediodorsal nucleus of the thalamus (Beckstead,1979).

With the use of the retrograde tracer Fast Blue it was demonstrated that also in the orbital parts of the prefrontal cortex neurons are located that sustain a transient CST projection during the first postnatal week (Schreyer and Jones,1988[a]). Furthermore, in the medial part of the prefrontal cortex a substantially larger number of cells was labelled after neonatal CST injections than after similar injections in adulthood. Especially in the ventral regions of the medial cortex, the pre- and infralimbic areas, this decrease in the number of cells was clear. This indicates that most probably also in these areas CST axons are eliminated from their parent cells in the course of development. The present study was undertaken to provide anterograde evidence for the occurrence of transient CS neurons in the medial prefrontal cortex of the rat. In addition the use of the anterograde tracer WGA-HRP enables the determination of the time course of the extension and subsequent retraction of these CST axons both qualitatively and quantitatively.

MATERIALS AND METHODS

In total 29 Wistar rats ranging in age from postnatal day 3 (P3) to postnatal day 14 (P14) were used, in which the day of birth was accounted P0. The ages of the animals given in the present paper are the ages at their respective day of injection. After anaesthetization with sodium pentobarbital (18 mg per kg body weight, i.p.) a single iontophoretic injection of 5% WGA-HRP (Sigma L7017) solution in distilled water was made through a glass micropipet (tip diameter 20-30 µm.) by applying a 5 uA positive current at 5 second intervals, for a period of 7-10 min., in the ventral parts of the medial prefrontal cortex (see Fig.1). The iontophoretic injections were made using a stereotactic procedure adapted for neonatal rats (van Eden and Uylings,1986). After a survival period of 24 hours the animals were reanesthetized and then transcardially perfused, under deep anesthesia, with saline followed by

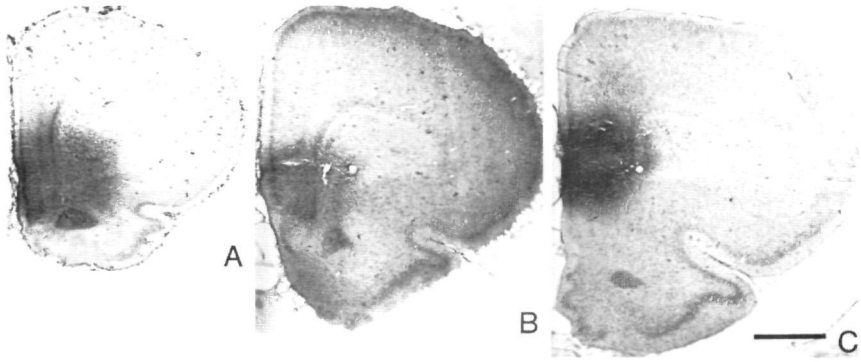


Fig.1 : Photomicrographs of representative examples of WGA-HRP labelling areas in the medial prefrontal cortex at different ages: P3 **(A)** ,P7 **(B)** ,P14 **(C)** . Bar= 1 mm.

1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M. phosphate buffer (pH 7.4) (PB). The brains and spinal cords were removed and postfixed by immersion overnight. After separation of both parts of the brain by cutting immediately caudal to the pyramidal decussation, they were embedded in a gelatin-sucrose mixture (15% gelatin and 20% sucrose in PB), stored overnight in 4% formaldehyde in 20% sucrose PB and washed in PB containing sucrose (PBS). The material then was frozen on dry ice and cut into 30 µm sections in the transverse plane on a freezing microtome. Every fifth (P3 and P7) or sixth (P14) section was immediately incubated with tetramethylbezidine (TMB) as a chromogen, according to Mesulam (1978). Control sections were processed identically but the incubation was carried out without TMB.

For each animal a reconstruction was made of the cortical injection area, including both the heavily labelled injection site and its diffusely labelled surroundings. Apart from the CST fibres WGA-HRP could be taken up by passing fibres which for instance project to the thalamus. These projections did not influence the results at spinal cord levels and therefore were not taken into account in the present investigation.

In each experiment a labelling distribution chart of the CST in the dorsal funiculus was made according to the method as described by Gribnau et al.(1986). In this way a rostrocaudal labelling curve could be deduced for each individual spinal cord. Drawings of the labelling in the spinal cord sections were made under darkfield illumination using a Zeiss microscope equipped with a drawing device.

Photomicrographs from the WGA-HRP labelled injection areas were made with an automatic Zeiss Photomicroscope II under brightfield illumination, using an Agfapan 25 film, whereas the spinal cord sections were photographed using the same equipment under darkfield illumination.

RESULTS

The extension of each injection area was controlled by reconstruction from serial sections of the cortex: within the prefrontal cortex the pre- and infralimbic areas were labelled as well as a part of the dorsal

anterior cingulate cortex. Representative sections of WGA-HRP injection areas in the medial prefrontal cortex at P3, P7 and P14 are shown in Fig.1. Based on the cytoarchitectonic characteristics of developing prefrontal cortex areas in the rat (van Eden and Uylings,1985) the medial prefrontal injection areas are conceived as correspondent for the ages studied. The same holds true for the sensorimotor injection areas.

After labelling the medial prefrontal cortex CST axons extend into the dorsal funiculus of the first thoracic segment (T1) at P3 and of the sixth thoracic segment (T6) at P7 (Fig.2,4A). No labelled CS axons originating in the medial prefrontal cortex were found either in the dorsal funiculus or in the spinal gray at any spinal cord level analyzed at P14. CST fibres with their originating neurons in the sensorimotor cortex extend much more caudally into the dorsal funiculus: the tenth thoracic segment (T10) at P3; the first sacral segment (S1) at P7 and up to the lowest sacral segments (S3/S4) at P14 (Fig.2,4A).

No outgrowth of labelled CST axons could be noted into the spinal cord gray matter at any spinal cord level at any age studied after medial prefrontal cortex injections (Figs.2,3A-3C). After labelling the sensorimotor cortex labelled spinal projections were present both at cervical (Fig.3D) and at lumbar levels at least two days after arrival of the first CST fibres at a given spinal cord segment.

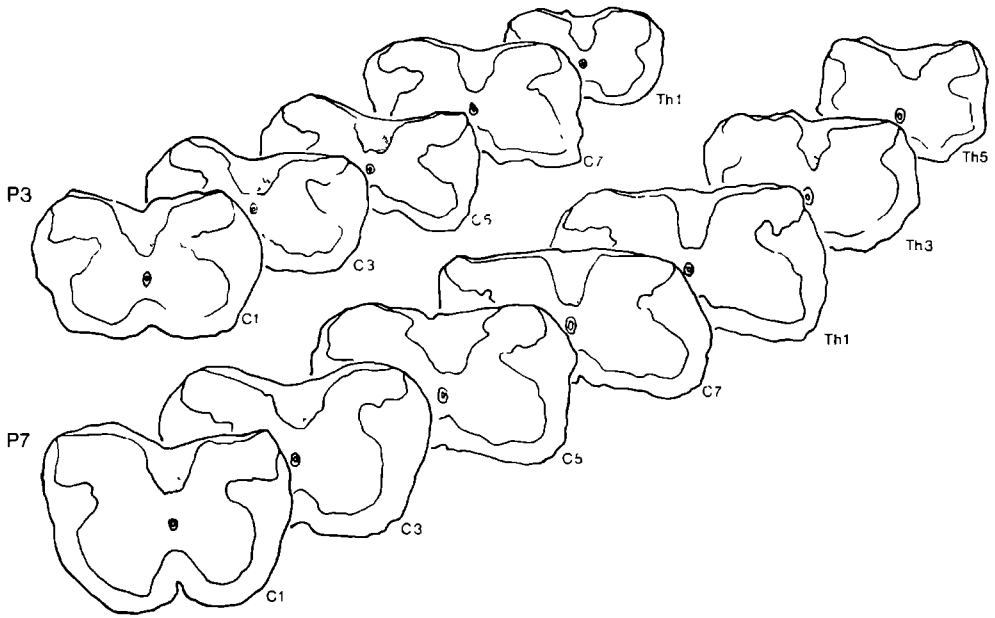


Fig.2 : Camera lucida drawings of transverse sections of spinal cord segments after iontophoretic WGA-HRP injection in the medial prefrontal cortex. No outgrowth of these axons into the adjacent spinal grey matter at any level either at P3 or at P7 can be noted.

A quantitative analysis of the amount of WGA-HRP label present in the most ventral part of the dorsal funiculus at different spinal cord levels shows remarkable differences after injections in the medial prefrontal cortex as compared with the sensorimotor cortex (Figs.4B1,4B2).
After labelling the medial prefrontal cortex a more or less gradual decrease in the amount of WGA-HRP can be noted from cervical to thoracic levels at P3 as well as at P7 (Fig.4B1). Contrastingly, after comparable injections in the sensorimotor cortex area two peaks in labelling density were found: a standing one at the cervical and a running one, which moves caudally to the lumbar intumescence between P3 and P14 (Fig.4B2), as was also noted earlier (Gribnau et al.,1986; Joosten et al.,1987[b]). Furthermore the amount of WGA-HRP label is much smaller after medial prefrontal cortex injections than after sensorimotor cortex labelling in all spinal cord sections observed (e.g. Figs.3B and 3D).

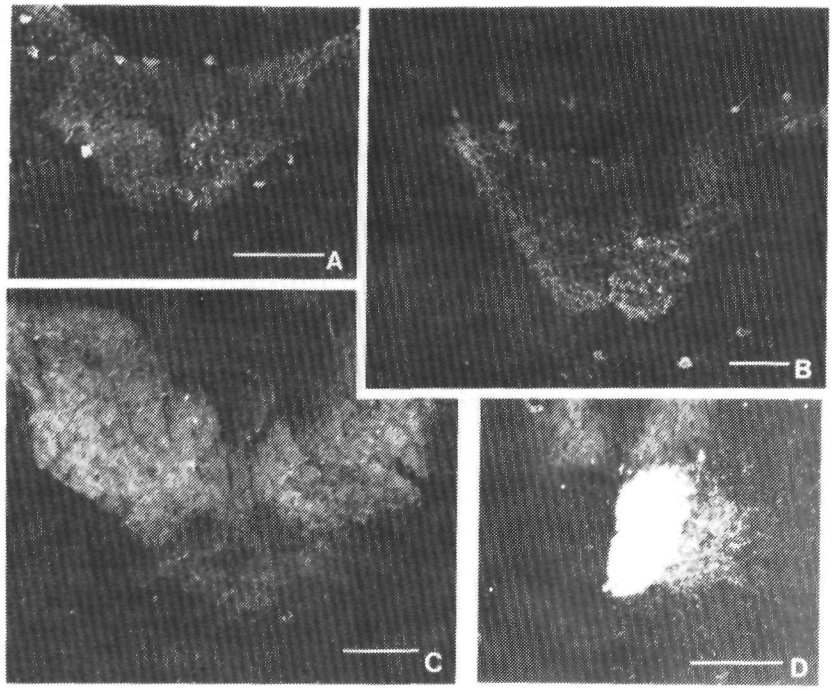


Fig.3 : Dark-field illuminated photomicrographs of WGA-HRP labelled corticospinal fibres in transversally sectioned spinal cord segments of the cervical intumescence (C5-C6). After medial prefrontal cortex labelling at P3 **(A)** ,P7 **(B)** or P14 **(C)** no outgrowth in the spinal grey matter can be noted. After sensorimotor cortex labelling at P7 **(D)** outgrowing labelled CS fibres in the spinal grey can be visualized. Bar = 100 µm.

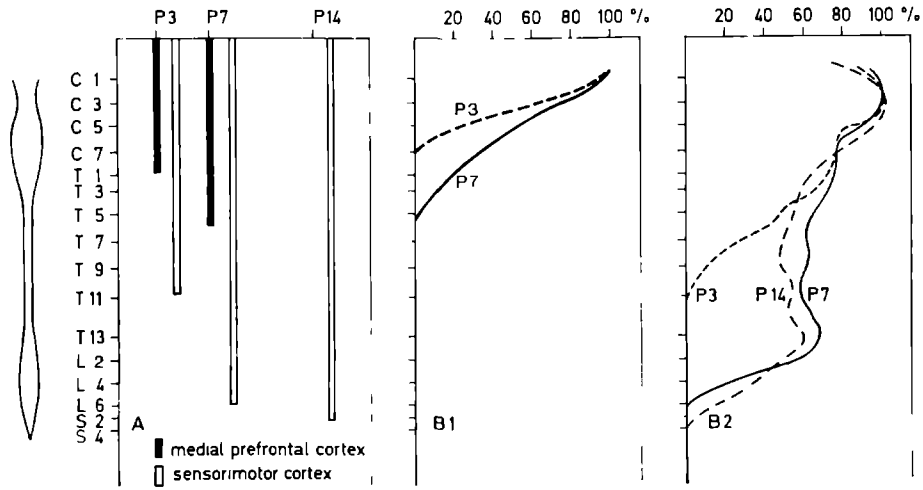


Fig.4 : Caudal extension of WGA-HRP labelled CST axons after iontophoretic injections in medial prefrontal or sensorimotor cortex **(A)** .Labelling distribution charts at different postnatal ages: after medial prefrontal cortex injection **(B1)** ;after sensorimotor cortex injection **(B2)** .

DISCUSSION

From our present results it can be concluded that: (1) the axons of medial prefrontal CS neurons reach their most caudal extension in the spinal cord (T6) at P7 and then can not be labelled at the end of the second postnatal week; (2) quantitative analysis of the amount of WGA-HRP label along the length CST after injecting the medial prefrontal cortex reveals a gradual rostro-caudal decrease; (3) the transient projections from the medial prefrontal cortex do not show any outgrowth into the spinal grey at any level at any age studied.

The transiency of CS projections from medial prefrontal cortex areas in young rats as presented in this anterograde tracer study is in line with retrograde tracer experiments using the fluorescent tracer Fast Blue in which a vast reduction in the number of labelled cells in the medial prefrontal cortex was demonstrated after injections in the cervical intumescence in the adult rats as compared with neonatal rats (preliminary results). However, a minority of the retrogradely labelled cells in the medial prefrontal cortex maintains their projections to cervical and thoracic spinal cord levels in the adult (Miller,1987). The CS neurons in the adult medial prefrontal cortex obviously represent a remnant of the numerous transient ones in young postnatal rats. In the present study, however, no CST fibres could be labelled at P14. The discrepancy between the results obtained after anterograde WGA-HRP and retrograde Fast-Blue tracing may be attributed to a lesser sensitivity of the former method and/or the uneven distribution of WGA-HRP-TMB crystals along the length of CS fibres (Joosten et al.,1987b).

Interestingly, the adult rat CST contains myelinated as well as unmyelinated axons, as was demonstrated by an ultrastructural anterograde HRP study (Joosten et al.,1987a; Joosten and Gribnau,1988). The CS axons originating in the medial prefrontal cortex may account for the unmyelinated profiles because physiological measurements on pyramidal tract neurons in the sensorimotor cortex always are in the range to be expected for myelinated axons (Takahashi,1965; Mediratta and Nicoll,1983)

The focussing of specific populations of neurons during normal development of the central nervous system can be explained either by naturally occuring cell death (Oppenheim,1986; Williams et al.,1986; Schreyer and Jones,1988a), or by the elimination of early-formed axon collaterals (Innocenti,1981; Stanfield,1984). The reorganization of cortical neurons during the first postnatal weeks is a general feature (Stanfield et al.,1982; Adams,1983a; Leong,1983; Mihailoff et al.,1984; Joosten et al.,1987b); the focussing of layer V CS neurons which are originally spread over the sensorimotor and occipital part of the cortex into the sensorimotor cortex of the rat and hamster was convincingly attributed to the elimination of early formed CS axon collaterals in double labelling experiments (Stanfield,1984; O'Leary and Stanfield, 1985,1986; Schreyer and Jones,1988a,1988b). Using anterograde labelling Joosten et al.(1987b) demonstrated a maximal caudal extension of occipital CST axons up to mid-thoracic levels at P7. These CS fibres gradually disappear from spinal cord levels during the second postnatal week. Besides, no outgrowth of the transient occipital CS projections into the spinal grey was detected (see Chapter 2B). Our present results demonstrate that the extension and subsequent elimination of CS axons from medial prefrontal neurons follow a similar time course as those from the occipital cortex.

The quantitative analysis of the amount of label along the length of the CST after medial prefrontal cortex injections shows a remarkable identical rostro-caudal distribution pattern as compared to that obtained after occipital cortex injections (Joosten et al.,1987b). In both cases no peaks of labelling could be observed either at the cervical or at the lumbar intumescence, but an almost linear decrease in the amount of WGA-HRP from cervical to thoracic levels was found. Contrastingly, two WGA-HRP labelling peaks can be observed after sensorimotor cortex labelling (Fig.4B2). These peaks presumably are related to the major spinal gray projection areas (Gribnau et al.,1986; Joosten et al.,1987b). It must be noted that the quantitative data are based on the assumption that the amount of label is related to the number of axons. The underlying aspects of this assumption were discussed in Capter 2B. The time course of the disappearance of the medial prefrontal as well as the occipital CS fibres is substantiated in a quantitative electron microscopic study on the number of axons present during the postnatal development of the rat pyramidal tract at the third cervical level

(Gorgels et al.,1989[a]), in which a gradual reduction of the total number of pyramidal tract axons during the second postnatal week was found.

From the present investigation it can be concluded that CS collateral elimination is a common feature of prefrontal and occipital cortical neurons and therefore is a general phenomenon in the development of the rat cortex.

Acknowledgements

The authors would like to thank Jos Dederen for technical assistance. We are grateful to Dr.A.Gribnau for critical reading of the manuscript.

Chapter 3

Corticospinal growth cones in the developing rat lumbar spinal cord

CHAPTER 3 :CORTICOSPINAL GROWTH CONES IN THE DEVELOPING RAT LUMBAR SPINAL CORD.

SUMMARY

In the present investigation we demonstrate the light microscopic characteristics of corticospinal growth cones in the lumbar spinal cord of the rat. Corticospinal growth cones were anterogradely labelled with horseradish-peroxidase (HRP) by application to the sensorimotor cortex of the left cerebral hemisphere. After 24 hours survival time the tracer was visualized by a new combination of HRP staining and intensification techniques, which in essence consists of three steps: 1. a tetramethylbenzidine (TMB) - ammoniumheptamolybdate (AHM) reaction, 2. a diaminobenzidine (DAB) - Nickel (Ni) stabilization, and 3. a glucose-oxidase intensification.

In the rat the first axons of the corticospinal tract, which is located in the ventralmost part of the dorsal funiculus, arrive at the lumbar intumescence at postnatal day 5 (P5). The growth cones of these pioneer axons do not exhibit a preferred alignment either at the periphery or at the centre of the prospective corticospinal area. Although the pioneer growth cone morphology varies considerably, four main categories were discerned: growth cones with fusiform, with clavate, with filopodial only, and with filopodial and/or lamellipodial endings.

The first corticospinal axons penetrating the termination area, being the lumbar grey matter, can be observed after a 'waiting' period of two days after the arrival of the pioneer axons, i.e. at postnatal day 7 (P7). These axons invading the target region of the corticospinal tract display considerably more elaborate growth cones as compared with those of the pioneer axons in the tract area. The growth cones invading the termination area are invariably characterized by the presence of numerous filopodial- and spiny extensions as well as a string of varicosities.

At least some of the corticospinal projections to the lumbar spinal grey arise from directly deflecting axons upon arrival at the level concerned. The entrance of the latter axons into the lumbar spinal grey occurs at an angle varying between 130 and 170 degrees; no acute angles were observed.

This study demonstrates that the configuration of the growth cones of outgrowing corticospinal axons varies with their position along their course, i.e. within the pathway, the spinal cord white matter, or within the target area, the spinal cord gray matter. The variations in growth cone morphology are discussed in the light of corticospinal outgrowth and guidance mechanisms.

Key words : Growth cones - morphology - corticospinal tract - guidance - outgrowth - rat.

INTRODUCTION

Ramon y Cajal (1890) first described the growth cone (GC) as an irregularly shaped enlargement at the tip of an outgrowing axon. The GC is present only during a short period of the neuron's life between birth and synaptogenesis and is responsible for directing the elongating neurite towards the target area, the branching as well as the subsequent recognition of the target neuron during synaptogenesis (for reviews see Johnston and Wessels,1980; Letourneau,1982; Lockerbie,1987). The GC can be identified as the focus of axonal growth (Landis,1983; Lockerbie,1987; Lasek and Katz,1987). The pathfinding of this thickening at the most distal end of a growing axon requires a substratum with which it necessarily interacts (e.g. Chapter 5).

In vitro experiments revealed the amoeboid motility of the GC (Kapfhammer and Raper,1987). In growth cones emission and retraction of fine projections (filopodia) as well as undulating membranes (veils, lamellipodia) was recorded using time-lapse video-enhanced microscopy in vitro (Burmeister and Goldberg,1988) as well as in vivo (Harris et al.,1987).

In vitro experiments on both vertebrate and invertebrate embryos demonstrated the type-specific response of GC's to different features of the environment (Letourneau,1982; Haydon et al.,1984;1985; Bray et al., 1987;Kapfhammer and Raper,1987). It was demonstrated that GC's exhibit different adhesive strengths between filopodia and the substratum (Letourneau,1982; Bray et al.,1987): strong adhesion pulls the GC forward towards a more adhesive substratum whereas if adhesion is weak the filopodial membrane will break its adhesive bonds and the filopodium is withdrawn. Hence, in vitro the direction and rate of axonal growth is correlated with varying substrates: GC's are supposed to be guided by local variations in the relative adhesiveness of its surrounding tissue to reach their targets (Letourneau,1975,1979,1982; Nardi,1983; Berlot and Goodman,1984). On the other hand, observations on developing chick embryonic nerve explants suggest the involvement of cues with an inhibitory action: the motility of particular GC's is shut down on contact with specific axons, which subsequently results in a total collapse of the GC (Kapfhammer and Raper,1987).

During the last decade it has become obvious that also in vivo GC's respond to the varying environment along their course in invertebrates (Goodman et al.,1984; Bastiani and Goodman,1984) as well as in vertebrates (Mason,1982,1985; Bovolenta and Mason,1987; Nordlander,1987). However, besides GC guidance by the relative adhesiveness of the tissue, various other mechanisms of axonal guidance have been described. For instance, GC's may be guided by the mechanism of stereotropism. This mechanism includes the formation of oriented extracellular spaces or channels prior to axonal outgrowth:"the blueprint hypothesis"(Singer et al.,1979; Silver and Robb,1979; Krayanek and Goldberg,1981).Furthermore axons might be guided by gradients of diffusable signals emanating from an axon's target. Up till now the trophic influence of the

protein nerve growth factor, NGF, (Levi-Montalcini and Booker,1960), although mainly restricted to the vertebrate peripheral nervous system, is the most clear example of the chemotropic guidance of outgrowing axons (Mensini-Chen et al.,1978; Gundersen and Barrett,1979).

Since GC-morphology is influenced by the environment the examination of GC morphology during pathway selection might reveal some features about their surroundings and/or the GC guidance mechanism (Roberts and Taylor,1983; Holley and Silver,1987; Bovolenta and Mason,1987; among others). Furthermore, the observation of GC morphology might disclose the way GC's branch in vivo (Bovolenta and Mason,1987; Harris et al.,1987; O'Leary and Terashima,1988). In vitro branching occurs by exertion of tensions along the lateral margins of the GC body resulting in GC bifurcation (Letourneau,1985). In vivo experiments with the fluorescent dye DiI demonstrated that terminal branches are formed by back branching rather than by bifurcation of leading growth cones as was found in the optic tectum of Xenopus (Harris et al.,1987) and the corticopontine projection in rats (O'Leary and Terashima,1988).

The corticospinal tract (CST) in the rat represents a good model to study the development of a long fibre system in mammals because of its postnatal outgrowth throughout the spinal cord as well as its convenient experimental accessibility (Schreyer and Jones,1982; Reh and Kalil,1982; Gribnau et al.,1986).

The rat CST is characterized by a staggered mode of outgrowth (Gribnau et al.,1986) in which pioneer axons reach cervical spinal cord levels at birth (P0), mid-thoracic spinal cord levels at postnatal day 2 (P2) and the lumbar enlargement at postnatal day 5 (P5) (Chapter 4B). Between the arrival of CST pioneer axons at a given spinal cord segment and their extension in the respective spinal gray matter a delay was noted of about two days (Donatelle,1977; Wise et al.,1979[a]; Schreyer and Jones,1982; Gribnau et al .,1986).

Our working hypothesis is that the shape of the CST GC's in the white and the grey matter of the spinal cord at least mirrors the interactions with the surrounding microenvironment. CST GC's were anterogradely labelled using horseradish-peroxidase (HRP) and subsequently visualized with a new combination of HRP-staining techniques. The variations in GC morphology as well as the way of outgrowth from white into lumbar grey matter are discussed in the light of CST outgrowth and guidance mechanisms.

MATERIALS AND METHODS

Twenty newborn Wistar rats aged postnatal days 5 (P5) or 7 (P7) were used. The day of birth was accounted as P0, whereas the age of the animals given in the present paper are always the ages at their respective days of HRP injection.

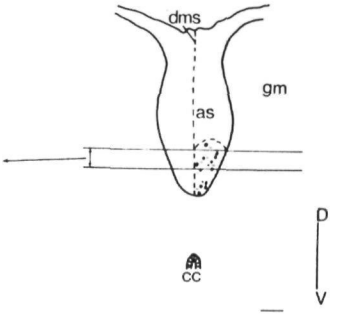
Rat pups were anaesthetized with sodiumpentobarbital (18 mg per kg b.w.). They were then held in a stereotactic apparatus, the skin over the skull was incised and with the use of a fine needle small holes were drilled into the skull and the underlying cerebral cortex. The entire sensorimotor and frontal cortex of the left hemisphere was labelled by implantation of three HRP-gels (Griffin et al.,1979). The pups were allowed to survive for twenty-four hours.

After a second injection of anaesthetic the animals were perfused transcardially with 30 ml 5% sucrose in 0.1 M phosphate buffer (PB), pH 7.2, followed by 40 ml 1% paraformaldehyde and 2% glutaraldehyde in the same buffer. After perfusion the brains and spinal cords were removed Thereafter, approximately 1 mm. thick transverse blocks were cut through the second and third lumbar spinal cord segment (L2-L3). Using a vibratome (Oxford Instruments) 50 µm horizontal and transverse sections were cut and collected in cold PB.

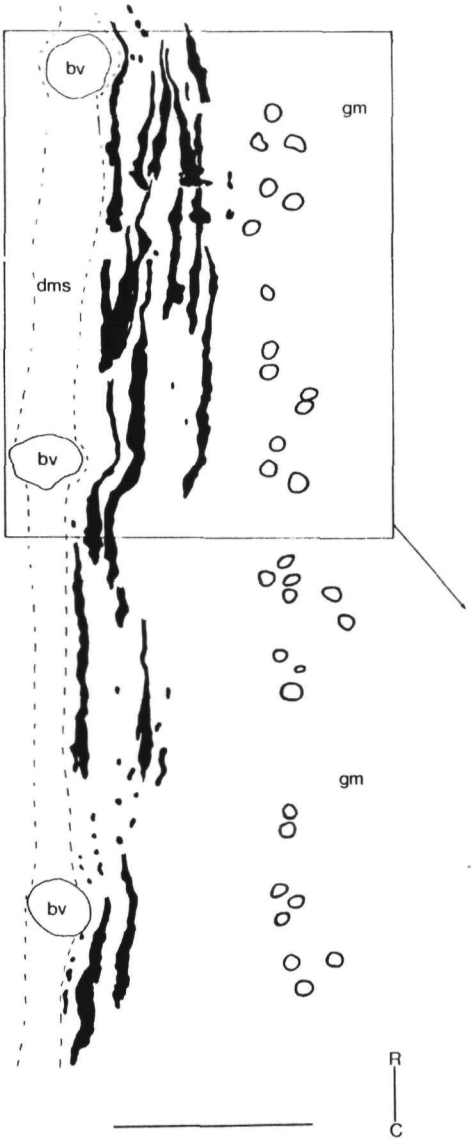
A very intense HRP staining was obtained after execution of the following procedure: the vibratome sections were first incubated with tetramethylbenzidine (TMB) as a chromogen and ammoniumheptamolybdate (AHM) as a stabilizing agent (Olucha et al.,1985). After rinsing twice in 0.1 M PB, pH 7.4, the HRP-TMB-(AHM) crystals were prevented from being washed out by an additional diaminobenzidine (DAB) incubation with the use of the intensifying agent Nickel (Ni) (Adams, 1981; Dederen and Joosten,1989) in 0.05 M Tris-buffer, pH 7.6. The latter reaction was terminated by rinsing the sections in two 5 min. baths of 0.1 M PB. In order to obtain an adequate light microscopic visualization of anterogradely HRP labelled CST growth cones the sections were subsequently incubated using a modified DAB-Cobalt Glucosidase (DAB-Co-GOD) reaction (Itoh et al.,1979; Oldfield et al.,1983). The DAB-Co-GOD reaction was carried out according to the following procedure: After two rinses in 0.1 M Tris-buffer, pH 7.6, the vibratome sections were incubated in 0.5% $CoCl_2$ in the same buffer for 10 min. After another 3-5 min rinses in the same Tris-buffer, and 0.1 M PB, pH 7.4, the vibratome sections were incubated in a mixture of 50 mg DAB, 200 mg ß-D-(+)-glucose 40 mg NH_4Cl and 0.3 mg glucose-oxodase in 100 ml PB, pH 7.4, for 1 hour at 37°C. Then, after two rinses in the same phosphate buffer sections were mounted on albumin-coated slides, dehydrated in alcohol, cleared in xylene and coverslipped with Depex.

Drawings of over more than 200 growth cone profiles were made using a Zeiss microscope equipped with a drawing device and 100x-oil immersion objective. To enhance the contrast a blue filter (Wratten 80 Å) was used. All photomicrographs were made with an automatic Zeiss-photo-microscope II using bright-field illumination and an Agfapan 25 film.

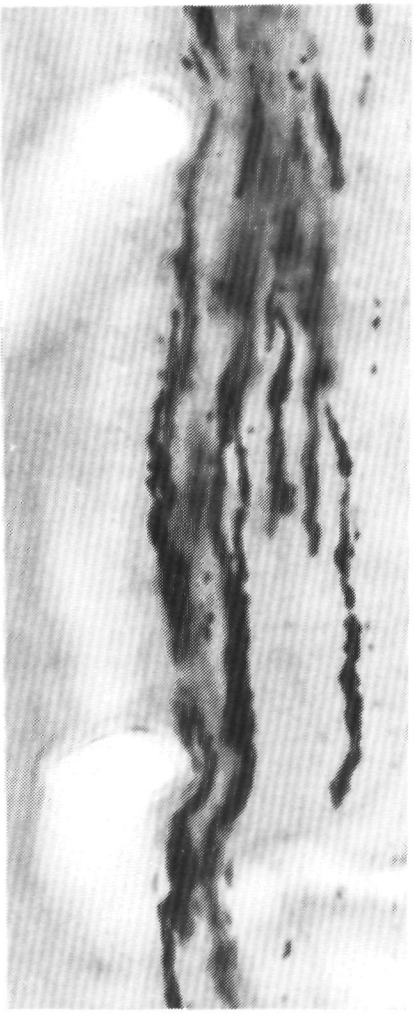
Figure 1 : 1A: Camera lucida drawing of the dorsal funiculus in the lumbar intumescence at P5. HRP-labelled CST pioneer axons are situated in the ventralmost part. AS=Ascending Systems; CC=Central Canal; DMS= Dorsal Median Septum; GM= Grey Matter. Bar= 50 µm.
1B: Camera lucida drawing of a horizontal section of the ventralmost part of the dorsal funiculus at P5. Note the variety of HRP-filled CST growth cone morphologies. Particularly near blood vessels (bv) the pioneer GC's show an undulatory aspect. Bar= 50 µm.
1C: Photomicrograph of part of Fig.1B. Bar= 25 µm.



A



B



RESULTS

The position of CST growth cones in the lumbar tract and target area

At lumbar spinal cord levels labelled growth cones can be observed within the ventralmost part of the dorsal funiculus at postnatal day 5 (P5). No preferred alignment of the labelled growth cones in the presumptive CST area was found either at the periphery or at the centre (Fig.1A). Although, in the tract area pioneer growth cones extend rather straightforward to more caudal spinal cord levels, they frequently show an undulatory aspect, particularly near blood vessels (Fig.1B,1C).

The staggered mode of outgrowth of the developing CST in the rat in which a small number of pioneer fibres takes the lead and additional fibres are successively added is illustrated in Figs.1A-1C and 3.

Two days after the entrance of the labelled outgrowing CST tip at the lumbar intumescence (at P5) the presence of the mass of axons is noted (at P7) (Fig.3).

As can be deduced from Fig.3 it is not possible to identify or to study the morphology of the growing tips of the later arriving CST fibres in the dorsal funiculus, at least within our experimental approach.

The first CST axons penetrating the lumbar grey matter can be observed at P7 (as visualized in Fig.3). Between the arrival of the first CST fibres at a particular spinal cord level and their extension into the adjacent spinal grey a 'waiting' period of about two days is noted.
With respect to the CST fibres situated in the dorsal funiculus the entrance of the labelled axons into the lumbar spinal grey occurs at an angle varying between 130 and 170 degrees (Fig.3). At this stage no growth cones orientated at a right angle to the CST in the lumbar dorsal funiculus were found in the lumbar spinal grey.

CST growth cone morphology

Pioneer axons within the CST lumbar pathway

The morphologies of labelled growth cones in the lumbar spinal cord situated within the confines of the pathway at postnatal day 5 were analysed. The length of CST pioneer growth cones generally approximated 30 - 50 µm, whereas they even might extend up to 70 µm. Between all growing tips of the pioneer fibres a very wide variation in morphology could be observed (Fig.2). In the variations observed four main categories could be discerned although intermediate forms did exist. Because of their slowly broadening tips without distinguishable swellings at their ends the first category of CST pioneer growth cones is called fusiform (Fig.2A). Sometimes, very small, stubby extensions can be observed at their leading tips (Fig.2A'). Fusiform growth cones in particular were large: their minimum length was 50 µm, whereas they

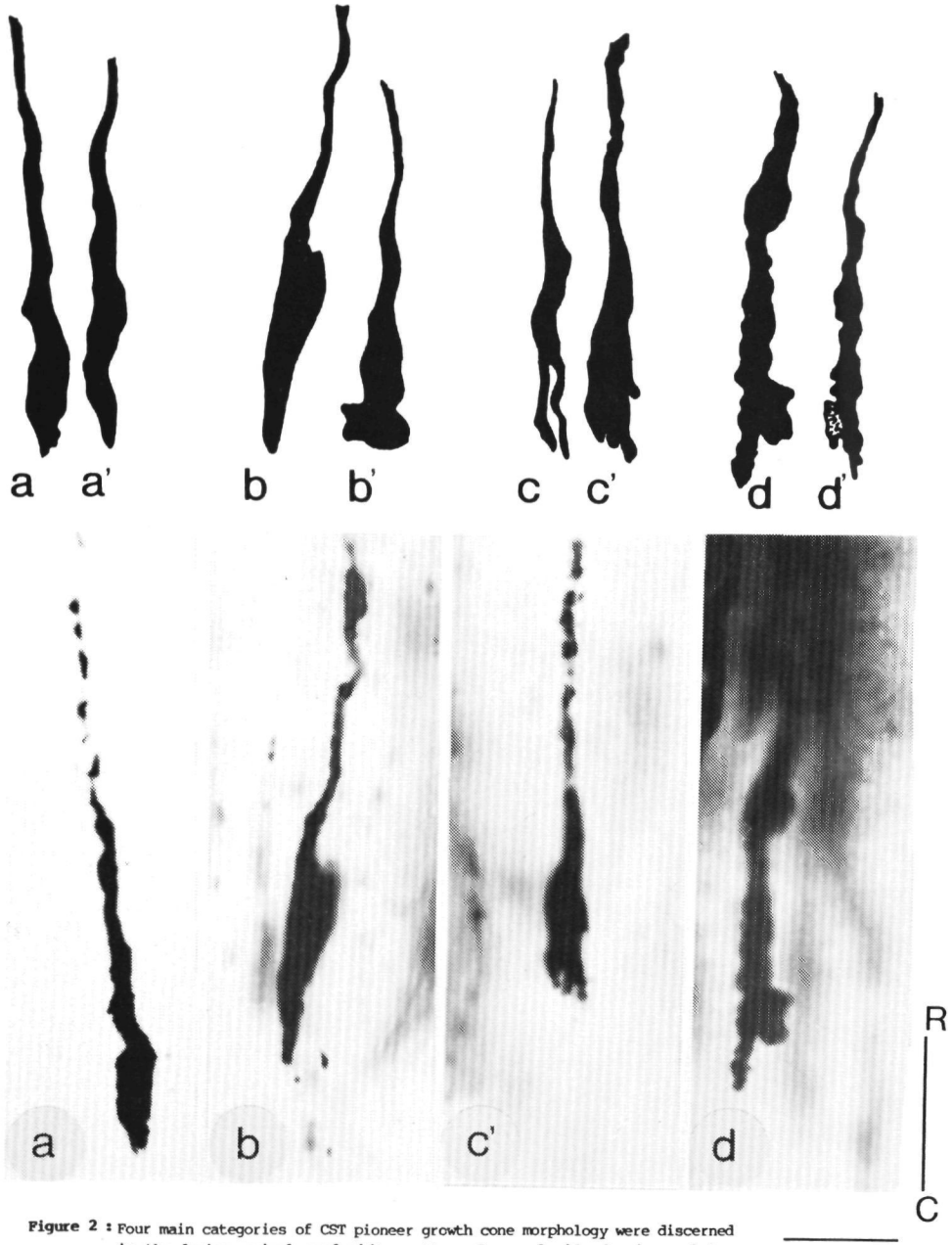


Figure 2 : Four main categories of CST pioneer growth cone morphology were discerned in the lumbar spinal cord white matter. Camera lucida drawings of two representatives out of each category are shown, whereas one of each is further illustrated by a photomicrograph at a magnification of 1000x. **Fig.2A:** fusiform type; **Fig.2B:** clavate type; **Fig.2C:** filopodial type; **Fig.2D:** lamellipodial type. Bar= 20 µm.

maximally reach up to 70 um (Fig. 1B,1C,2A).

The second group of the clavate growth cones were characterized by their bulbous irregularities at the tip (Fig.2B).

Other growth cones were more complex and showed fingerlike extensions (filopodia) at their distal ends (Fig.2C). These filopodia were usually aligned parallel to the direction of axonal growth and vary in length considerably: they sometimes extend for 15 µm (Fig.2C). The number of filopodial extensions per growth cone varied between one and four (Fig.2C').

The fourth type showed thin veil-like extensions (ruffled membranes) or lamellipodia (Fig.2D). Besides lamellipodia this type of growth cones displayed filopodia which only extend to a maximum of 10 µm.

Pioneer axons entering the CST lumbar target area

Within the lumbar spinal cord the CST axons invading the spinal grey displayed considerably more elaborate growth cones as compared to the pioneer axons in the tract area (e.g. Figs. 4 and 5). Besides, the length of the body plus the extensions of the former are smaller: namely ranging from 10 to 40 um. Growth cones with a length of 50 um or more, as were

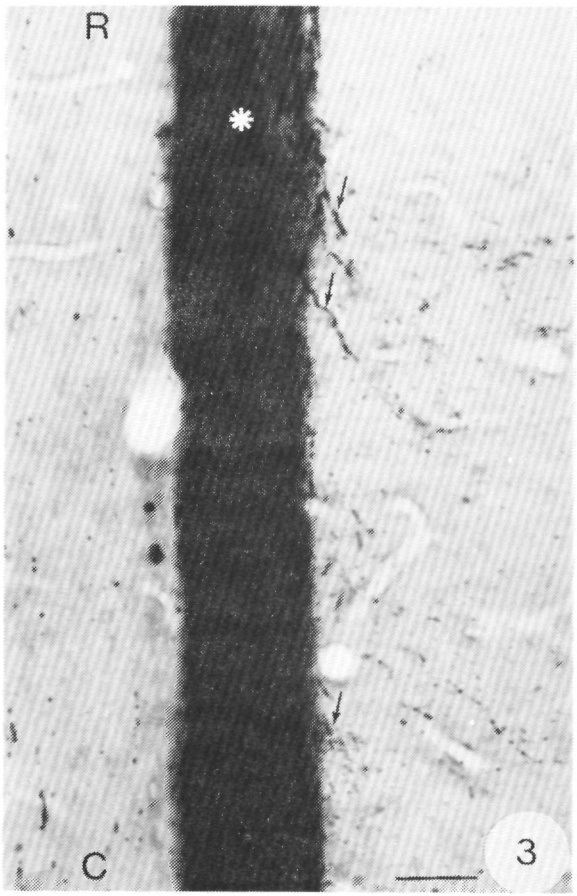


Figure 3 :

Photomicrograph of a horizontal vibratome section at lumbar spinal cord at P7. The CST tract area is heavily labelled because of the presence of the bulk of later arriving CST axons, whereas the first CST axons can be noted penetrating the lumbar grey at an an angle varying between 130 and 170° (arrows). R= rostral;C=caudal

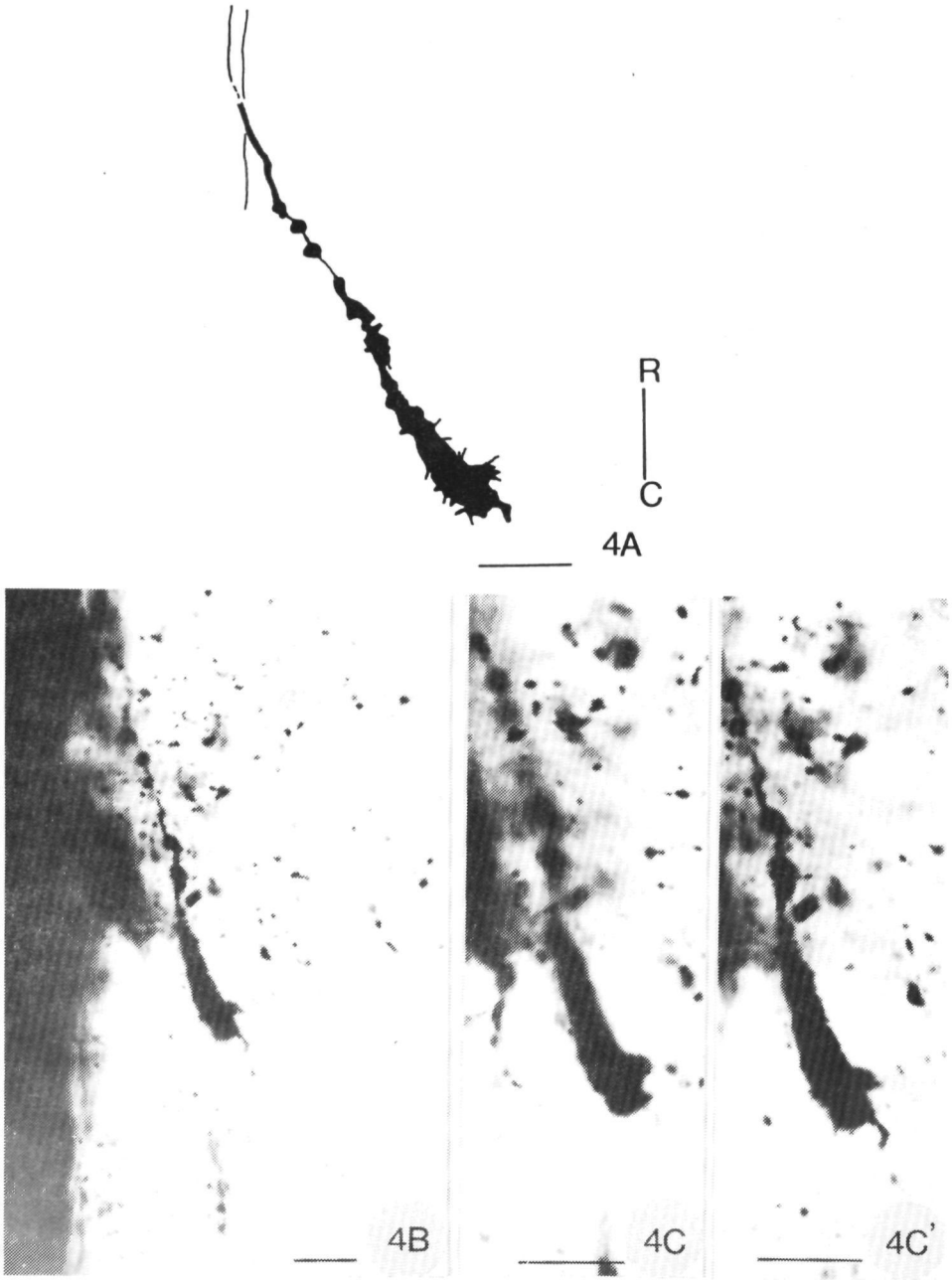


Figure 4 : CST axon invading the lumbar spinal grey at P7. **Fig.4A:** Camera lucida drawing of the growth cone. Note its fingerlike extensions and spiny protrusions. **Fig.4B:** corresponding photomicrograph. **Fig.4C,4C':** photomicrographs at higher magnification at two different phocal planes. Bar= 20 µm.

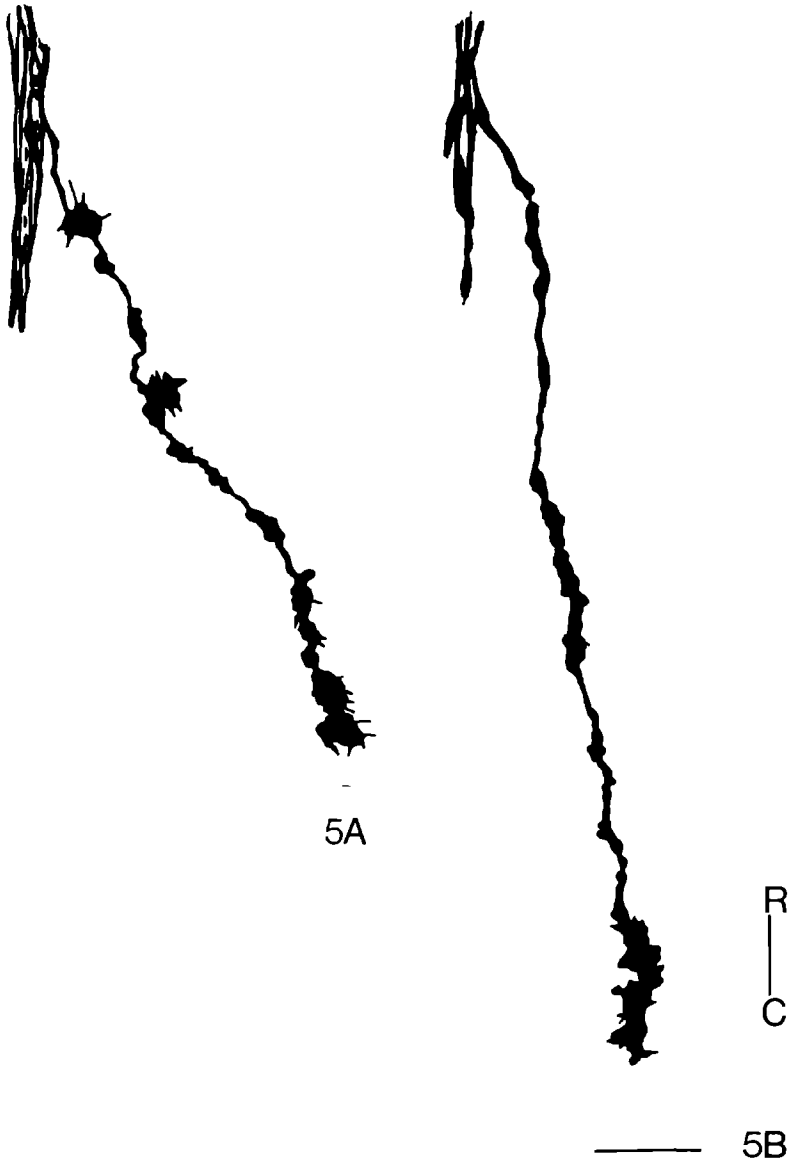
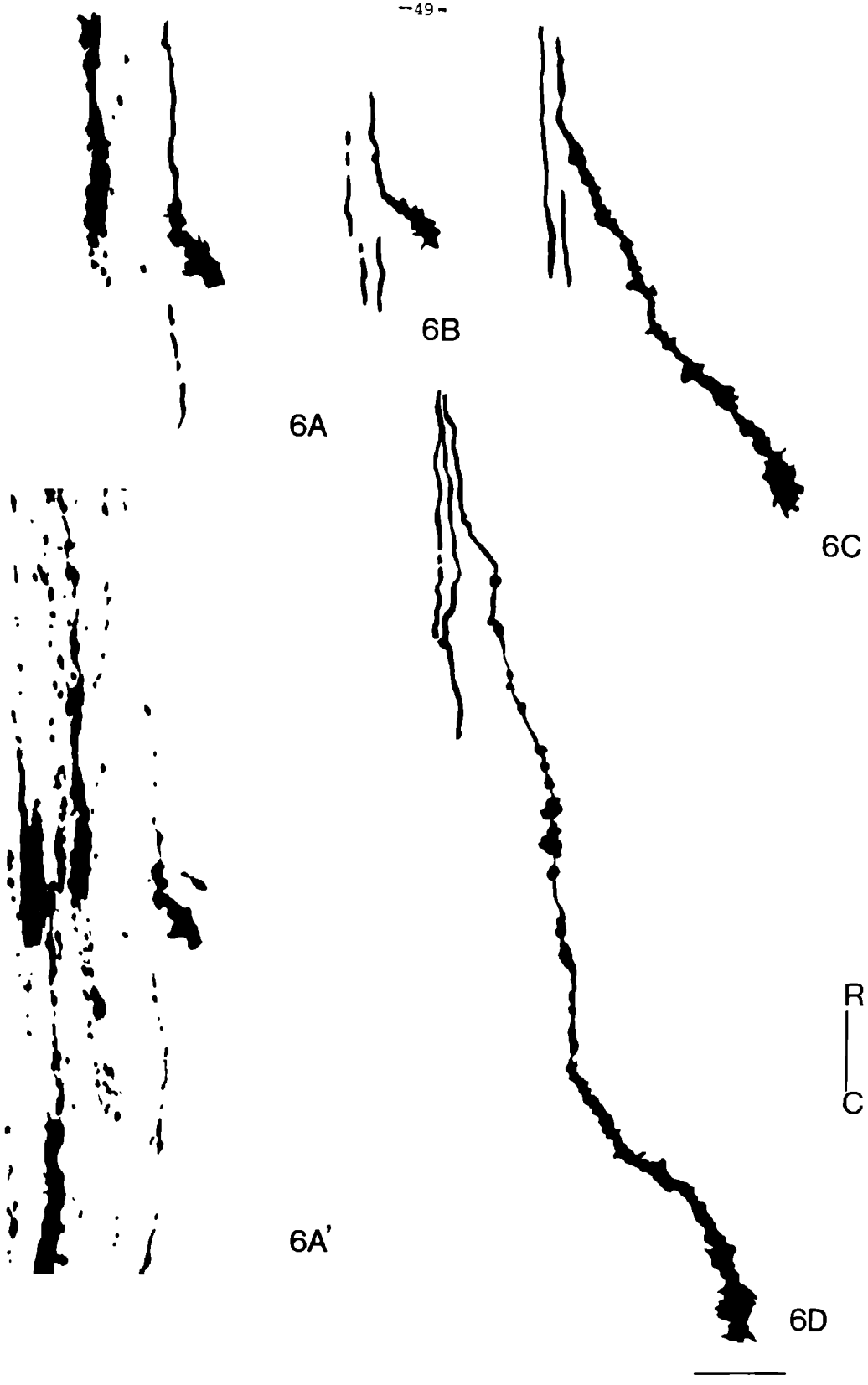


Figure 5 : Camera lucida drawings of two CST axons penetrating the lumbar grey matter at P7. Note the accumulation of varicosities proximal to the growth cone. Spines were found occasionally on large varicosities (arrow). Bar= 20 µm.

Figure 6 : Directly outgrowing CST axons from the CST area into the grey matter at P7. **Figs.6A-6D:** Camera lucida drawings. Note the accumulation of varicosities (6C,6D) and the many numerous extensions at the tip of the axon. **Fig.6A':** Photomicrograph of Fig.6A. Bar= 20 µm.


6B
6A
6C
6A'
R
C
6D

observed in the ventralmost part of the dorsal funiculus, particularly the fusiform growth cones (Fig.2A), have not been found.

The labelled CST growth cones invading the lumbar grey matter invariably were characterized by the presence of both numerous finger-like and veil-like extensions as well as a great number of small spiny protrusions (Figs.4,5). In addition to the great number of extensions, target area CST growth cones were also characterized by an accumulation of varicosities proximal to the growth cone (Figs.4,5), in contradistinction to tract area growth cones.

Whereas the filopodial type of pioneer growth cones in the tract area (Fig.2C) contains filopodia up to a maximum of four, spinal grey target area CST growth cones displayed numerous spiny extensions, sometimes even more than twenty (Fig.4).

Interestingly, some varicosities exhibited spines and/or filopodial extensions (Fig.5B). These particular varicosities may be potential axonal branching sites in which axon collaterals might develop (see Discussion).

Direct outgrowth of CST axons can be observed from the white into the adjacent grey matter (Fig.6). Clearly, these spinal grey CST projections were not developing from interstitial budding of collaterals. However, the formation of collaterals of CST axons by the process of budding, which occurs days after the parent axons grow caudally past the lumbar intumescence, cannot be excluded. The accumulation of tracer HRP prevents a clear determination of possible branching sites situated within the dorsal funiculus (see Fig.3).

The outgrowth of labelled CST axons into the lumbar spinal grey always occurs at an angle varying between 130 and 170 degrees (Figs.3,4,5,6).

DISCUSSION

Technical considerations

In order to visualize growing axons in the rat corticospinal tract system, the cells of origin in layer V of the sensorimotor cortex (Leong,1983; Wise et al.,1979[a]) were labelled with HRP.

Because of its superior sensitivity we used the TMB to visualize HRP (Mesulam and Rosene,1979; Rye et al.,1984). An additional diaminobenzidine-Nickel (DAB-Ni) incubation prevented the HRP-TMB-(AHM) crystals from washing out and preserved the high TMB-sensitivity (Dederen and Joosten,1989). Absence of the TMB-AHM reaction considerably reduced the amount of labelled fibres. Furthermore, to obtain an adequate LM visualization of the anterogradely HRP labelled CST growing tips at lumbar levels it was necessary to further incubate the sections in a modified DAB-Co-GOD reaction mixture (Itoh et al.,1979). The

visualization of the HRP labelled growth cones by using a single DAB-Co/GOD reaction did not allow a clear intense staining.

The question remains whether the staining method used reveals an accurate growth cone morphology. Although this question cannot be answered with full certainty, there are strong indications for a positive and complete labelling and subsequent visualization. First of all comparison with findings as described previously in literature (Mason,1982,1985; Bovolenta and Mason,1987; Nordlander,1987) makes the CST growth cone morphology, as observed in this study, rather likely. Nevertheless, the strongest argument in favor of a complete staining of the CST growth cones is obtained on the results of serial sectioning of unlabelled pyramidal tract pioneer fibres at the cervical intumescence. Reconstructions of these growth cones revealed a very similar morphology (personal communication T.Gorgels) as compared with the lumbar CST pioneer growth cones as presented in this paper.

CST growth cone morphology and its implications

This study reveals striking variations among corticospinal growth cones related to their position in the lumbar spinal cord of the rat. CST pioneer growth cones are characterized by their long and rather simple configuration in the white matter (tract area), whereas the tips of the CST axons invading the spinal grey matter (target area) are more complex.

This change in morphology may reflect the changing architecture of the surroundings, on the one hand, and the altering challenges the CST growth cones encounter, on the other. Alterations in growth cone morphology as axons pass from one region of the vertebrate CNS to another have previously been observed in the Xenopus spinal cord (Nordlander and Singer,1982, 1987) and in the mouse retinotectal system (Reh and Constantine-Patton, 1985; Harris et al.,1985,1987; Bovolenta and Mason,1987).

A rather common feature of growth cones situated within straight pathways is their highly elongated form as well as their simple morphology, not only in invertebrates (grasshopper leg segment, Caudy and Bentley, 1986) but also in vertebrate fibre systems (the chicken peripheral nervous system, Tosney and Landmesser,1985 ; the mouse visual pathway, Bovolenta and Mason,1987 ; the rat corpus callosum, Kalil,1988). Furthermore, both in invertebrates as well as in vertebrates growth cones become more elaborate (filopodial) when they diverge or come to decision points (Tosney and Landmesser,1985; Caudy and Bentley,1986; Bovolenta and Mason, 1987; Nordlander,1987; Van Mier and Ten Donkelaar,1989).

The factors responsible for changes in growth cone shape and behavior are still somewhat tentative. One of the most important hypotheses on this subject is based on differential affinity: growth cones are guided and subsequently change their form as they register and react on to variations in the relative adhesiveness of the local environment

(Letourneau,1975; Nardi,1983). In vitro experiments conclusively demonstrated growth cones exhibiting differential adhesive strenghts between their filopodia and the substratum (Letourneau,1979;1985). In vivo observations (Bastiani and Goodman,1984; Bovolenta and Mason,1987) support these in vitro experiments. The increase of filopodial extensions at the moment CST growth cones enter the spinal grey indicates a decision point in the development of this tract at which an extensive exploration and/or attraction of or repulsion by the local environment is necessary.

The elongated and rather simple morphology of the growth cones of CST pioneer fibres in the tract area suggests a less extensive exploration and/or attraction and repulsion, respectively,to their microenvironment. Whereas these pioneer fibres probably do not intensely examine the local environment but nevertheless are clearly permanently guided in one (caudal) direction one would suggest the involvement of one dominant guidance cue (adhesive or inhibitory) and/or the occurrence of a channel-like guidance system.A channel-like guidance system, consisting of glial or presumptive glial cells was substantiated in the developing mouse optic nerve (Silver and Sidman,1980; Silver,1984) and corpus callosum (Silver et al.,1982). However the directed growth of the pioneer CST axons in the tract by the process of stereotropism is very unlikely: up till now a channel-like system could not be detected in developing rat CST (Schreyer and Jones,1982; Joosten et al.,1989).
Nevertheless,vimentin-immunoreactive (VIM-ir) precursor-astrocytes are oriented in longitudinal tiers with their processes perpendicular to the outgrowing pioneer CST axons (Joosten and Gribnau,1989[b]). The VIM-ir precursor astrocytes, as stepping stones, might direct the outgrowing pioneer CST axons by means of chemical attraction and/or inhibition. These processes of neuron-glia interaction might be initiated or mediated by either attractive molecules (for reviews see Edelman,1984; Purves and Lichtman,1985; Dodd and Jessell,1988) on the one hand and/or repulsive proteins on the other (Caroni and Schwab,1988[a], 1988[b]).

The first CST axons entering the lumbar spinal grey are characterized by the appearance of many filopodia, lamellipodia and spines. Other studies on growth cones in situ support our observations that in decision regions growth cones are characterized by elaborate forms (Mason,1985; Harris et al.,1985,1987; Bovolenta and Mason,1987).

Whereas differential adhesion may account for the guidance of pioneer axons in the tract area, more soluble factors (chemotrophic factors) may play an essential role in attracting the growth cones within the target area (Purves and Lichtman,1985; Dodd and Jessell,1988). Little is known about the molecules that exert chemotrophic guidance in the nervous system. Nerve growth factor (NGF) is the only defined molecule for which a chemotrophic role has been postulated (Mensini-Chen et al.,1978; Gundersen and Barrett,1979).

Concludingly, the change in CST growth cone morphology from lumbar tract to target area not only reflects the varying interactions with the

microenvironment but possibly also indicates a change of the predominant guidance mechanism.

Development of the CST projection to the lumbar grey matter

Recent findings on the development of the corticopontine projection in the rat (O'Leary and Terashima,1988) demonstrate the occurrence of interstitial budding of collaterals from the parent axons. Furthermore, growth cone bifurcation appears to be the exclusive mechanism of axon branching as shown in vitro (Shaw and Bray,1977; Bray et al.,1987) or in vivo during early neurogenesis (Raper et al.,1983; Fujisawa,1987). Neither of the latter two mechanisms can be excluded from our results because of the masking effect of the very intense labelling in the tract itself (Fig.3). However, the development of collaterals by the mechanism of budding mainly occurs at sharp turning angles of 90° perpendicular to the parent axons (Reh and Constantine-Patton,1985; O'Leary and Terashima 1988). The CST axons enter the lumbar spinal grey at an angle varying between 130 and 170 degrees (Figs.3,5,6): a result which clearly is not in favor of the budding-hypothesis. On the other hand the fact that at lumbar spinal cord levels only a small number of axons eventually will grow further caudally implicates a clear difference as compared with more rostral levels of this particular tract (as for instance at the pons).

Concludingly, it can be deduced from our results that at least some CST axons change their direction and directly grow out into the spinal grey.

It is an intriguing question whether the development of CST projections to the lumbar spinal cord is representative for the entire pre- and postnatal CST formation.

Acknowledgement

The author wants to thank Jos Dederen for excellent technical assistance.

Chapter 4

An electron microscopic tracer study on the postnatal development of the corticospinal tract in the rat

CHAPTER 4A: ULTRASTRUCTURAL VISUALIZATION OF ANTEROGRADELY TRANSPORTED HORSERADISH PEROXIDASE IN DEVELOPING CORTICOSPINAL TRACT OF RAT.

SUMMARY

Until now a satisfactory method for electron microscopic (EM) detection of anterogradely transported horseradish peroxidase (HRP) in developing neural tissue, using sensitive chromogen tetramethyl-benzidine (TMB), has not been described. Use of the stabilizing agent ammoniumheptamolybdate, in combination with a modified prolonged osmication (4 hr at pH 5.0 in 0.1 M phosphate buffer (PB)) made possible visualization of HRP-TMB-(AHM) reaction product at the ultrastructural level in outgrowing corticospinal (CST) fibers of young postnatal rat. This reaction product appeared to be very distinctive and clearly detectable, making ultrastructural identification of HRP-labeled outgrowing CST fibers in rat spinal cord rather easy. In addition, the procedure described in this report preserves the ultrastructural details of developing neural tissue.

Key words :horseradish peroxidase - tetramethylbenzidine - ammonium-heptamolybdate - electron microscopy - anterograde transport - corticospinal tract - development - rat.

INTRODUCTION

Horseradish peroxidase (HRP) can be very successfully employed for tracing fiber connections within the central nervous system by use of anterograde transport techniques (Mesulam and Mufson,1980).
Tetramethylbenzidine (TMB) proved to be the most sensitive chromogen available for demonstration of HRP-labeling at the light microscopic level (Hardy and Heimer,1977; Mesulam,1978; Mesulam and Rosene,1979). The HRP-TMB reaction product is most stable in strongly acidic buffers (pH 3.3), but appeared to be progressively less stable at increasing pH (Mesulam,1978; Sakumoto et al.,1980).

Up till now, a satisfactory method for EM detection of anterogradely transported HRP in developing neural tissue using the sensitive chromogen TMB has not yet been described. Such studies have been carried out using diaminobenzoic acid (DAB) which, however, displays some obvious limitations as compared with TMB. DAB is much less sensitive than TMB (Mesulam and Rosene, 1979) and it often produces a rather diffuse reaction product (Itoh et al.,1979; Adams,1981; Murray and Edwards,1982; Edwards and Murray,1985). For detection of HRP-TMB at the ultrastruc-

tural level, the presence of osmium black is necessary. The rate of formation of osmium black from the blue HRP-TMB product during osmication, however, depends on the pH of the osmication solution: increasing pH of this solution provides an increasing rate of osmium black formation (Sakumoto et al., 1980). Apparently, the HRP-TMB product must destabilize before it can react with osmium and the conversion to osmium black can commence (Sakumoto et al., 1980; Henry et al., 1985[a], 1985[b]).

By using an overnight osmication (12 hours) in 0.1 M phosphate buffer (PB) pH 5.0, Henry et al.(1985[a], 1985[b]) achieved a highly detectable and stable electron-dense HRP-TMB reaction product in adult nervous tissue. This overnight osmication procedure (Henry et al.,1985[b]) and other modified protocols (Carson and Mesulam, 1982; Naus et al., 1985; Westman et al., 1986),however, were not suitable in developing postnatal tissue because of loss of reaction product and/or ultrastructural detail. The effect of strongly acidic buffer (pH 3.3) in which the HRP-TMB reaction must be carried out appears to be very destructive on loosely packed postnatal nervous tissue, in contradistinction to its effect on the more compact tissues of the adult central nervous system.

Recently, Olucha et al. (1985) showed that using ammoniumhepta-molybdate (AHM) as a stabilizing agent the HRP-TMB reaction can be accomplished at an optimal pH of 6-8, at least at the LM level. Use of this stabilizing agent might be essential to preservation of ultrastructural details in developing postnatal neuronal tissues. The object of the present investigation, therefore, was to develop a suitable procedure for the ultrastructural visualization of the HRP-TMB reaction product in outgrowing corticospinal fibers of young postnatal rats that would be comparable to the intense LM labeling achieved by Gribnau et al.(1986).

MATERIALS AND METHODS

In the present study, five 7-day-old Wistar rats, bred at the Central Animal Laboratory of Nijmegen were used. After IP anesthetization with sodium pentobarbital (18 mg./kg. body weight) the entire sensorimotor and frontal cortex of the left cerebral hemisphere was labeled by the implantation of three HRP-gels (Griffin et al., 1979), which were placed after opening the skin and making small holes in the skull using a fine needle. The postinjection survival times were kept constant at 24 hours. The animals then were reanesthetized and perfused transcardially with 30 ml 5% sucrose in PB (pH 7.2) followed by 40 ml 1% paraformaldehyde and 2% glutaraldehyde in the same buffer.

After perfusion, the brains and spinal cords were immediately removed and the spinal cords only were further immersed for about 1 hr before being transferred into cold (4°) PB with 5% sucrose. Using a vibratome (Oxford Vibratome) 100-µm sections were cut and collected in cold PB

without sucrose.

Sections were immediately incubated with TMB as a chromogen and AHM as stabilizing agent according to the following procedure: After rinsing twice for 1 min in distilled water, each section was pre-soaked for 20 min in a mixture containing 0.25% AHM in 0.1 M PB (pH 6.0) and 0.005% TMB (dissolved in absolute alcohol). The incubation was started by adding 50 ul of 33% H_2O_2 per 100 ml pre-incubation bath. This addition was repeated every 5 min for 20 min. The HRP reaction was terminated by rinsing the sections in two 1-min baths of 0.1 M PB (pH 5.0). Control sections were incubated with TMB as chromogen, at pH 3.3 or pH 6.0, but without AHM.

Further processing for EM was carried out using a 1% OsO_4 solution in 0.1 M PB (pH 5.0) for 1,2,4 or 16 hr at room temperature. Osmication was followed by dehydration: 80% ethanol for 3 min, 95% ethanol for 3 min, 100% ethanol twice for 3 min, and 100% acetone twice for 3 min. The sections were very quickly infiltrated by Epon 812: 1 hr 1:1 Epon:acetone; 3 hr 3:1 Epon:acetone, followed by embedding in Epon.

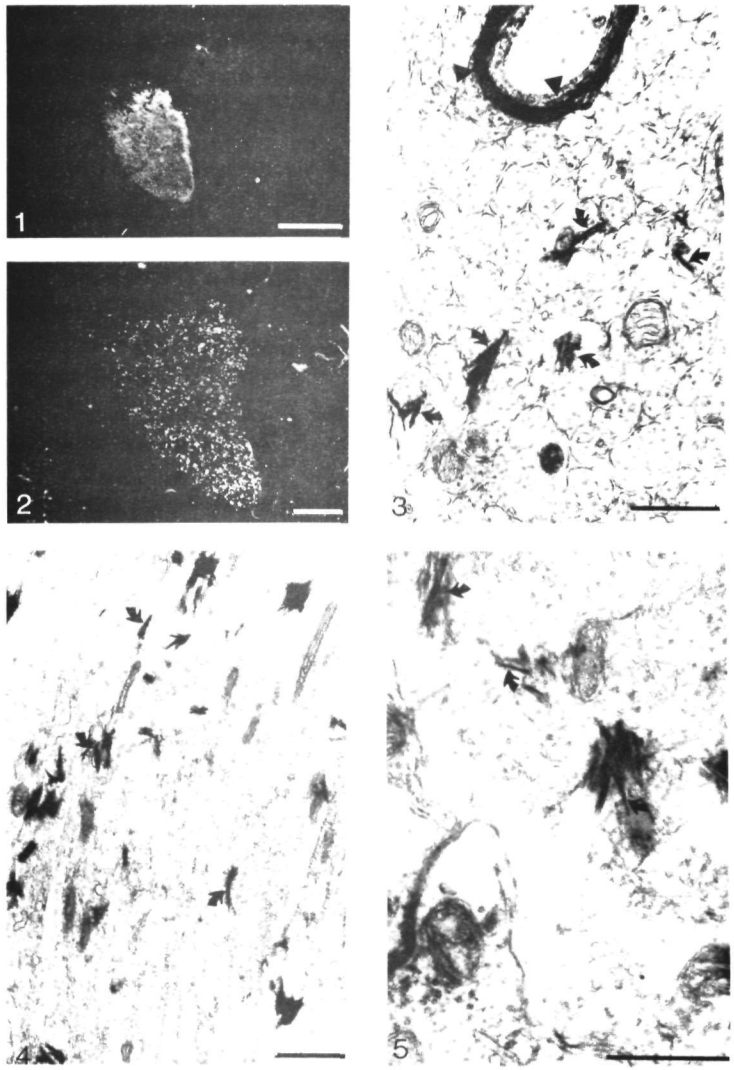
Semi-thin sections were counterstained in 0.5% toluidine blue solution and examined by LM to select blocks for ultra-thin microtomy. Ultra-thin sections were mounted on 75-mesh formvar (0.8%)-coated copper grids, contrasted with uranyl acetate (20 min) and lead citrate (5 min), respectively, and were studied in a Philips EM 300 at an accelerating voltage of 60 kV.

For comparative LM studies, freeze-microtome sections of the same tissue were reacted with TMB-AHM according to the method of Olucha et al. (1985). These sections were then mounted on chrome-aluin coated slides, counterstained with 0.5% neutral red, rapidly dehydrated in alcohol, cleared in xylene, and enclosed in Depex. All LM photographs were made with an automatic Zeiss photomicroscope-II using darkfield illumination.

RESULTS

For LM visualization of outgrowing CST projections during postnatal development of the rat, the anterograde transport of HRP, in conjunction with TMB histochemistry (Mesulam, 1978; Mesulam and Rosene, 1979),proved to be a very sensitive method (Gribnau and Dederen, 1984; Gribnau et al., 1986). Figure 1 shows a darkfield micrograph of a transverse freeze-microtome section through the spinal cord at the cervical enlargement (C5) of a 7-day-old rat, containing anterogradely labeled corticospinal fibers in which AHM was used as a stabilizing agent.

Ultrastructural visualization of outgrowing fibers using TMB-histochemistry proved to be rather difficult, especially in early postnatal tissues. In our first approach, it became evident that even the modified TMB protocols, as developed earlier for ultrastructural visualization of anterogradely transported HRP were not suitable for young postnatal animals in contrast to adults (Carson and Mesulam, 1982; Naus et al.,



Figs.1-5 : Fig.1 : Photomicrograph of a 30 µm transverse freeze-microtome section of the cervical enlargement (C5) of the spinal cord of 7 days postnatal rat showing unilaterally labeled CST fibers. Note the amount of HRP-TMB-(AHM) reaction product under darkfield illumination.Bar=100 µm. **Fig.2** : Photomicrograph of a 1 µm transverse semithin section of the cervical enlargement (C5) at P7 under dark-field illumination.The unilaterally HRP-TMB-(AHM) labelled CST fibers are clearly visible.Bar=50 µm. **Fig.3** : Electron microscopic picture of the labeled CST at C5. The electron-dense HRP-TMB-(AHM) reaction product (arrows) generates a clearly visible intracellular marker in the developing nonmyelinated CST fibers. Myelination processes (arrowheads) are clearly discernable as compared with the crystalline reaction product. Transverse section. Original magnification x 38,700. Bar=0.5 µm. **Fig.4** : Electron micrograph of CST axons labeled with the electron-dense HRP-TMB-(AHM)reaction product (arrows) after 4 hours osmication. Longitudinal section.Original magnification x 15,500.Bar=1 µm. **Fig.5** :Electron micrograph of a transversely cut control section:HRP-TMB reaction product at pH 3.3 in combination with prolonged osmication (4hr). Note the poor ultrastuctural tissue preservationas compared with Figure 3. The electron-dense HRP-TMB reaction product (arrows) is clearly visible. Original magnification x 49,500.Bar=0.5 µm.

1985, Henry et al.,1985a,1985b; Westman et al.,1986). In sections incubated with TMB as chromogen at pH 3.3, in combination with prolonged osmication, the ultrastructure of the tissue was poor (Figure 5). Higher pH (6.0) resulted in improved ultrastructural preservation but also in a total decrease of HRP-TMB reaction product (not shown). We therefore employed and improved an very sensitive TMB method using AHM as a stabilizing agent (see Materials and Methods),especially suitable for the outgrowing CST fibers in young postnatal rats. In this way, an extensive pattern of labeling was established at the semi-thin level (Figure 2) which is highly comparable with the LM picture (Figure 1).

At the ultrastructural level, the HRP-TMB-(AHM) product, can easily be identified in the nonmyelinated CST fibers in spinal cord (Figures 3 and 4). This reaction product generates a highly discernable and clearly visible intracellular marker, confirming descriptions by other authors (Carson and Mesulam,1982; Henry et al., 1985a,1985b; Marfurt et al., 1986), with only minor loss of ultrastructural details (see Figures 3 and 4).

With this procedure, the length of the osmication procedure was of utmost importance. Increasing osmication time resulted, under the conditions described above, in enhancement of the ultrastructurally visible HRP-TMB-(AHM) reaction product. After 1 hour osmication, no reaction product could be detected. On the other hand, when osmication was extended to 16 hr, accumulation of the approximately 1 um great HRP-TMB-(AHM) crystals in the nonmyelinated CST-fibers resulted in their masking of fine ultrastructural features. Optimal osmication time appeared to be 4 hr (see Figures 3 and 4).

DISCUSSION

The present investigation deals with the ultrastructural detection of the HRP-TMB reaction product in anterogradely labeled outgrowing CST fibers in young postnatal rats at a level comparable to the LM pattern (Mesulam,1978; Mesulam and Rosene,1979; Gribnau and Dederen,1984).
Conversion of the HRP-TMB reaction product, at least in adult neuronal tissues, into an electron-dense complex during osmication is closely related to the pH of the phosphate buffer (Sakumoto et al.,1980; Carson and Mesulam,1982; Henry et al., 1985a,1985b; Naus et al.,1985). Our observations showed that the ultrastructural visualization of anterogradely transported HRP by use of TMB as chromogen in adult tissues (Carson and Mesulam,1982; Henry et al.,1985a,1985b; Naus et al.,1985; Westman et al.,1986) was not suitable for use in early postnatal tissues because it led to loss of reaction product and/or ultrastructural details (Figure 5).

Improvement of the ultrastructural detail could be achieved by the use of the stabilizing agent AHM, which allows the HRP-TMB reaction to be carried out at pH 6.0 (Olucha et al.,1985) instead of the before mentioned (see Introduction) optimal pH 3.3 (Mesulam,1978; Sakumoto et

al., 1980). The combination of a 4 hr osmication in combination with the use of the stabilizing agent AHM appeared to be essential, in developing postnatal tissue, to obtain acceptable ultrastructural preservation on the one hand and a sensitive HRP-TMB-(AHM) labeling pattern on the other. The TMB reaction product also proved able to resist the solvents used in rapid EM tissue processing (see Materials and Methods). Moreover the reaction product appeared to be very distinctive and clearly detectable making identification of the HRP-labeled outgrowing CST fibers in rat spinal cord rather easy. Particularly in outgrowing fibers where myelination as well as degradation processes occur, as described in CST (Schreyer and Jones,1982) or optical nerve (Williams et al.,1986), correct interpretation of labeling is very important. Also, the lamellated and multivesicular bodies which normally are present in outgrowing optic fibers (Hildebrand and Waxman,1984) are easily distinguishable from the HRP-TMB-(AHM) crystals in contrast to the diffuse HRP-DAB reaction product as described by Murray and Edwards (1982) among others. Moreover, the HRP-DAB reaction is disadvantageous because of its lesser sensitivity than TMB (Mesulam,1978; Mesulam and Rosene,1979; Carson and Mesulam, 1982).

In summary, use of the stabilizing agent AHM, in conjunction with a modified prolonged osmication procedure, results in a very clearly discernable HRP-TMB-(AHM) reaction product at the ultrastructural level in outgrowing corticospinal fibers of the young postnatal rat.

Acknowledgements

The authors are grateful to Dr. Peter van Mier and Dr. Paul Jap for helpful discussion, to Miss Margreet Rieck for typing the manuscript, and Theo Hafmans for photographic assistance.

CHAPTER 4B: POSTNATAL DEVELOPMENT OF THE CORTICOSPINAL TRACT IN THE RAT. AN ULTRASTRUCTURAL ANTEROGRADE HRP STUDY.

SUMMARY

Horseradish-peroxidase was used to anterogradely label and trace the growth of corticospinal axons in rats ranging in age from one day to six months. Three to eight HRP-gels were implanted in the left cerebral hemisphere of the cortex. In each spinal cord three levels were studied: the cervical intumescence (C5), the mid-thoracic region (T5) and the lumbar enlargement (L3). The technique used for the electron-microscopic visualization of HRP was described earlier (see Chapter 4A).

The outgrowth of unmyelinated labelled corticospinal tract axons in rat spinal cord primarily occurs during the first ten postnatal days. The outgrowth of the main wave of these fibres is preceded by a number of pathfinding axons, which are characterized by dilatations at their distal ends: the growth cones. By contrast, later appearing unmyelinated axons, which presumably grow along pathfinding axons, do not exhibit such growth cones. The first labelled pioneer axons can be observed in the cervical intumescence at the first postnatal day (P1), in the mid-thoracic region at day three (P3) and in the lumbar enlargement at day five (P5).

Prior to the entrance of axons, the prospective corticospinal area or pre-arrival zone is composed of fascicles consisting of unlabelled unmyelinated fibres surrounded by lucent amorphous structures. During the outgrowth phase of corticospinal fibres some myelinated axons could be observed within the outgrowth area even before day 14. These axons, however, were never labelled. These findings strongly suggest that the outgrowth area, which generally is denoted as pyramidal tract, contains other axons besides corticospinal fibres (and glial cells). The process of myelination of labelled corticospinal tract axons in the rat spinal cord starts rostrally (C5) at about day 14 and progresses caudally during the third and fourth postnatal weeks. Although myelination seems to be largely complete at day 28 at all three spinal cord levels, labelled unmyelinated axons are still present in the adult stage.

Key words :corticospinal tract - development - myelination - pyramidal tract - anterograde tracing - electronmicroscopy - rat.

INTRODUCTION

The pyramidal tract (PT) is defined as a set of fibres passing through the medullary pyramids. One of its major components is the corticospinal tract (CST). Besides a major CST-component the rat PT-area at spinal cord levels probably contains corticobulbar, aberrant cuneatus and gracilis fibres (Dunkerley and Duncan,1969; Matthews and Duncan,1971). Additionally, glial cells are present in the PT-area (Ralston et al.,1987, among others).

The CST originates in the cerebral cortex and projects to the spinal gray via the medullary pyramids; after decussation it continues within the ventral part of the dorsal funiculus. In rodents, the arrival of corticospinal fibres in the spinal cord occurs at birth whereas their outgrowth throughout the cord comes about postnatally (Brown,1971; Jones et al.,1982; Schreyer and Jones,1982; Leong,1983; Gribnau et al.,1986). On the basis of quantitative electron microscopic studies of the PT-area in normal hamster spinal cord it was concluded that the bundle grows with a compact front of fibres (Reh and Kalil,1982), whereas in the rat a more staggered growth was proposed, such that significant numbers of axons are added to the tract within the first two weeks after its initial outgrowth (Schreyer and Jones,1982). Light microscopic anterograde tracer studies (Schreyer and Jones,1982; Gribnau et al.,1986), however, indicated that during outgrowth of the rat CST through the spinal cord new fibres are probably continually added to the first leading fibres, even beyond day 9. An understanding of the mode of CST outgrowth is essential for the interpretation of the effect of lesions and eventual regeneration of these fibres.

The present experimental ultrastructural study was undertaken in order to provide more insight into the the postnatal development of the rat PT and particularly its CST component. We employed the anterograde transport of horseradish peroxidase (HRP) as a marker for outgrowing corticospinal axons and tetramethylbenzidine (TMB) in conjunction with ammoniumheptamolybdate (AHM) to visualize labelled CST fibres at the electron microscopic level (Joosten et al.,1987[a]). Some of the results were presented earlier in preliminary form (Joosten et al.,1987[c]).

MATERIALS AND METHODS

The anterograde transport of the enzyme HRP (Sigma type IV) was used to label corticospinal axons in neonatal and adult Wistar rats, bred at the Central Animal Laboratory of Nijmegen. A total of 29 rats ranging in age from postnatal day 1 (P1) to six months (adult(A)) were used (Table 1). The day of birth is termed postnatal day 0 (P0); the ages of the animals given in the present paper are the ages at their respective day of HRP-gel implantation.

After intra-peritoneal anaesthetization with sodium pentobarbital (18 mg./kg. body weight) and additional cooling, if necessary, the greater

Table 1 :Material used in the present investigation

Age	Number of animals	Number of HRP gels implanted in the left hemisphere	Survival time (hours)
P1	2	3	24
P3	5	3	24
P5	2	3	24
P7	5	3-4	24
P10	4	3-4	24
P14	3	5	24
P21	3	5	24
P28	3	5	24
YA	2	6	24
	2	6	48
A	2	8	48

part of the cortex of the left cerebral hemisphere was labelled by implantation of at least three HRP-gels (Griffin et al.,1979) (Table 1). The gels were implanted after opening the skin and making small holes in the skull using a fine needle. The survival time was 24 hours.
Some young adult (YA) animals (aged three months; weighing ca 200 g.) and adult rats (aged six months; weighing ca 260 g.) were allowed to survive 48 hours after the gels had been implanted. After reanaesthetization the animals were transcardially perfused with 5% sucrose in 0.1 M. phosphate buffer (PB), pH 7.2, followed by 1% paraformaldehyde and 2% glutaraldehyde in the same buffer.

The brain and spinal cord were dissected from the skull and vertebral column and postfixed by immersion with the same fixative for 1 hr at 20°C. The spinal cord was then placed into cold (4°C.) PB with 5% sucrose and samples of the cervical intumescence (C5), the mid-thoracic region (T5) and the lumbar enlargement (L3) were cut transversely on an Oxford Vibratome at 30 or 100 µm . Further processing was carried out according to the method recently developed for electron microscopic detection of anterogradely transported HRP in outgrowing CST of the rat (Joosten et al.,1987a). Briefly the procedure consists of the following steps: 100 um vibratome sections are incubated with 0.005% tetramethyl-benzidine (TMB) as chromogen and 0.25% ammoniumheptamolybdate (AHM) as stabilizing agent in 0.1 M PB, pH 6.0. The HRP-TMB-(AHM) reaction was started by adding 50 µl of 33% H_2O_2 per 100 ml incubation mixture; this addition is repeated every 5 min. for 20 min. The incubation in combination with a modified prolonged osmication (1% OsO_4, during 4 hours at pH 5.0 in 0.1 M PB) and an accelerated dehydration before embedding in epon, enabled the ultrastructural visualization of the crystalline HRP-TMB-(AHM) reaction product. Control sections were processed in the same way without addition of the chromogen TMB to the incubation mixture.

Semithin (0.5-1.0 µm) and ultrathin transverse or longitudinal sections

were cut using a Reichert OM-4 ultramicrotome. The latter were viewed on 0.8% formvar coated 75-mesh copper grids with a Philips EM-300 electron microscope at an accelerating voltage of 60 kV.

For comparative light microscopic (LM) studies, 30 µm vibratome sections of the same tissue were reacted with TMB-AHM as described for electron microscopic detection. The sections were then mounted on glass slides, counterstained with 0.5% neutral red and embedded in Depex.

Photomicrographs of the vibratome section were taken with an automatic Zeiss photomicroscope II under brightfield illumination.

RESULTS

General aspects

The postnatal developmental process of the CST in the spinal cord was subdivided into three phases:(1) an outgrowth phase; (2) a myelination phase and (3) the adult stage. The description of these three phases will be preceded by a section in which attention is paid to the respective regions prior to the entry of the first CST axons: the pre-arrival zone.

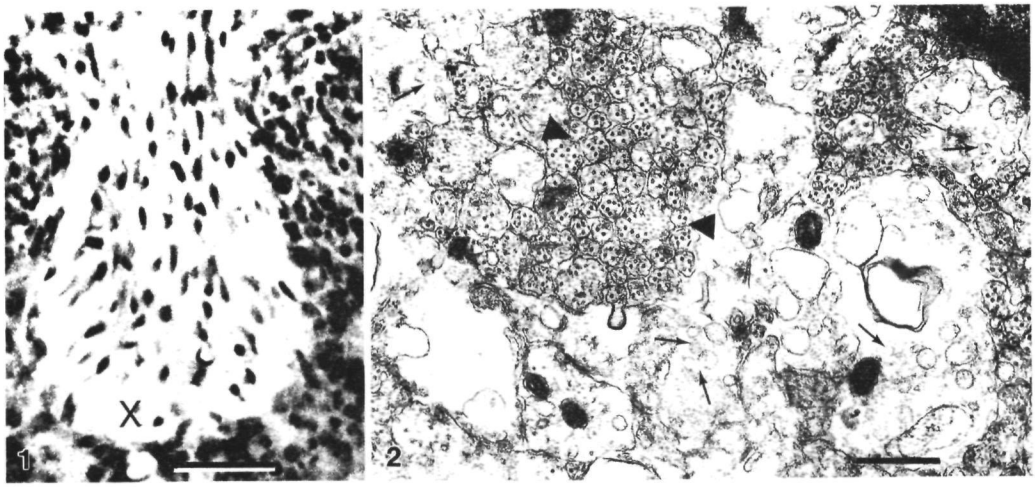
Camera lucida drawings of the injection areas revealed that they invariably encompassed both the somatosensory and motor areas of the cerebral cortex as well as the adjacent cortical regions, at all ages studied.

The pre-arrival zone

Electronmicroscopic observations of the pre-arrival zone in the present study are limited to levels T5 at P1 and L3 at P1 or P3 animals. In a 30 um thick LM-section of the mid-thoracic region at P1 (Fig.1) the dorsal funiculus could be very clearly delimited from the adjacent gray matter. By contrast, within the dorsal funiculus an indistinct boundary existed between the region in which the CST eventually will develop in the ventralmost part of the dorsal funiculus (the pre-arrival zone) on the one hand and the fasciculus gracilis and fasciculus cuneatus on the other (Fig.2). Examination of the pre-arrival zone (this region is marked X (Figs.1-2)) and funiculus gracilis at the electron microscopic level yielded quite different aspects of these two parts of the dorsal funiculus. The fasciculus gracilis was mainly composed of small unmyelinated fibres (with diameters ranging between 0.1 and 0.3 µm.) with a few intervening electron-light areas, whereas region X exhibited several fascicles of unmyelinated fibres (each fascicle contained about 20-50 axons of approximately 0.2 µm. diameters) surrounded by lucent amorphous structures (Fig.2).

The outgrowth phase

The exact timetable of the outgrowing CST axons in the rat spinal cord



Figs.1-2 :The pre-arrival zone. **Fig.1** :Photomicrograph of a 30 µm transverse section of the dorsal funiculus and its adjacent grey matter of the mid-thoracic region (T5) at P1. The region in which the CST eventually will develop is marked X. Bar=60 µm. **Fig.2** :Electron micrograph of the pre-arrival zone (region X) (T5 at P1). Note the fascicles of unmyelinated axons (arrowheads) which are surrounded by watery profiles containing large vacuole-like structures, mitochondria and clusters of round vesicles (arrows). Transverse section.Bar=1 µm.

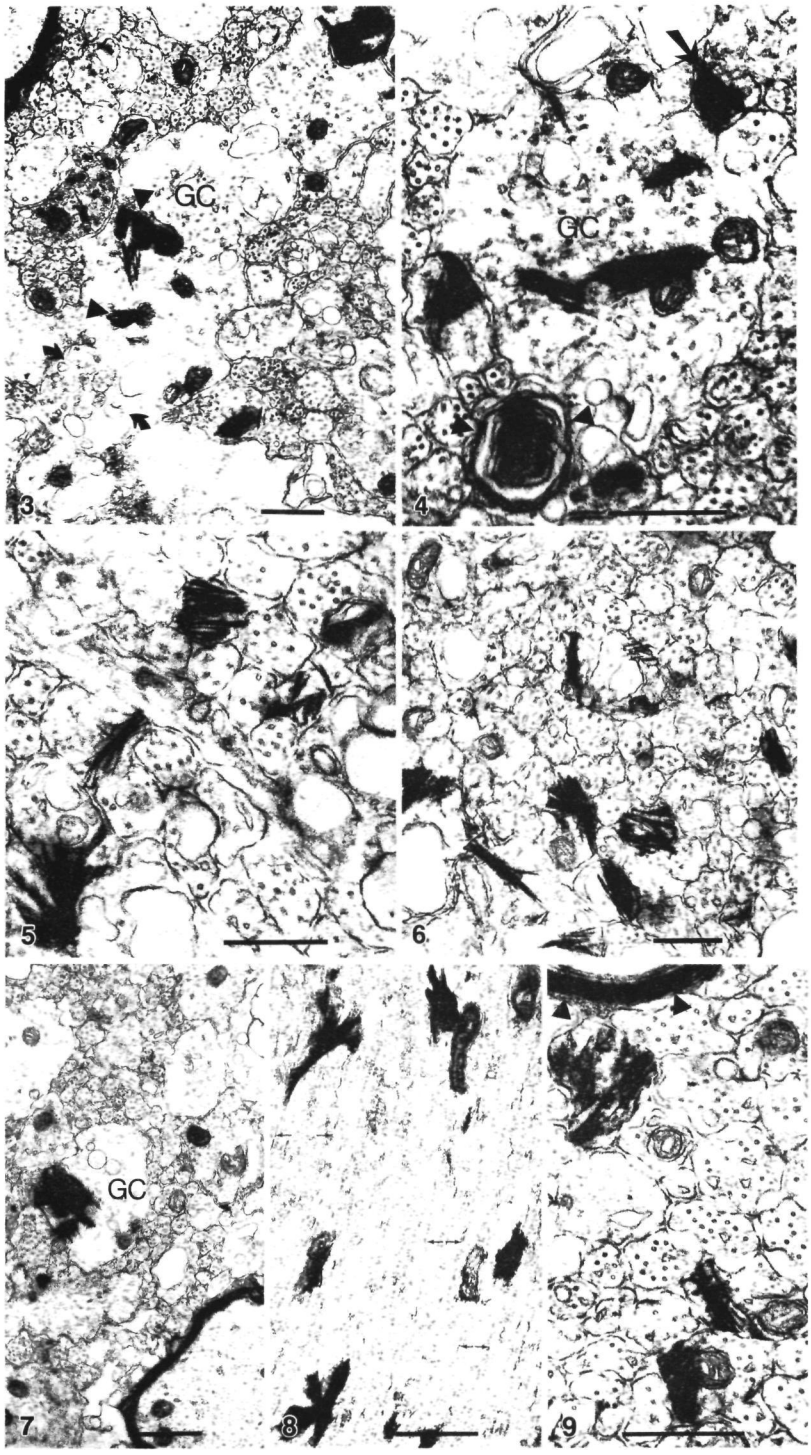
(Gribnau et al.,1986) is used as a basis for our electron microscopic investigations. At the EM level the tip of the outgrowing labelled CST (C5 at P1; T5 at P3; L3 at P5) mainly consisted of large profiles (1-2 µm wide) (Figs.3,4,7),which, according to their morphological features, were classified as growth cones. Numerous small axonal profiles easily recognizable with their almost circular outline, measuring about 0.1-0.3 µm in diameter and provided with distinct microtubules, were present but they rarely were labelled (Fig.4).

The morphological qualities of labelled growth cones at C5 (P1) (Fig.4), T5 (P3) or L3 (P5) (Fig.7) were very similar. Labelled growth cones in the CST ingrowth area (region X) did not show a preferred alignment either at the periphery or at the central part (at the LM as well as at the EM-level). Within the labelled growth cone profiles electron-dense figures were commonly found, occasionally presenting themselves as a series of loosely packed concentric lamellae (Fig.4). However, no specific morphological attachments or membrane specializazations of the labelled profiles in the tip of the outgrowing CST could be detected.

Two days after the arrival of the labelled outgrowing tip of the CST at a given spinal cord segment (C5 at P3; T5 at P5) the labelled CST consisted of unmyelinated small axons (with diameters ranging between 0.1-0.3 µm) (Fig.6).

Large profiles either labelled or unlabelled could no longer be detected.

From P5 up to P10 the labelled CST consisted exclusively of unmyeli-


GC
3
GC
4
5
6
GC
7
8
9

Figs.3-9: :The outgrowth phase. **Fig.3** :P1-C5· initial growth of 'pathfinding' axons with their growth cones (GC) into the pre-arrival zone. Growth cones are labelled by the HRP-TMB-(AHM) crystals (arrowheads). The growth cones penetrate the area between the lucent amorphous structures (arrows) and the fascicles of unmyelinated axons. Transverse section. Bar=1 µm. **Fig.4** :P1-C5: CST growth cone (GC) containing smooth endoplasmatic reticulum, microtubuli, mitochondria, a few vesicles and several distinct HRP-TMB-(AHM) crystals. Note the labelled unmyelinated axon (arrow). The distinctive electron-dense myelin-like structure figure (arrowheads), probably represents a degenerating axon.Transverse section. Bar=1 µm. **Fig.5** :P5-C5, and **Fig.6:** P5-T5: the labelled CST exlusively consists of unmyelinated axons with varying diameters. Transverse section. Bar=0.5µm. **Fig.7** :P5-L3: labelled growth cone (GC) at the tip of the outgrowing CST containing many HRP-TMB-(AHM) crystals. At this age no labelled unmyelinated axons were present at this level. Transverse section. Bar=1 µm. **Fig.8** :P7-C5: longitudinal section of labelled CST exclusively consisting of unmyelinated axons. Note the uneven presence of HRP-TMB-(AHM) crystals within the unmyelinated axons. Axon diameters are indicated by arrows. Bar=1 µm. **Fig.9** :P10-L3: unmyelinated CST axons containing the intracellular reaction product are clearly discernable as compared with myelinating processes (arrowheads). Transverse section. Bar=0.5 µm.

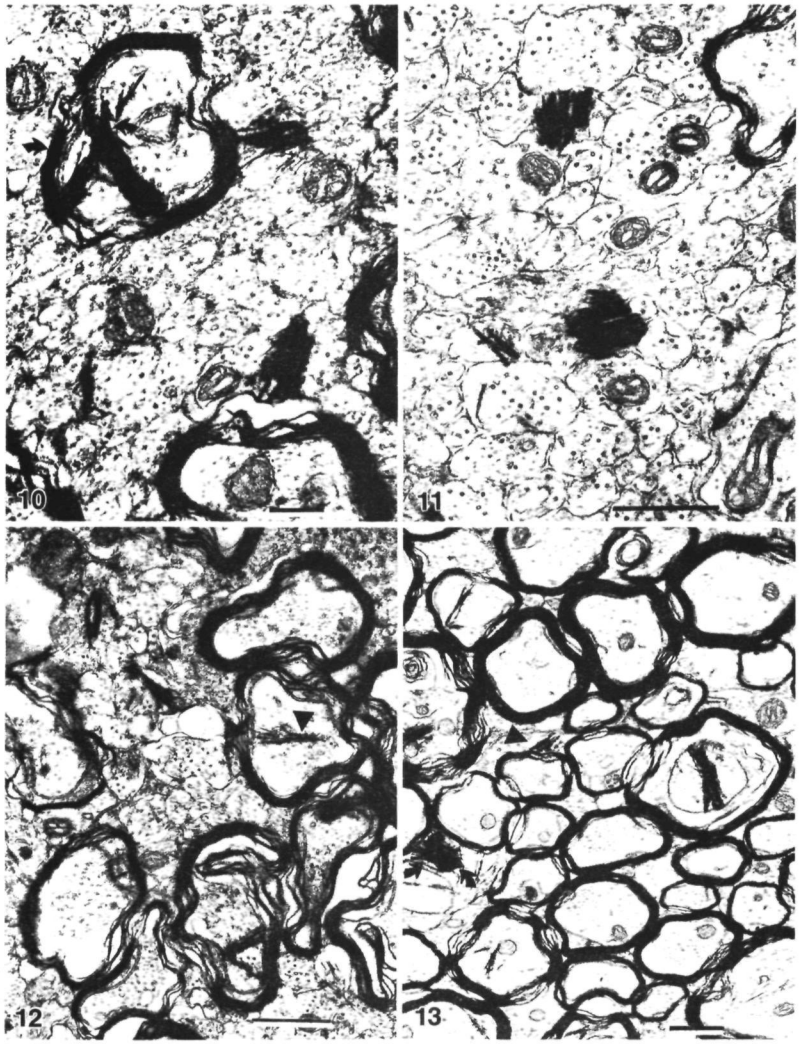
nated axons (0.1-0.4 um) at C5 (Figs.5,8), T5 (Fig.9) and L3. At P10 a few myelinated profiles within the ventral part of the dorsal funiculus were present (Fig.9) but they never exhibited HRP-TMB-(AHM) crystals.

Transversely sectioned unmyelinated axons generally showed one single HRP-TMB-(AHM) crystal (Figs.5,6) whereas in growth cones occasionally several distinct HRP-crystals were present (Figs.3,4,7). Longitudinal sections of the developing tract demonstrated a number of HRP-crystals both in growth cones (not shown) and in unmyelinated axons (Fig.8).

The myelination phase

During the first ten postnatal days no labelled myelinated CST-axons could be detected in any of the spinal cord segments studied. Although some myelinated axons were present in the ventral part of the dorsal funiculus at P7 or P10 (Fig.9), the first labelled myelinated CST-fibres appeared in C5 at P10 (Fig.10).

In addition to the myelinated profiles, the CST contained a great number of labelled unmyelinated axons (with diameters ranging between 0.1 and 0.4 µm.) in C5 at P14 (Fig.10). In a transverse section of P14-C5 a variety of myelinated axon diameters (approximately 0.5-3.0 µm) containing the HRP-TMB-(AHM) crystals could be seen (Fig.10). In contrast to the P14-C5 segment, both the T5 as well as the L3 (Fig.11) spinal cord levels contained labelled unmyelinated axons at P14 exclusively. The



Figs.10–13 :The myelination and adult phase. **Fig.10** :P14-C5: besides labelled unmyelinated axons also labelled myelinated profiles can be noted (arrows). Transverse section. Bar=0.5 µm. **Fig.11** :P14-L3: the labelled CST exclusively consists of unmyelinated profiles. Transverse section. Bar=1 µm. **Fig.12** :P21-L3: the labelled myelinated axons is enwrapped by a myelin sheath consisting of only a few myelin layers (arrowhead). Other labelled profiles are unmyelinated. Transverse section. Bar=1 µm. **Fig.13** :A-L3; 48 hours survival time. The labelled CST contains myelinated as well as unmyelinated axons (arrowheads). The labelled myelinated fibres are varying in diameter whereas occasionally a relatively large unmyelinated axon, probably starting its myelination, appears (arrows). Transverse section. Bar=1 µm.

levels did contain myelinated profiles but they were all devoid of HRP labelling (Fig.11).

At P21 HRP labelled myelinated axons were present at more caudal levels than at P14. In addition to C5, now also T5 and L3 segments contained labelled myelinated profiles (Fig.12).

At the end of the fourth postnatal week (P28) all three spinal cord levels studied consisted mainly of labelled mature myelinated axons (diameters ranging between 1.0 and 3.0 μm).

The adult stage (3-6 months)

Because of the lengthened spinal cord of young adult and adult animals, as compared with that of rats aged P21 or P28, double the standard 24 hour survival time was used. The results acquired after a 48 hours survival time were similar to those obtained after 24 hours as far as the proper CST was concerned.

In both the three and the six month old animals, the CST contained labelled myelinated as well as labelled unmyelinated axons at C5,at T5 and at L3 (Fig.13). In spite of the occurrence of some labelled unmyelinated axons at those spinal cord segments of YA and A animals, most of the labelled profiles were myelinated. Considering their morphological characteristics (Peters et al.,1976; Angevine,1986) the labelled myelinated axons apparently were in a mature state. The diameters of the labelled myelinated axons varied considerably (0.5-3.0 μm) (Fig 13).

DISCUSSION

For descriptive reasons the postnatal period of the development of the CST in the rat has been subdivided into three phases:(A) outgrowth, (B) myelination, and (C) adult. It must be emphasized that at a given point in time such a separation is arbitrary since it is obvious that several developmental processes are taking place simultaneously. The postnatal development and maturation of CST fibres in rat spinal cord should in fact be regarded as a continuum. Figure 14 presents a diagrammatic survey of the postnatal development of the rat CST,in which the three phases mentioned are indicated. This Figure also illustrates the fact that the timing of the three subsequent phases varies with the rostrocaudal progress of CST development. The discussion will be focused on the main events which occur during CST development: i.e. outgrowth, myelination, and the adult CST.

CST outgrowth

The outgrowth of CST axons through the spinal cord in rodents has been extensively studied, at least at the LM level (Donatelle,1977; Hicks and D'Amato,1975,1980; Schreyer and Jones,1982; Kalil,1984; Kalil and Norris

1985; Gribnau et al.,1986). We used the anterograde HRP-TMB technique, as Schreyer and Jones,1982; Gribnau et al.,1986) since TMB proved to be the most sensitive chromogen available for the demonstation of HRP labelling at the LM level (Hardy and Heimer,1977; Mesulam and Rosene,1979). At the EM level combination of the HRP-TMB technique with the use of the stabilizing agent AHM and a prolonged osmication proved to be essential to obtain acceptable ultrastructural preservation of developing CST on the one hand and a sensitive HRP-labelling pattern on the other (Joosten et al.,1987[a]). In the outgrowing CST where myelination (Figs.10-13) as well as degradation processes (Fig.4) occur (Joosten et al.,1987[a]), as is the case in developing optic nerve (Williams et al.,1986), the very distinctive HRP-TMB-(AHM) labelled fibres are easily detectable.

On the other hand the HRP-TMB-(AHM) crystals are irregularly distributed within the labelled profiles. In transverse sections unmyelinated axonal profiles predominantly contain a single HRP-TMB-(AHM) crystal (Figs.,5,6,9), whereas myelinated axonal profiles as well as the larger unmyelinated ones, such as growth cones (Figs.3,4,7) often contain several distinct crystals. Longitudinal sections clearly demonstrate the uneven distribution of the reaction product along the unmyelinated (Fig.8) and myelinated axons. Because of the uneven presence of HRP-TMB-(AHM) crystals in labelled profiles, quantification of the results should be carried out very carefully not only at the EM but also at the LM level.

The outgrowth of CST axons in rat spinal cord occurs mainly during the first ten postnatal days. At birth the first CST axons reach upper cervical levels (Donatelle,1977; Gribnau et al.,1986). According to our electron microscopic observations the outgrowth of the main wave of corticospinal axons is preceded by a number of 'pioneer' axons. These pathfinding axons are characterized by growth cones at their most distal ends. On the contrary, the later arriving fasciculating axons, which presumably are growing along the pathfinding axons, do not exhibit such growth cone-like thickenings.

Comparison of the development of the PT between hamster and rat shows some remarkable species differences. Based on light microscopic studies, using both ^{3}H-proline and HRP as anterograde tracers, and electron microscopic examination of normally fixed unlabelled tissue, Reh and Kalil (1982) suggested that the PT (and probably its CST component) in the hamster grows out as a compact front of fibres. This suggestion, however, was not substantiated by a combined HRP tracing and electron microscopic analysis of the developing CST in the hamster. Besides, O'Leary and Stanfield (1986) found that the outgrowth of these axons in the hamster is protracted. In the rat, we noted an increase of the number of labelled fibres after the arrival of the first pioneer axons. From our results combined with the quantitative electron microscopic data on the development of the rat cervical spinal cord (Gorgels et al,1989) as well as those reported by Schreyer and Jones (1982) and Stanfield and O'Leary

(1985b) it can be concluded that in the rat CST a staggered mode of growth occurs: axons are added to the CST several days after the initial arrival at a given spinal cord level of the leading fibres.

Prior to the arrival of the leading CST axons the region in which the CST eventually will develop (the pre-arrival zone) is composed of fascicles of unmyelinated profiles surrounded by lucent amorphous structures (Fig.2). Whether these small unmyelinated profiles (0.5 µm in diameter) represent axons or astroglial processes (Ralston et al.,1987) can not unambiguously be determined. However, the presence in these profiles of neurofilaments, neurotubules, small dense mitochondria, a smooth endoplasmatic reticulum and particularly their regular contour strongly suggest that they represent unmyelinated axons (Peters et al.,1976; Thomas et al.,1984; Angevine,1986), belonging to either the fasciculus gracilis or the fasciculus cuneatus.

According to Schreyer and Jones (1982) the lucent amorphous structures in the PT-area enwrapping the fascicles of unmyelinated profiles are large, irregularly mainly rostro-caudal oriented glial processes with 'watery' cytoplasm. However, most glial precursors are not 'watery' and they usually contain glycogen granules and free ribosomes (Peters et al.,1976; De Kort et al.,1985; Angevine,1986). Moreover, these lucent

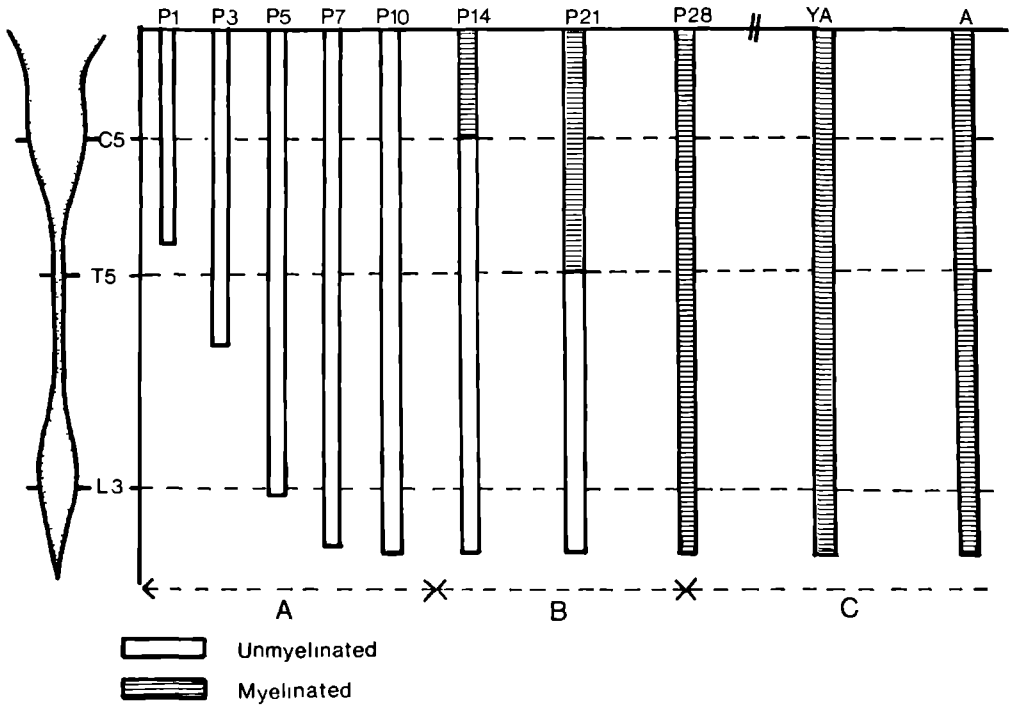


Fig.14 : Postnatal development of the rat corticospinal tract. A: outgrowth phase (P1-P10);B: myelination phase (P10-P28);C: maturation phase (P28-A). During myelination the ratio between myelinated and unmyelinated axons gradually increases, but a number of unmyelinated axons remains present up to the adult stage.

amorphous stuctures are longitudinally oriented parallel to the fibre bundles (Fig.2) (De Kort et al.,1985), whereas pale processes belonging to astrocytes in the perinatal rat spinal cord show a randomness of orientation (Phelps,1972). Similar clear processes of radial glia in the embryonic mouse spinal cord (Henrikson and Vaughn,1974) are characterized by their radial orientation. These characteristics are not in favour of Schreyer and Jones' (1982) interpretation. On account of the additional electronmicroscopic features, such as the amorphous structures containing clusters of round vesicles, mitochondria and large vacuole-like stuctures (Fig.2), these profiles might also represent preterminal varicosities of growing ascending axons (De Kort et al.,1985).

During the outgrowth phase,labelled growth cones and unmyelinated axons sometimes contain electron-dense stuctures, occasionally appearing as a series of loosely packed lamellae (Fig.4). These dense lamellar inclusions are disintegrating mitochondria, which might be signs of axon-elimination (Williams et al.,1986; Lasek and Katz,1987). The elimination of early formed corticospinal axon collaterals , either by withdrawal or by degeneration, was demonstrated for layer V neurons in the occipital part of the cortex in the rat (Stanfield et al.,1982; Stanfield and O'Leary,1985b). These axons attain their most caudal extension in the rat spinal cord at P7 and then gradually disappear up to P14 (see Chapter 2B).

Myelination

Myelination of the CST axons in the rat spinal cord starts rostrally (C5) at about P14 and moves caudally during the third postnatal week (Fig.14). Although at C5 the first labelled myelinated axons could be observed at P14, a number of unlabelled myelinated axons were present in the ventralmost part of the dorsal funiculus at P7 or P10 (Fig.9). Myelination of the fibres in the fasciculus gracilis as well as the fasciculus cuneatus starts at P5 and is completed between P15 and P20 (Matthews and Duncan,1971). During the period P5-P10, some relatively large myelinated fibres (2-3 µm) were seen in the PT-area, but they were found close to the border of the fasciculus cuneatus and probably represent aberrant cuneate fibres. At the end of the fourth postnatal week most of the labelled CST fibres at all three levels studied are myelinated.

Myelination is of obvious behavioural significance. However, the CST in rat can fulfil a behavioural function before myelination. There exists a close temporal relationship between the appearance of fore- and hindlimb placing responses and the arrival of corticospinal axons and their presumed formation of functional synapses within the spinal gray at the appropriate levels of the rat spinal cord (Donatelle,1977). Placing responses are less well coordinated in young postnatal animals than in adult rats (Huttenlocher,1970; Stelzner,1971; Donatelle,1977).

The adult stage

Although previous studies (Langford and Coggeshall,1981; Leenen et al.,1982,1985; Chung and Coggeshall,1987) demonstrated the presence of numerous unmyelinated fibers in the rat PT, the origin, destination and function of these fibers was unknown. Our results indicate that at least a number of these unmyelinated axons in the adult rat PT have their origin in the cortex and are apparently CST fibers. Due to the uneven distribution of HRP-TMB-(AHM) crystals along the length of unmyelinated axons (Fig.8), the question whether the population of unmyelinated profiles in the adult PT exclusively consists of CST axons remains unanswered.

Acknowledgement

The authors are grateful to Drs.E.De Kort, T.Gorgels, P.van Mier and R.Nieuwenhuys for critical reading of this manuscript.
We also want to thank Theo Hafmans for his excellent photographic assistance.

CHAPTER 4C: UNMYELINATED CORTICOSPINAL AXONS IN ADULT RAT PYRAMIDAL TRACT AN ELECTRON MICROSCOPIC TRACER STUDY.

SUMMARY

The aim of the present study was to provide experimental ultrastructural evidence for a corticospinal component in the adult rat pyramidal tract (PT). For this purpose the entire sensorimotor and frontal cortex of the left hemisphere was labelled using the anterograde tracer horseradish-peroxidase (HRP). Six months old rats were sacrificed 24 or 48 hours after implantation of six to eight HRP-gels. The detection of anterogradely transported HRP at the cervical as well as the lumbar intumescence was carried out as described earlier (J.Histochem.Cytochem. 35 (1987):623-626).
Our results demonstrate the occurrence of labelled myelinated as well as labelled unmyelinated axons within the adult rat PT at both spinal cord levels analyzed. This implicates that at least part of the unmyelinated profiles in the adult rat PT belong to fibres originating in the cortex and therefore must be interpreted as corticospinal axons. The findings are discussed in the light of their physiological significance.

Key words :Pyramidal tract - corticospinal tract - unmyelinated axons - anterograde tracing - rat

INTRODUCTION

The pyramidal tract (PT) is a major descending fibre tract of the central nervous system and is known to be an important motor pathway, which is only present in mammals. It is defined as a set of fibres passing through the medullary pyramids. One of its major components is the corticospinal tract (CST). The CST in the rat is a substantial pathway which after passing through the medullary pyramids decussates and subsequently proceeds via the contralateral dorsal column through the whole length of the spinal cord (Dunkerley and Duncan,1969; Brown,1971; Schreyer and Jones,1982; Gribnau et al.,1986). The PT has been shown to contribute to the control of fine manipulatory movements of the hand in primates (Lawrence and Kuypers,1968; Beck and Chambers,1970; Hepp-Reymond et al.,1970). Impairment of motor function of the forepaw has been demonstrated in rats and hamsters following PT lesions (Castro,1972; Kalil and Schneider,1975).

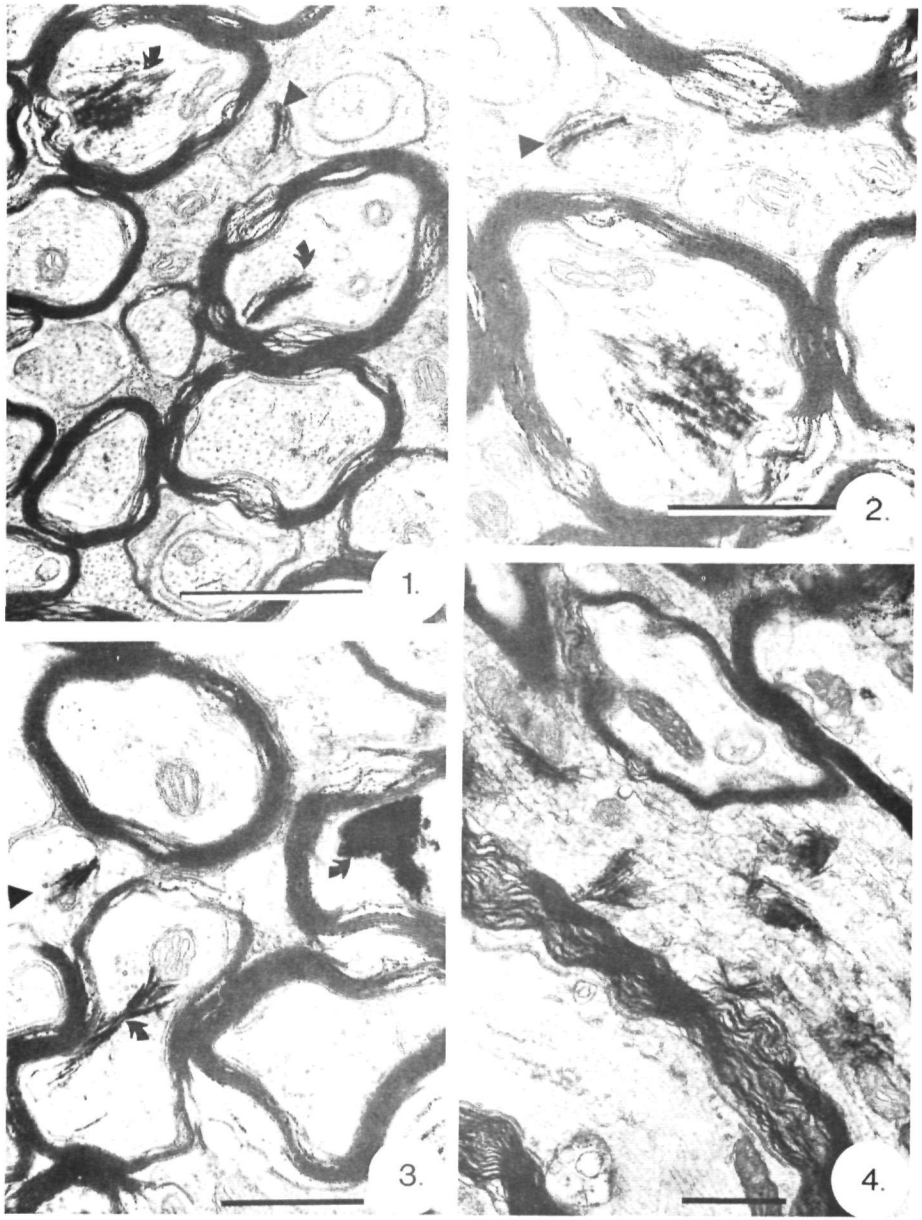
Electron microscopical observations provided evidence that the PT

contains a substantial amount of unmyelinated profiles, not only in rodents (Langford and Coggeshall,1981; Leenen et al.,1982; Reh and Kalil,1982), but also in cat (Thomas et al.,1984) and monkey (Thomas, 1985), although the presence of unmyelinated axons in the monkey is questioned by Ralston et al.(1987). Leenen et al. (1985) counted a total number of 43,000 ± 2,000 myelinated and 35,000 ± 8,000 unmyelinated fibres at the level of the second cervical segment in the rat; at the second sacral segment still 2,800 unmyelinated fibres could be detected (Chung and Coggeshall,1987). The site of their originating neurons still is a a matter of speculation and therefore the object of the present study.

MATERIALS AND METHODS

Three Wistar rats aged six months (weighing appr. 250 g.) were anesthetized by IP-injections with Nembutal. The entire sensorimotor and frontal cortex of the left hemisphere was labelled by implantation of six to eight HRP-gels (Griffin et al.,1979). Using this technique a depot of tracer is established for protracted HRP transport and therefore its enhanced detection along the length of the axons. On the other hand for quantitative studies probably the use of the more sensitive tracer wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) is more appropriate in order to label greater numbers of axons. The post-implantation survival times were either 24 or 48 hours. After reanesthetization the animals were transcardially perfused with 50 ml. 5% sucrose in phosphate buffer (PB) (pH 7.2) followed by 1% paraformaldehyde and 2% glutaraldehyde in the same buffer. After perfusion, the brains and spinal cords were immediately removed and the spinal cords were further immersed for about 1 hour before being transferred into cold (4°C) PB with 5% sucrose. Then 100 µm vibratome sections of the cervical (C5/C6) and lumbar (L2/L3) intumescence were cut transversely on an Oxford Vibratome. Anterogradely transported HRP was visualized with the use of the chromogen tetramethylbenzidine (TMB) in combination with the stabilizing agent ammoniumheptamolybdate (AHM) in 0.1 M PB at pH 6.0 as introduced by Olucha et al.(1985) for light microscopy and adapted for electron-microscopy (Joosten et al.,1987[a]). In essention the vibratome sections were presoaked for 20 min. in a mixture containing 0.005% TMB (dissolved in absolute ethanol) and 0.25% AHM in 0.1M PB (pH 6.0). The incubation was started by the addition of 50 µl. of 33% H_2O_2 per 100 ml. pre-incubation bath, which was repeated every 5 min. for 20 min. Control sections were processed identically but the incubation was carried out without TMB. After the HRP-TMB-(AHM) reaction, osmication was carried out using 1% OsO4 solution in 0.1M PB (pH 5.0) for 4 hours at room temperature. Osmication was followed by an accelerated dehydration and subsequent embedding in Epon.

Transverse or longitudinal ultrathin sections were mounted on 75-mesh formvar (0.8%)-coated grids contrasted with uranylacetate (20 min.) and



Figs.1-4: **Fig 1.:** Cervical Intumescence; 48 hours survival time. The labelled CST comprises myelinated as well as unmyelinated axons (arrowhead). Note the typical crystalline appearance of the HRP-TMB-(AHM) reaction product (arrows). Transverse section. Bar = 1 µm. **Fig.2.:** Cervical Intumescence; 48 hours survival time. Detail of Fig.1. Transverse section. Bar = 0.5 µm.

Fig.3.: Lumbar Intumescence; 24 hours survival time. Detail of a labelled unmyelinated axon (arrowhead) surrounded by labelled myelinated axons (arrows). Transverse section. Bar = 0.5 µm.

Fig.4.: Lumbar Intumescence; 48 hours survival time. Longitudinal section of labelled unmyelinated axons. Note the uneven distribution of HRP-TMB-(AHM) crystals within the unmyelinated axons. Bar = 0.5 µm.

lead citrate (5 min.) respectively, and were studied in a Philips EM-300 at an accelerating voltage of 60 kV.

RESULTS

Our results demonstrate that the adult rat PT contains many HRP-TMB-(AHM) labelled myelinated and a number of unmyelinated axons originating in the cortex, at both the cervical as well as the lumbar intumescence (Figs.1-4). The occurrence of labelled profiles is restricted to the PT-area of the dorsal funiculus. The results obtained after 24 hours survival time were identical to those obtained after 48 hours, at least as far as the white matter is concerned. Control sections always were negative.

At the ultrastructural level the HRP-TMB-(AHM) reaction product generates a clearly visible and highly discernable intracellular crystalline marker, which makes the identification of HRP-labelled fibres rather easy (Figs.1-4). Besides, TMB proved to be the most sensitive chromogen available for the demonstration of HRP labelling (Mesulam, 1979). Longitudinal sections clearly demonstrate the uneven distribution of the reaction product along myelinated as well as unmyelinated axons (Fig.4). Because of the uneven distribution of HRP-TMB-(AHM) crystals in labelled profiles, quantification of the results should be carried out very carefully. Transversely sectioned unlabelled profiles might contain the HRP-TMB-(AHM) reaction product at more distal or proximal levels (Fig.4). Because of this uneven distribution pattern it is very easy to underestimate the quantity of labelled unmyelinated CST axons.

Whereas the diameters of the labelled unmyelinated corticospinal axons remain fairly constant at about 0.2 µm, labelled myelinated CST axons considerably vary with respect to their diameters, namely between 0.5 and 3 µm. (Figs.1 and 3).

DISCUSSION

Although previous studies (Langford and Coggeshall,1982; Leenen et al.,1982,1985; Chung and Coggeshall,1987) demonstrated the presence of numerous unmyelinated profiles in the adult rat PT at several spinal cord levels, the origin, destination and function of these profiles still are unknown. Based on longitudinal sections Ralston et al.(1987) concluded that most of the profiles which might be interpreted as unmyelinated axons in the primate PT (Thomas,1985) at medullary levels are actually astroglial processes. They stated that less than 1% of the PT in the old world adult primate are unmyelinated axons. Our results demonstrate that at least part of the unmyelinated axons in the adult rat PT have their origin in the cortex.

From physiological studies on the conduction velocities of PT axons it was concluded that all measurements are within the range of myelinated axons (Mediratta and Nicoll,1983). Based on their different biophysical

properties, their different connections as well as their varying conduction velocities PT cells are subdivided into two distinct populations (Takahashi,1965; Deschenes et al.,1979): first, slow conducting PT cells with conduction velocities below 21 m/s. (the slowest velocities reported are 4-6 m/s.), and secondly, fast PT cells whose axons conduct at velocities of 21-90 m/s. Fast conducting PT neurons discharge phasically and are involved in the initiation and control of brief, quick movements. On the other hand slowly conducting PT neurons discharge in tonic phase whereas they are implicated in the determination of muscle tone and control of small, fractioned movements (Humphrey and Corrie,1978; Wiesendanger,1981; Biedenbach et al.,1986). Although Leenen et al. (1985) suggested that the unmyelinated axons in the adult rat PT might have a similar function as the slowly conducting PT neurons the conduction velocities measured (Takahashi,1965; Mediratta and Nicoll,1983) are not in the range to be expected for unmyelinated axons. Remarkably, even the slowest conduction velocities reported (4-6 m/s.) (Takahashi,1965; Deschenes et al.,1979; Mediratta and Nicoll,1983; Landry et al.,1984) do not correspond with the category of myelinated axons with fibre diameters of 0.5-1.0 µm. in the rat PT (Dunkerley and Duncan,1969). Possibly, the unknown mode of collateralization of CST axons may hamper an accurate determination of conduction velocities.

The unmyelinated CST axons in the adult rat PT at least in part may represent collaterals of myelinated axons, which either reach out into the spinal gray or, being recurrent collaterals, even might return to more proximal destinations. On the other hand, the unmyelinated CST axons also might represent axons which not yet have acquired myelin sheaths. This would implicate that myelination of CST axons still occurs at a low level during adulthood, because myelination appears to be completed at postnatal day 28 at the cervical as well as at the lumbar intumescence (see Chapter 4B). Unilateral pyramidal lesions in monkeys revealed that recovery occurs by local sprouting of CST fibres of the intact bundle into contralateral areas of spinal neurons with severed homolateral CST connections (Kucera and Wiesendanger,1985). Probably unmyelinated CST axons in the adult PT account for this recovery function.

A recent study on the corticospinal projection neurons in adult rats (Miller,1987) using retrograde HRP-techniques revealed, besides great concentrations of HRP-positive neurons in the sensorimotor cortex, less dense concentrations in anterior cingulate and prefrontal cortical areas. Neurons from the latter regions also might account for the unmyelinated CST projections. Hence, we currently are dealing with the ultrastructural visualization of CST axons in the adult rat PT, with their originating neurons in (pre-) frontal cortex areas.

Acknowledgement

We are grateful for the technical and photographic assistance from Jos Dederen and Theo Hafmans, respectively.

Chapter 5

Outgrowth and guidance of pyramidal tract axons in rat

CHAPTER 5A INTRODUCTORY NOTE: OUTGROWTH AND GUIDANCE

The highly integrative functional capacity of the CNS is based upon the very complex network of its neuronal connections. The efficiency of the CNS depends on the accurate formation of neuronal connections generated during development. One of the main events during CNS development is the outgrowth and subsequent target finding of the axons through diverse and changing environments. The accuracy in which outgrowing axons select their pathways and create neuronal connections is an intriguing phenomenon.

Anatomical and physiological experiments on the development of neural circuits in the insect limb revealed that all axons recognize the appropriate signals along their route: not only the initial or pioneer axons are capable of navigational feats but also later differentiating axons have similar characteristics (Edwards et al.,1981; Ho and Goodman, 1982;Bentley and Caudy,1983;Bentley and Keshishian,1982). The elimination of the appropriate signals along the route alters the formation of a pathway (Bentley and Caudy,1983). The fact that all developing axons in insect appendages obey the same rules of outgrowth leads to the hypothesis that invertebrate neural circuits are internally programmed. Recent experiments (Chiba et al.,1988), however, demonstrated the extrinsic refinement of initial synaptic contacts during insect sensory system development, indicating that invertebrate neuronal circuits are not as definitely intrinsically programmed as one once thought.

Although outgrowing vertebrate axons might as well be internally programmed, the guidance to their targets clearly appeared to depend on precise and coordinated interactions with their environment. Outgrowing axons respond to their environment by means of a motile sensory apparatus at their tip, termed the growth cone (Landis,1983; Lockerbie,1987; Lasek and Katz,1987 among others). In vitro as well as in vivo experiments revealed the striking changes of growth cone morphology in different cellular environments (see Chapter 3). This indicates that growth cones are able both to explore and to respond to their local environment. The fact that growing axons separated from their cell bodies continue to grow proves that growth cones must contain the machinery needed to sense their specific paths and eventually to direct towards their targets (Harris et al.,1987).

At present, a number of cues or mechanisms are considered to play a prominent role in the guidance of outgrowing axons to their final destinations (Purves and Lichtman,1985; Edelman,1985; Dodd and Jessell,1988). For instance mechanical factors such as paths of less resistance, interfaces or aligned tracts of cells might direct outgrowing axons to the right place (Horder,1978). A representative of the concept of mechanical routing of axons or stereotropism is the so-callled blue-print hypothesis as developed in lower vertebrates (Singer et al.,1979): extracellular spaces in the embryonic neural epithelium may

form channels through which growth cones migrate. The occurrence of a channel-like guidance system, consisting of glial or presumptive glial cells was substantiated in the developing optic nerve (Silver and Rutishauser,1984; Silver,1984) and corpus callosum (Silver etal.,1982) of the mouse.

In vitro experiments demonstrated the clear response of outgrowing axons on electrical influences (Jaffe and Poo,1979; Bray,1979). In developing Xenopus embryo's voltage gradients are commonplace and can influence axonal growth (Jaffe,1981). Nevertheless, electrical influence or galvanotropism is, up till now, considered to be not a very important guidance cue for outgrowing axons.

Axonal guidance also can be explained by more or less direct chemical interactions between the environmental structures and the growth cones: differential adhesiveness. Already in 1963, Sperry (1963) put forward the chemoaffinity hypothesis on a direct interaction between growth cones and their micro-environment. This concept was based on the proposition that axon guidance and target recognition are achieved by the operation of highly specific chemical affinity cues between individual neurons. Athough different molecules were identified that provide permissive substrates for axon extension, the complexity inherent to Sperry's model hampered its validity. The molecules involved in the adhesive type of axon extension now can be subdivided in two categories the general CAM's and the local CAM's (see Table 1).

In the developing vertebrate nervous system three general adhesive molecules were identified, which are expressed on early neuro-epithelial and mesenchymal cells as well as on the extracellular matrix (ECM) and which might be important in initial axon extension. In addition to these general adhesive molecules acting in the guidance of growth cones, the

Table 1 : Molecules involved in adhesive axon extension in developing CNS

Category	Name	Ref.
General	N-CAM	Rutishauser et al.,1976
various species	N-Cadherin	Takeichi,1987
	Laminin	Hynes,1987
Local rat/mouse	L1 (NILE/69A1)	Rathjen and Schachner,1984; Stallcup and Beasley,1985; Pigott and Kelly,1986
	TAG-1	Dodd et al.,1988
chicken	Ng-CAM (G4/8D9)	Grumet et al.,1984[a]; Lagenauer and Lemmon,1987; Rathjen et al.,1987[a]
	Neurofascin	Rathjen et al.,1987[a]
	F11	Rathjen et al.,1987[b]

latter phenomenon also depends on the recognition of more local adhesive cues (see Table 1). For example, cell surface molecules located on oligodendrocytes inhibit axon extension and therefore contribute to the local selection of axonal pathways (Sommer and Schachner,1981; Caroni and Schwab,1988a,1988b; for a review see Patterson,1988). Other glycoproteins,which are restricted to axonal surfaces of later differentiating neurons, as for instance L1 (Rathjen and Schachner,1984), probably are involved in axon outgrowth and guidance by means of selective fasciculation (Rathjen,1988; Dodd and Jessell,1988; Rutishauser and Jessell, 1988).

Besides the mutual adhesive interactions among axons or between axons and micro-environment, growth cones may be guided by the mechanism of chemotropism. Although diffusable gradients of specific molecules probably play a prominent role in the attraction or repulsion of outgrowing axons to their targets (Kapfhammer and Raper,1987) the only well-defined molecule for which a chemotrophic role has been demonstrated in the nervous system is nerve growth factor (NGF) (Mensini-Chen et al.,1978).

Although several mechanisms as presented above are involved in the guidance of outgrowing axons, none of them solely provides a satisfactory explanation. It is most likely that during axon-outgrowth and guidance a number of cues act together to create adequate neuronal connections. Given this perspective, the involvement of two different putative guidance cues was studied in the developing CST: the role of astrocytes (Chapter 5B) on the one hand and that of the cell adhesion molecule L1 (Chapter 5C1,5C2) on the other.

CHAPTER 5B: ASTROCYTES AND GUIDANCE OF OUTGROWING CORTICOSPINAL TRACT AXONS IN THE RAT. AN IMMUNOCYTOCHEMICAL STUDY USING ANTI-VIMENTIN AND ANTI-GLIAL FIBRILLARY ACIDIC PROTEIN.

SUMMARY

In the present study the role of astrocytes and their precursors in guidance of outgrowing corticospinal tract axons in the rat is studied. Antibodies against glial fibrillary acidic protein and vimentin are used to analyse immunogen expression of glial cells, whereas the postnatal outgrowth of corticospinal axons through the spinal cord was studied using anterogradely transported horseradish-peroxidase. The first, leading corticospinal axons, being the objective of the present study, are characterized by dilatations at their distal ends, the growth cones. Growth cones of pioneer corticospinal axons are randomly distributed in the presumptive corticospinal tract area of the ventralmost part of the dorsal funiculus. A dramatic change in glial cell labelling is found from the majority being vimentin immunoreactive and glial fibrillary acidic protein negative at birth to almost all being the reverse at the end of the fourth postnatal week. From double labelling experiments it can be concluded that the vimentin-glial fibrillary acidic protein transition occurs within astrocyte precursor cells. The absence of glial fibrillary-immunoreactive glial cells during the outgrowth period of pioneer corticospinal axons indicates that they cannot play a role in the guidance of outgrowing corticospinal pioneer axons.

Vimentin-immunoreactive glial cells are present throughout the presumptive corticospinal tract area at the time of arrival of the leading corticospinal fibres. The vimentin-immunoreactive glial cells which themselves are orientated perpendicular to the outgrowing corticospinal tract axons, are mainly arranged in longitudinal tiers parallel to the rostrocaudal axis. Electronmicroscopically, growth cones of pioneer corticospinal tract axons frequently exhibit protrusions into vimentin-immunoreactive glial cell processes, suggesting an adhesive type of contact. Therefore, in addition to a positional role, vimentin-immunoreactive glial cells probably play a chemical role in guidance of pioneer corticospinal tract axons.

A prominent vimentin-immunoreactive glial septum was noted during corticospinal tract outgrowth in the midline raphe of the medulla oblongata and spinal cord whereas it is absent in the decussation area of corticospinal tract fibres. After the first postnatal week the major vimentin-immunoreactive glial barrier either completely disappears (medullary levels) or gradually reduces to a minor glial fibrillary acidic protein-immunoreactive one (spinal cord levels).

This glial septum is suggested to play a physical role in guidance of outgrowing CST axons in preventing them from decussation.

Key words: Guidance - Glia - Corticospinal tract - Development - Immunoelectron microscopy - Anterograde tracing.

INTRODUCTION

Different roles have been proposed for glia during development of the central nervous system (CNS). Initially, radial glial cells of the embryonic CNS were advocated to play an important role in guiding migrating neuronal cells (Rakic,1971; Rakic et al.,1974). Later on, radial glial cells and their astrocytic derivatives (Choi,1981), were associated with guidance of axon outgrowth. The discovery of glial channels (Silver and Robb,1979; Singer et al.,1979), the glial sling (Silver et al.,1982) and the glial barricade (Silver and Rutishauser, 1984; Hankin and Silver,1986) represent the first evidence that glial structures along the pathway of developing CNS fibre tracts are organized in such a way that the glial substrate may play an important role in guiding outgrowing axons. In a number of studies, however, the presence and the guidance role of such glial channels was questioned (Schreyer and Jones,1982; Valentino and Jones,1982; Valentino et al.,1983). Although Schreyer and Jones (1982) could not rule out the possibility that pioneer axons in the rat pyramidal tract (PT) grew along some preferred glial channels, they were not able to detect an orderly configuration of glia at times prior to the arrival of the axons.

Using immunocytochemical staining a palisading pattern of subpial astroglial processes in the adult rat brain (Bitner et al.,1987) as well as of the radial glia in the CNS of the embryonic mouse (Dupouey et al.,1985) could be observed. This palisading organization may have a role in the construction of the developing CNS.

From previous studies (Gribnau et al.,1986; see Chapter 4B) it was concluded that the process of CST outgrowth can be subdivided into two subsequent phases: the first,leading fibres are succeeded by the main wave of developing CST axons. The latter are growing in a fasciculative way. The guidance of the first pioneer axons is the objective of the present study.

Pathfinding axons, having growth cones at their distal ends (Schreyer and Jones,1982; Mason,1985; Holley and Silver,1987), may well be conducted by chemical cues released by mediating glia e.g. neuron-glia cell adhesion molecules (NgCAM) as suggested by Grumet et al.(1984^{b}).

The use of specific glial markers may provide more conclusive evidence on the localization and orientation of glial cells and their processes in relation to outgrowing pioneer CST axons. Glial fibrillary acidic protein (GFAP) is a 51 kDa intermediate filament protein (Dahl,1981; Bovolenta et al.,1984, 1987) and a chemical marker of protoplasmic (Ludwin et al., 1976) and fibrous astrocytes (Bignami and Dahl,1973,1974; Bignami et al.,1972; 1982). Using the immunofluorescence method GFAP-ir astrocytes have been demonstrated in developing rat pyramidal tract (Bignami and Dahl,1973). Vimentin (VIM) is a 57-58 kDa intermediate filament protein and the major cytoskeletal component in immature astroglia (Dahl et al., 1981;Bovolenta et al.,1984). Immunofluorescence methods using polyclonal

antibodies to VIM showed subpopulations of astrocytes in the adult and immature rat (Dahl et al.,1981; Shaw et al.,1981; Bignami et al.,1982).

Whereas the presence of GFAP has been demonstrated in primate radial glia (Choi,1981; Levitt et al.,1983), rodent radial glia do not contain GFAP (Bignami and Dahl,1973,1974; Schnitzer et al.,1981) but the intermediate filament protein VIM (Dahl et al.,1981; Schnitzer et al,1981; Bovolenta et al.,1984; Pixley and DeVellis,1984). Contrastingly, mature astrocytes in the rat predominantly exhibit GFAP. The VIM-GFAP transition in rat brain occurs at the second to third postnatal week as was biochemically determined by Dahl (1981).Immunocytochemical results of Pixley and De Vellis (1984) substantiated the VIM-GFAP transition in rat glial cytoskeleton to occur at the time of myelination, as was affirmed by Bovolenta et al.(1984,1987) in mouse cerebellum and visual pathway. Double labelling experiments have shown that during this transition astrocytes both in culture and in vivo express two different types of intermediate-sized filaments, e.g. VIM-filaments and filaments containing GFAP (Schnitzer et al.,1981; Bovolenta et al.,1984). Bovolenta et al.(1987) demonstrated the presence of intermediate filaments composed of VIM subunits in radially oriented glial precursor cells in mouse optic nerve. At the time outgrowth of primitive glial processes occurs VIM disappears and GFAP is expressed with abundant filament assemblage.

In the present study the possible role of astrocytes in the guidance of outgrowing pioneer CST fibres is analysed using glial VIM and GFAP expression in conjunction with HRP labelling of outgrowing CST axons.

MATERIALS AND METHODS

Tissue preparation

Thirty-five Wistar rats ranging in age from embryonic day 18 (E18) to postnatal day 29 (PND29) were used in this study. In our colony litters were usually born on the 22nd day of gestation (E22=PND0). For HRP labelling all postnatal rats were anesthetized with sodium pentobarbital (18 mg per kg body weight, i.p.). Then the entire sensorimotor and frontal cortex of the left cerebral hemisphere was labelled by implantation of HRP-gels (Griffin et al.,1979), which were placed after opening the skin and making small holes in the skull using a fine needle. The postimplantation survival times were kept constant at 24 hours. The ages of the animals given in the present paper are the ages at their respective days of perfusion.

After anesthetization rats were either perfused with paraformaldehyde-lysine-periodate-glutaraldehyde (PLPG) (McLean and Nakane,1974) in 0.1 M phosphate buffer (PB, pH 7.2) or freshly prepared. For electronmicroscopy the rats were perfused with 1% paraformaldehyde and 2% glutaraldehyde (Joosten et al.,1989). The brains and spinal cords were resected from the skulls and vertebral columns respectively, and then postfixed by

immersion during 2 hours. PLPG-fixed tissue was cryoprotected with sucrose (8.5% sucrose in 0.1 M PB, pH 7.2 during 15 hours at 4°C and then frozen by immersion in liquid nitrogen-cooled isopentane (2-methyl butane), whereas freshly prepared tissue was not cryoprotected before freezing. Samples of the medulla oblongata (MO), the CST decussation area, the cervical intumescence (fifth and sixth cervical segment C5/C6) and the lumbar enlargement (second and third lumbar segment L2/L3) were cut transversely on a Reichert cryostat. The 10 um. sections were collected on albumin/glycerin (1:1) slides.

HRP-visualization

Anterogradely transported HRP was visualized using tetramethylbenzidine (TMB) in combination with the stabilizing agent ammoniumheptamolybdate (AHM) in 0.1 M PB at pH 6.0 (Olucha et al.,1985; see Chapter 4A). Briefly: after rinsing twice for 1 min. in distilled water, each 10 µm. frozen section was presoaked for 20 min. in a mixture containing 0.25% AHM in 0.1 M PB (pH 6.0) and 0.005% tetramethylbenzidine (TMB, dissolved in absolute ethanol). The incubation was started by adding 50 ul of 33% H_2O_2 per 100 ml pre-incubation bath. This addition was repeated every 5 min for 20 min. The HRP-reaction was terminated by rinsing the sections in two 1 min. baths of 0.1 M PB (pH 6.0). The sections were counterstained with 0.5% neutral red, rapidly dehydrated in alcohol, cleared in xylene and enclosed in Depex. Control sections were processed identically but the incubation was carried out without TMB.
For EM visualization of anterogradely transported HRP 100 µm. vibratome sections (Oxford vibratome) were used, which were treated similarly as for LM visualization. Osmication then was carried out using a 1% OsO_4 solution in 0.1 M PB (pH 5.0) for 4 hours at room temperature. Further processing was accomplished by an accelerated dehydration procedure and embedding in Epon 812. Ultrathin sections were mounted on 75-mesh formvar (0.8%)-coated copper grids, contrasted with uranyl acetate (20 min) and lead citrate (5 min) (Joosten et al.,1987a).

Antisera

Anti-GFAP serum (DAKO,no Z334 as specified by Tascos et al.(1982) and Baumal et al. (1980) was used in a dilution of 1/100. The detection of VIM immunoreactive glial cells was carried out with a monoclonal anti-VIM serum (DAKO,no M725 as described by Osborn et al.(1984), diluted 1/10.
For double labelling both the mouse monoclonal anti-VIM and the polyclonal anti-GFAP were applied simultaneously.

The following secondary antisera were used: sheep-anti-mouse conjugated with fluorescein-isothiocyanate (FITC) (Sigma no F6257), diluted 1/100 and a goat-anti-rabbit conjugated with tetramethylrhodamine-isothiocyanate (TRITC) (Statens Bakteriologiska Lab), diluted 1/20. For immunoelectronmicroscopy a rabbit-anti-mouse serum conjugated with HRP (DAKO,no P260) was used.

Additionally, reference incubations were carried out with either a

polyclonal antiserum against GFAP (diluted 1/65) or a polyclonal antiserum against VIM (diluted 1/20) (both generous gifts from Dr.F.Ramaekers), as described by Ramaekers et al.(1981,1983).

Indirect immunofluorescence.

Sections were incubated with the primary antiserum for 1 hour at room temperature. After rinsing twice in 0.1M PB (pH 7.2, 10 min each) incubation was started with the second antibody, and was continued for 1 hour at room temperature in the dark. Sections were then rinsed in 0.1M PB (pH 7.2) and mounted in glycerol/aqua dest.(3:1). For double labelling both antisera were applied simultaneously, e.g. after application of the first antibodies, sections were washed with 0.1M PB (pH 7.2) and then treated with fluorochrome-coupled second antibodies. Under these conditions, each second antibody reacts specifically with its corresponding primary antiserum. Control sections were processed identically but without addition of the primary antiserum. For each antiserum used control sections were incubated with the respective normal antiserum or 0.1 M PB (pH 7.2).

Immunoelectronmicroscopy.

Transverse, longitudinal or sagittal 100 µm vibratome sections were incubated with the monoclonal anti-VIM serum (DAKO) (dilution 1/2400) for 16 hours at 4°C. After rinsing twice in 0.1.M PB (pH 7.2) the sections were treated with a rabbit-anti-mouse serum conjugated with HRP (dilution 1/50) for 1 hour at room temperature. After rinsing twice, once again in 0.1 M. PB (pH 7.2) the HRP was visualized using the chromogen diaminobenzidine (DAB) and Nickel (Ni) as a stabilizing agent (Adams,1981). The specificity of the immunoreaction was tested by the addition of normal rabbit serum instead of the primary antibody.

After the HRP-DABNi reaction the sections were osmicated (1% OsO_4 in 0.1 M PB, pH 7.2 for 45 min), rapidly dehydrated in ethanol and embedded in Epon 812 on repelcoated slides. Semi-thin sections were counterstained in a 5% toluidine blue solution. Ultrathin sections were mounted on 75-mesh formvar (0.8%)-coated copper grids and contrasted with uranyl acetate (20 min) and lead citrate (5 min).

Photomicroscopy.

Photomicrographs of HRP-TMB-AHM sections were made with an automatic Zeiss-photomicroscope-II using dark field illumination and an Agfapan-25 film. Immunofluorescent sections were examined using a Zeiss microscope with epifluorescent equipment using the filter system appropriate for FITC or TRITC. Photographs were taken on a Kodak-Tri-X film (400 ASA).

Ultrathin EM-sections were viewed with a Philips-300 electron microscope at an accelerating voltage of 60 kV.

RESULTS

The present light and electron microscopic study of the development of the rat CST in medulla oblongata and spinal cord coherent with the expression of vimentine- and/or GFAP-ir glial cells comprises the period between embryonic day 18 (E18) and the 30th postnatal day (PND 30).

CST outgrowth

Pyramidal cells with their somata in layer V of cerebral cortical areas give rise to CST axons (Wise et al.,1979[a]; Neafsey et al.,1986). The outgrowth of the CST during the period studied is schematically shown in Fig.1.. This figure resulted from our present and earlier (Gribnau et al.,1986; Joosten et al.,1987[b]) light microscopic studies.
The timing of prenatal CST development is based upon results obtained on normal Rager-material. The latter analysis showed that the CST could be positively identified at the level of the pontine nuclei at E18 and at the pyramidal decussation at E21.

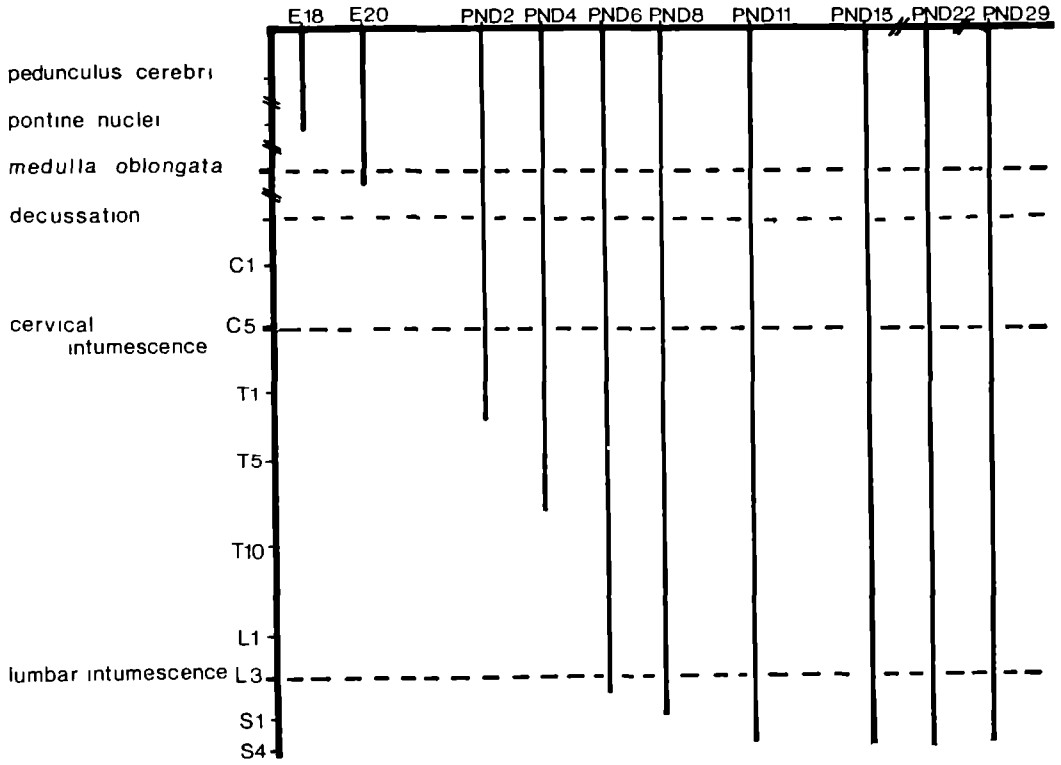


Figure 1 : Schematic representation of pre- and postnatal CST outgrowth. Prenatal observations are based on Rager-stained material. The postnatal CST outgrowth is experimentally studied with the use of anterogradely transported HRP.

The postnatal development of rat CST was studied using anterograde HRP labelling, in which HRP-gel implantations always encompassed both the somatosensory and the motor areas of the cerebral cortex of the left cerebral hemisphere. The outgrowth of labelled CST-fibres in rat spinal cord mainly occurs during the first ten postnatal days. As schematically shown in Fig.1 labelled CST axons caudally extend into the third thoracic segment at PND 2; into the tenth thoracic segment at PND 4; into the were third lumbar segment at PND 6 and finally into sacral segments at PND 8. Based on electron microscopic observations (Joosten et al.,1989) the outgrowth of the corticospinal component of rat pyramidal tract can be subdivided into two subsequent phases: the outgrowth of the main wave of labelled CST axonsis preceded by a number of pathfinding axons. Pioneer axons show labelled growth cones at their distal ends (Figs.2B and 2C). These growth cones are characterized by their relatively large diameters (1-3 μm), as compared with surrounding axons (approx. 0.2 μm.) as well as the fact that they were larded with organelles such as microtubuli, smooth endoplasmatic reticulum, mitochondria , and a large number of vesicles (Fig. 2B). This part of the growth cone was called vesicular-reticular zone (De Kort et al.,1985). The distal zone of the CST-growth cone, called fine granular zone (De Kort et al.,1985), contained a filamentous matrix. The crystalline HRP-TMB-(AHM) reaction product appeared to be present both at the distal as well as at the more proximal region (Fig.2B) of the CST growth cone. One or two days after the arrival of the labelled outgrowing tip of the CST at a given spinal cord segment,

Table 1: Vimentin and glial fibrillary acidic protein expression during corticospinal tract development in the rat.

	Age				
	E18	PND2	PND6	PND11	PND29
Medulla Oblongata					
Vimentin-IR	++	-	-	-	-
GFAP-IR	-	+	++	++	++
Lumbar Intumescence					
Vimentin-IR	++	++	+	-	-
GFAP-IR	-	-	-	++	++

The immunoreactivity within the CST outgrowth area at medullary levels is compared with that in the lumbar spinal cord. -: no reaction; +: positive reaction; ++: strong positive reaction. The transition period in medulla oblongata was between E18 and PND2, whilst that in the lumbar intumescence was between PND6 and PND11.

i.e. at the arrival of the mass of the axons (Chapter 4B), the labelled CST mainly consisted of unmyelinated profiles (their diameters ranging from 0.1 to 0.5 µm (Fig.2D)). As far as their morphology is concerned, these distal ends of the later arriving axons considerably differ from the large growth cones of pioneer axons as was also noted earlier in the rat pyramidal tract (Gorgels et al.,1989[a]) as well as in leading axons in developing amphibian spinal cord (Nordlander, 1987). Preliminary observations obtained on serial sectioning of pioneer growth cones and those of later arriving pyramidal tract axons corroborate these results (personal communication T.Gorgels).

The pioneer axons with their growth cones have entered an area which consisted of fascicles of unlabelled unmyelinated fibres surrounded by a composition of lucent amorphous structures (Fig.2A).The latter structures were characterized by their light cytoplasmic density, an irregular outline and often contained clusters of small lucent vesicles with diameters of about 100 nm. Occasionally, a few mitochondria and cisterns of smooth endoplasmatic reticulum were present within these structures (Figs.2A,2C). Structures with typical glial characteristics such as dense cytoplasm and rosettes of free ribosomes could be easily recognized (Fig.2C). Besides, profiles with a concentration of intermediate filaments incidentally were noted (Fig.2C), which probably may also be interpreted as glial processes.

VIM and/or GFAP-IR in relation to CST outgrowth

The immunoreactivity found in PLPG fixed tissue was identical with that obtained after methanol fixation. As far as LM observations are concerned only the results of the PLPG fixed tissue will be described in the present paper because of its superior tissue preservation. Control incubations, using either normal antisera or PB always were negative. Incubations with the polyclonal antisera against GFAP and VIM demonstrated an identical pattern of labelling as that obtained after the use of the DAKO-antisera (polyclonal anti-GFAP and monoclonal anti-VIM). Although during pre- and postnatal development VIM antisera clearly labelled blood vessels as well as meningeal tissues these structures could be easily discriminated against immunopositive glial cells because of their distinct morphology.

Table 1 presents a rough survey of the VIM and GFAP expression in the CST area between E18 and PND30. This table clearly demonstrates a level-dependent VIM-GFAP transition period. At medullary levels this transition occurred between E18 and PND2, whereas at lumbar spinal cord levels it occurred later, i.e. between PND6 and PND11.

At E18 and E20 the monoclonal VIM antibody revealed an intense labelling of numerous glial cells in the midline raphe of the medulla oblongata (Figs.3A,3C). Besides, this antiserum also positively reacted on radial glial processes during prenatal stages (not shown). At the same age, i.e. prior to and at the arrival of the first pioneer CST axons, the

medullary pyramids showed numerous dispersely situated VIM-ir glial cell processes (Figs.3A,3C). During embryonic stages only a few GFAP-ir processes could be observed at medullary levels, almost exclusively situated near the meningeal surface (Fig.3D).
Within the decussation area no accumulation of VIM-ir glial cell labelling could be observed in the midline raphe whereas scattered VIM-IR was present between the decussating axon bundles (Figs.3E,3F).

The first CST-axons reach upper cervical spinal cord levels at the day of birth (Fig.1). The lucent amorphous structures, as described in the CST outgrowth section did not show any VIM-IR. Prior to the arrival of CST axons in the ventralmost part of the dorsal funiculus of the cervical intumescence at E20, the presumptive CST area was characterized by the occurrence of VIM-ir glial cells (Fig.3B). By contrast the surrounding grey matter contained many VIM-ir glial cells, whereas an accumulation of VIM-ir glial cells could be noted in the midline between the central canal and the dorsal funiculus (Fig.3B).

During the first postnatal week pioneer CST axons have extended caudally up to sacral spinal cord levels. Prior to the arrival of the first labelled CST axons, i.e. PND4 for the lumbar enlargement (Fig.4C), the dorsal funiculus is characterized by the absence of GFAP-IR (Fig.4A; Table 1) and the presence of strong VIM-IR (Fig.4B; Table 1). At the time the first scattered HRP-labelled CST axons entered the lumbar intumescence (Figs.1 and 5C), no preferred localization of these axons either at the periphery or at the center of the ventralmost part of the dorsal funiculus can be noted (Fig.5C). At PND5 the dorsal funiculus still exhibited high VIM-IR, consisting of a major VIM-ir glial structure in the midline raphe whereas the entrance zone of the first CST-axons contained dispersely situated VIM-ir glial processes (Fig.5B). At this age and level no GFAP-IR could be noted (Fig.5A).
One day after the arrival of pioneer CST axons the main wave of labelled CST axons has entered the lumbar intumescence (Fig.6C). Between PND5 and PND6 the VIM-IR in the CST growth zone has decreased considerably (Figs. 5B,6B). Although during this period the GFAP-IR obviously has increased in the midline, the entrance zone of CST axons remained almost negative (Fig.6A).Double labelling experiments revealed the occurrence of both GFAP-IR and VIM-IR in the same cells in the dorsal median septum at PND6 (Figs.6A,6B).

Based on transverse cryo- and vibratome sections the VIM-ir glial cells seemed to be dispersely situated in the entrance zone at the lumbar enlargement at PND5 (Figs.5B,7A). Horizontal vibratome sections of the CST-area at this spinal cord level revealed a longitudinal alignment of the VIM-ir glial cells (Figs.7B,7C). The VIM-ir glial cells were mainly arranged in longitudinal tiers orientated parallel to the rostrocaudal axis (Figs.7B,7C). The distance between the VIM-ir palisades varied between 5 and 10 µm. (Fig.7C). This typical palisade-organization may have a role in the construction of the developing CST (see discussion).

VIM-ir glial processes were situated adjacent to growth cones (Figs.

Figure 2 : Electron microscopic visualization of postnatal CST outgrowth with the use of anterogradely transported HRP. **Fig.2A:** PND4-L3: The prospective CST region called pre-arrival zone. Within this zone fascicles of unmyelinated axons are surrounded by electron-light profiles (asterisks).Transverse section.Bar=0.5 µm. **Fig.2B:** PND5-L3: Entrance of the first CST axons with their growth cones (GC). Note the crystalline HRP-TMB-(AHM) labelling (arrows).Transverse section.Bar=0.5 µm. **Fig.2C:** Labelled CST growth cones (GC) surrounded by unmyelinated fibres as well as profiles which contain accumulations of intermediate filaments (thin arrow). Besides electron-light profiles (asterisks), structures with typical glial characteristics such as dense cytoplasm and rosettes of free ribosomes (gross arrows) can be seen. Transverse section. Bar=0.5 µm. **Fig.2D:** PND6-L3: Later arriving labelled CST axons (arrows). Note the presence of an astrocytic process (arrowheads).Transverse section. Bar=0.2 µm.

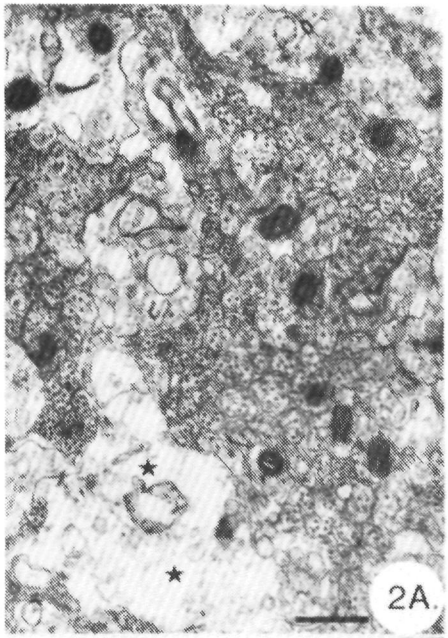
Figure 3 : Identification of astrocytes and/or their precursors using immunofluorescence techniques with anti-VIM and anti-GFAP at prenatal stages. **Top:Dorsal; Bottom:Ventral** .The CST area is marked with an asterisk (Figs.3A,3C,3D,3F) **Fig.3A:** anti-VIM, medulla oblongata (E18). Note the accumulation of VIM-ir glial cells in the midline raphe and the scattered VIM-ir glial cells in the pyramids. Bar=25 µm. **Fig.3B:** anti-VIM, cervical intumescence (E18). The presumptive CST area is designated by X. Bar=25 µm. **Fig.3C:** anti-VIM, medulla oblongata (E20). The medullary pyramids show numerous dispersely situated VIM-ir glial cells. **Fig.3D:** anti-GFAP, medulla oblongata (E20). Absence of GFAP-ir glial cells within the medullary pyramids; only a few GFAP-ir processes can be noted near the meningeal surface. Bar=25 µm. **Fig.3E:** anti-VIM, decussation area (PND0), the midline raphe is indicated by an arrow. Bar=50 µm. **Fig.3F:** detail from 3E, various axon bundles are marked with asterisks.

Figure 4,5 and 6 : Dorsal funiculus at the lumbar intumescence between PND4 and PND6. Double immunofluorescence staining with polyclonal anti-GFAP **(A)** and monoclonal anti-VIM **(B)** Adjacent sections are stained for HRP **(C)** . Top:Dorsal; Bottom:Ventral. **Fig.4:** PND4, i.e. prior to the arrival of the first labelled CST axons; the presumptive CST is marked with X.. **Fig.5:** PND5, i.e. the time the first labelled CST axons are present. **Fig.6:** PND6, i.e. the time of entrance of the main wave of labelled CST axons. Bar=50 µm.

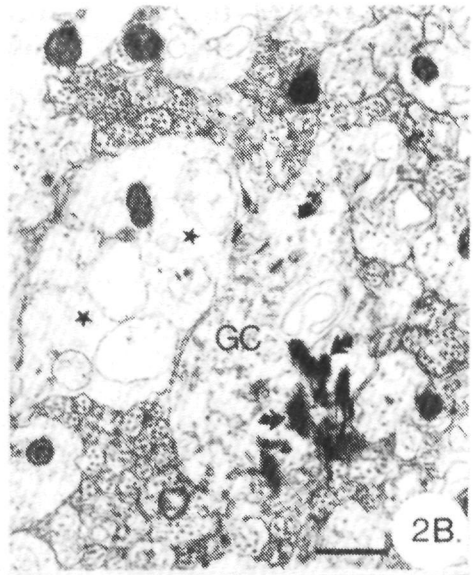
Figure 7 : **Fig.7A:** Camera lucida drawing of a transverse vibratrome section at the lumbar intumescence at PND5 stained with anti-VIM. CST= Corticospinal tract; FC=Fasciculus cuneatus; FG=Fasciculus gracilis; GM=Grey matter; WM=White matter. **Fig.7B:** Camera lucida drawing of a horizontal section of the CST area at PND5 stained with anti-VIM. Palisade pattern of the VIM-ir glial processes. **Fig.7C:** Photomicrograph of part of Fig.7B.

Figure 8 : Pre-embedding immunoelectronmicroscopy with monoclonal anti-VIM serum at the lumbar intumescence at PND5. Dark stained VIM-ir glial processes are situated adjacent to growth cones (GC) of pioneer CST axons (**Figs.8A** and **8B**) .Besides growth cones and VIM-ir glial cell processes this area contains unmyelinated profiles. Growth cones of CST axons frequently exhibit characteristic protrusions (arrows) into the VIM-ir glial processes. Bar represents 0.1 µm.

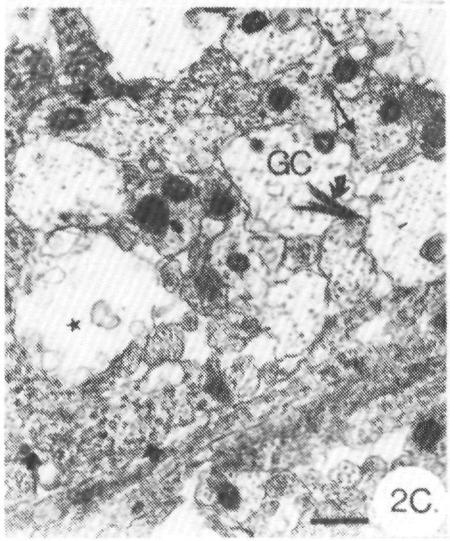
Figure 9 : Double immunofluorescence labelling with the use of anti-VIM and anti-GFAP on cryosections of PND11 (upper lane) and PND29 (lower lanes). **Top:Dorsal; Bottom:Ventral. Fig.9A:** anti-VIM,L3 (PND11). Note the absence of VIM-ir glial cell processes in the CST area. Bar=100 µm. **Fig.9B:** anti-GFAP, L3 (PND11). Bar=100 µm. **Fig.9C:** detail of 9B. Bar=25 µm. **Fig.9D:** anti-VIM, L3 (PND29). **Fig.9E:** anti-GFAP, L3 (PND29). Bar=50 µm. **Fig.9F:** detail of 9E. Bar=25 µm. **Fig.9G:** anti-GFAP, medulla oblongata (PND29). Bar=50 µm.



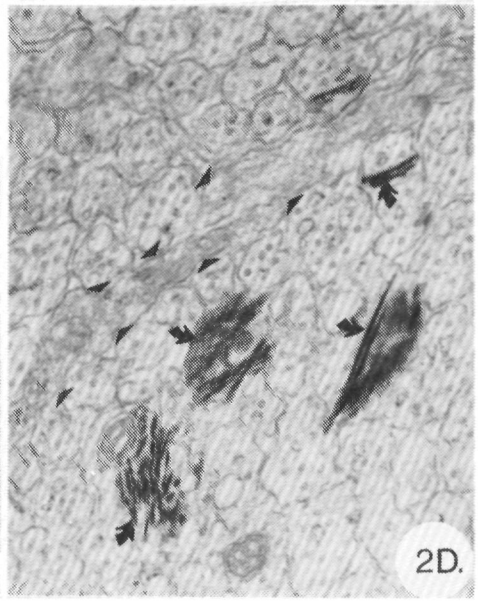
2A.



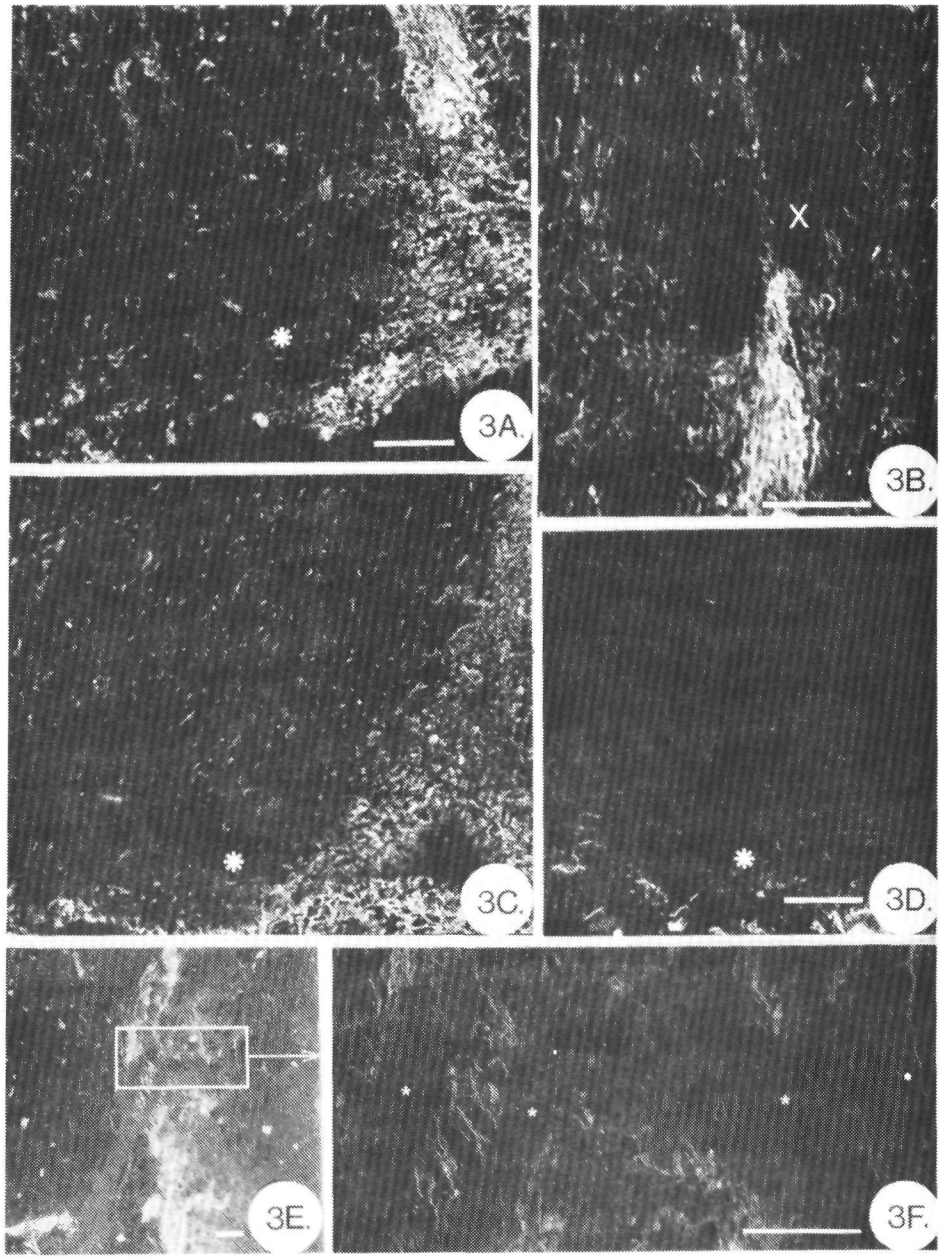
2B.

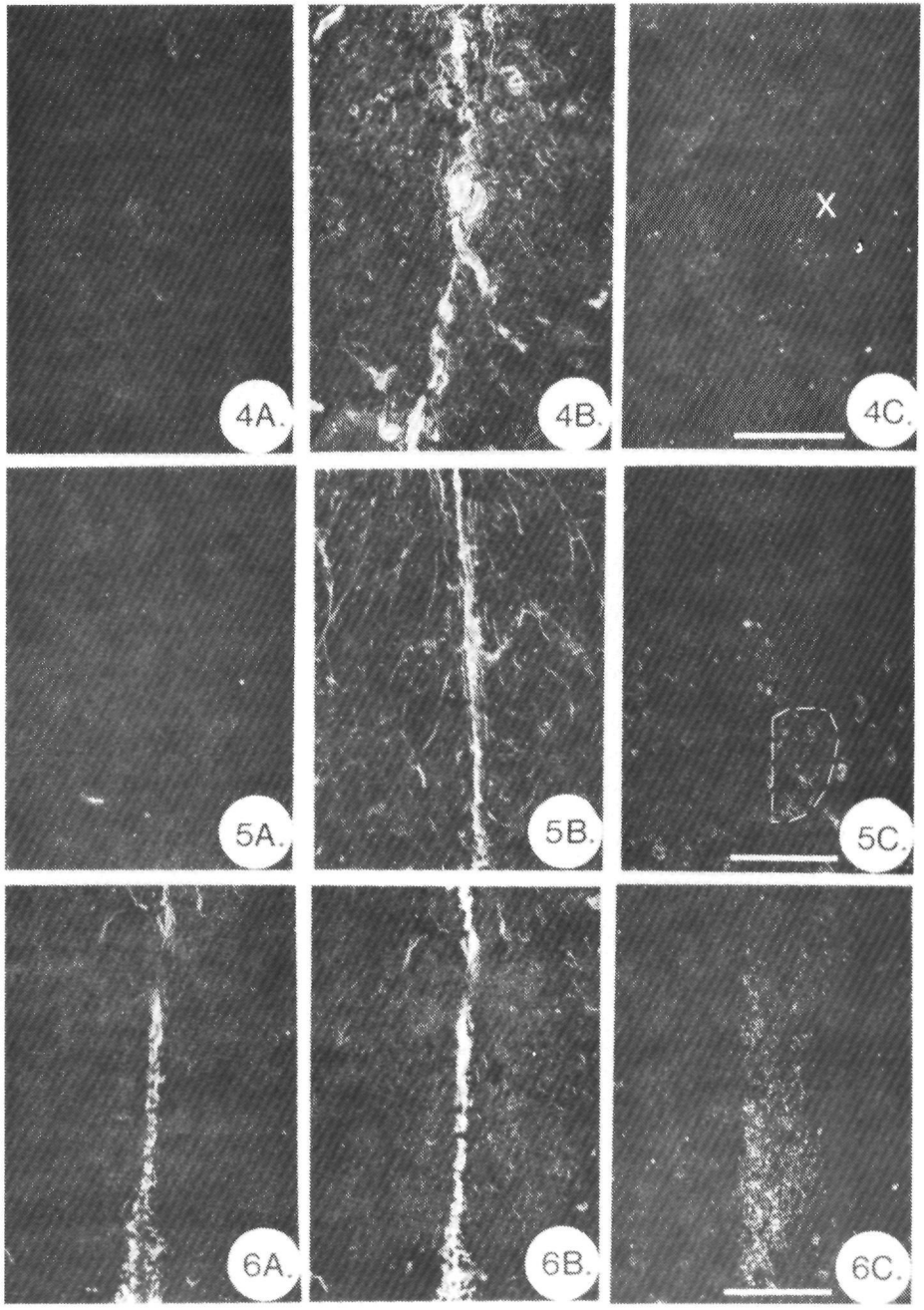


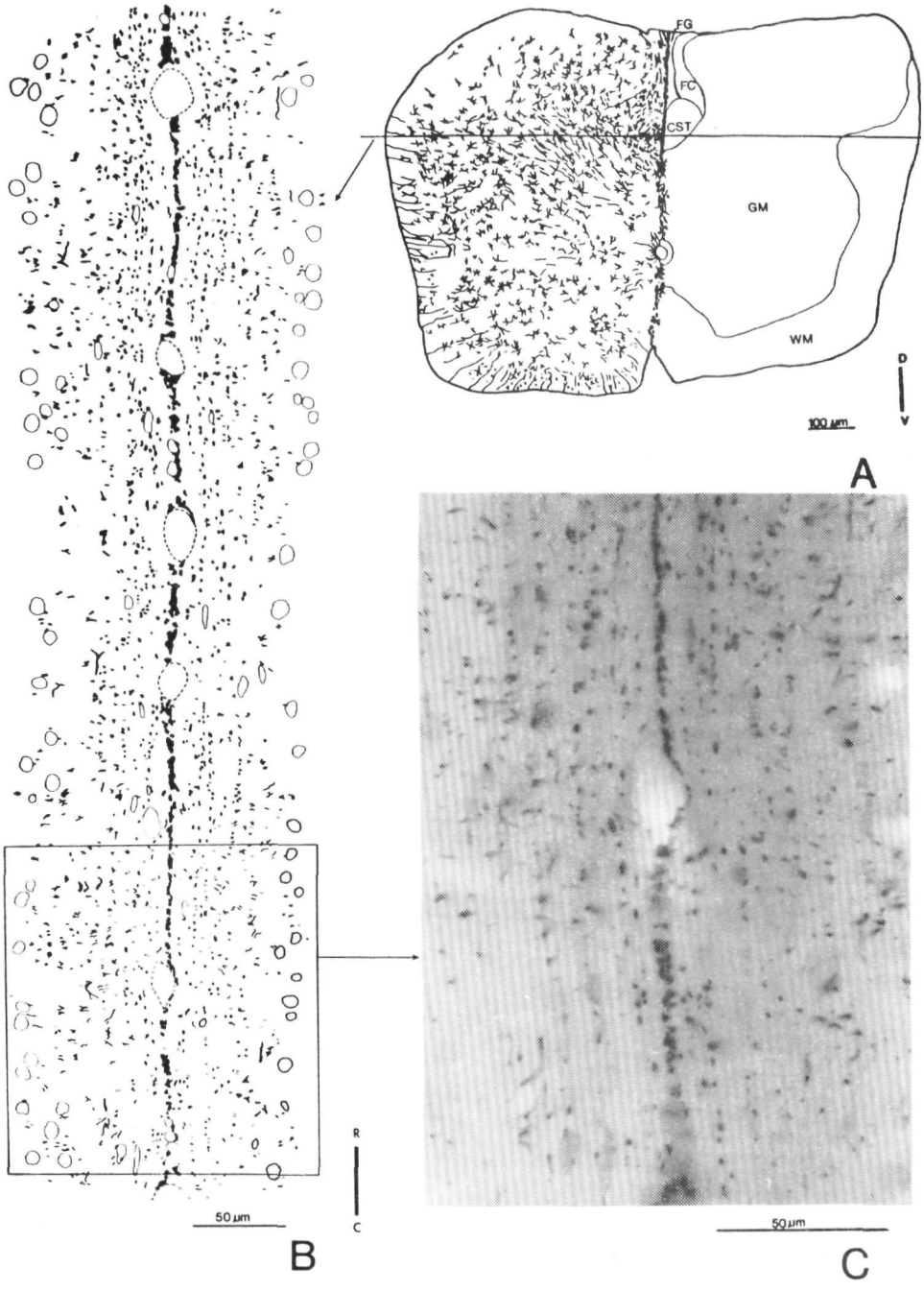
2C.

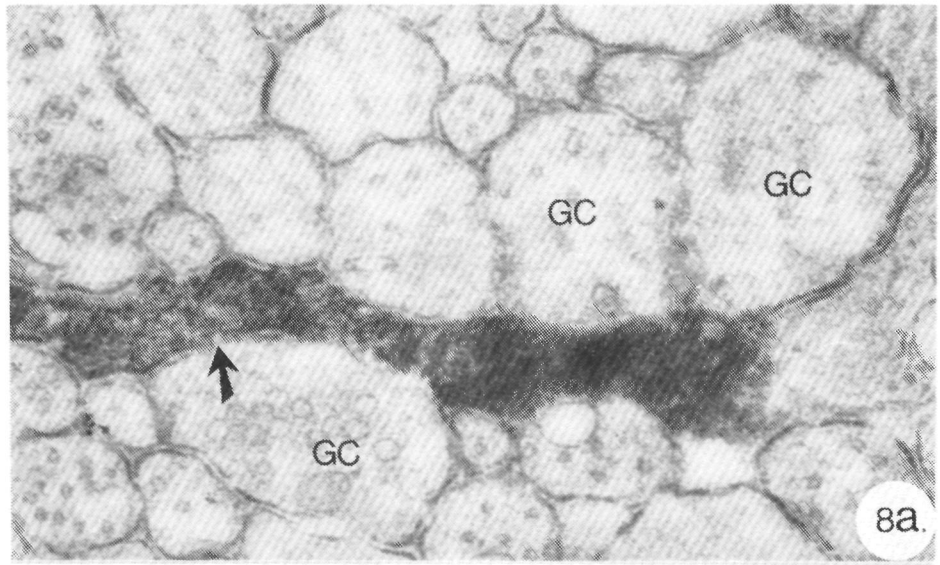


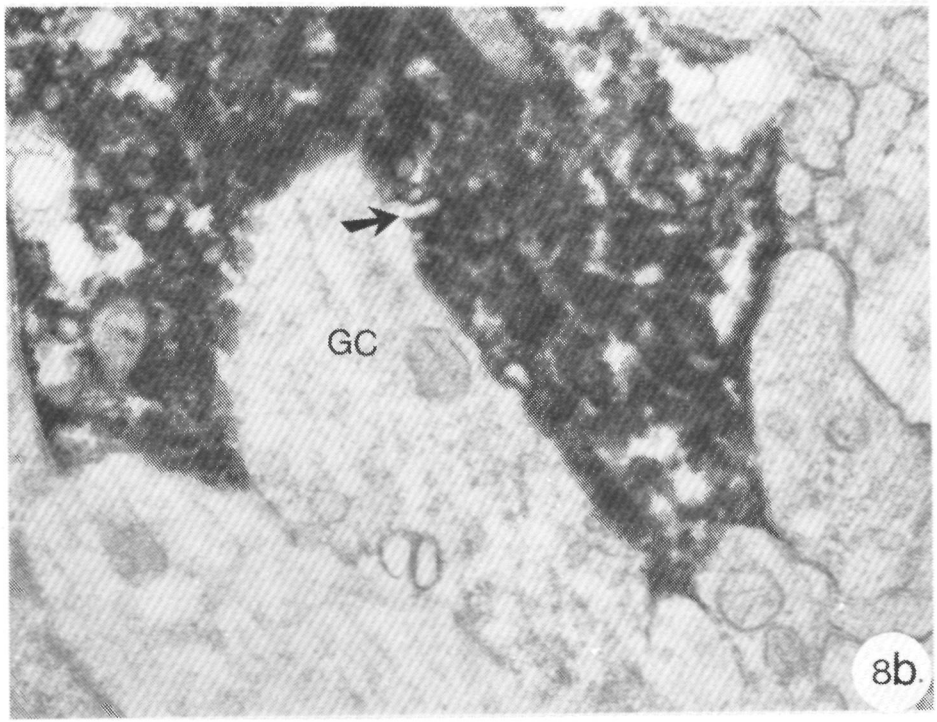
2D.

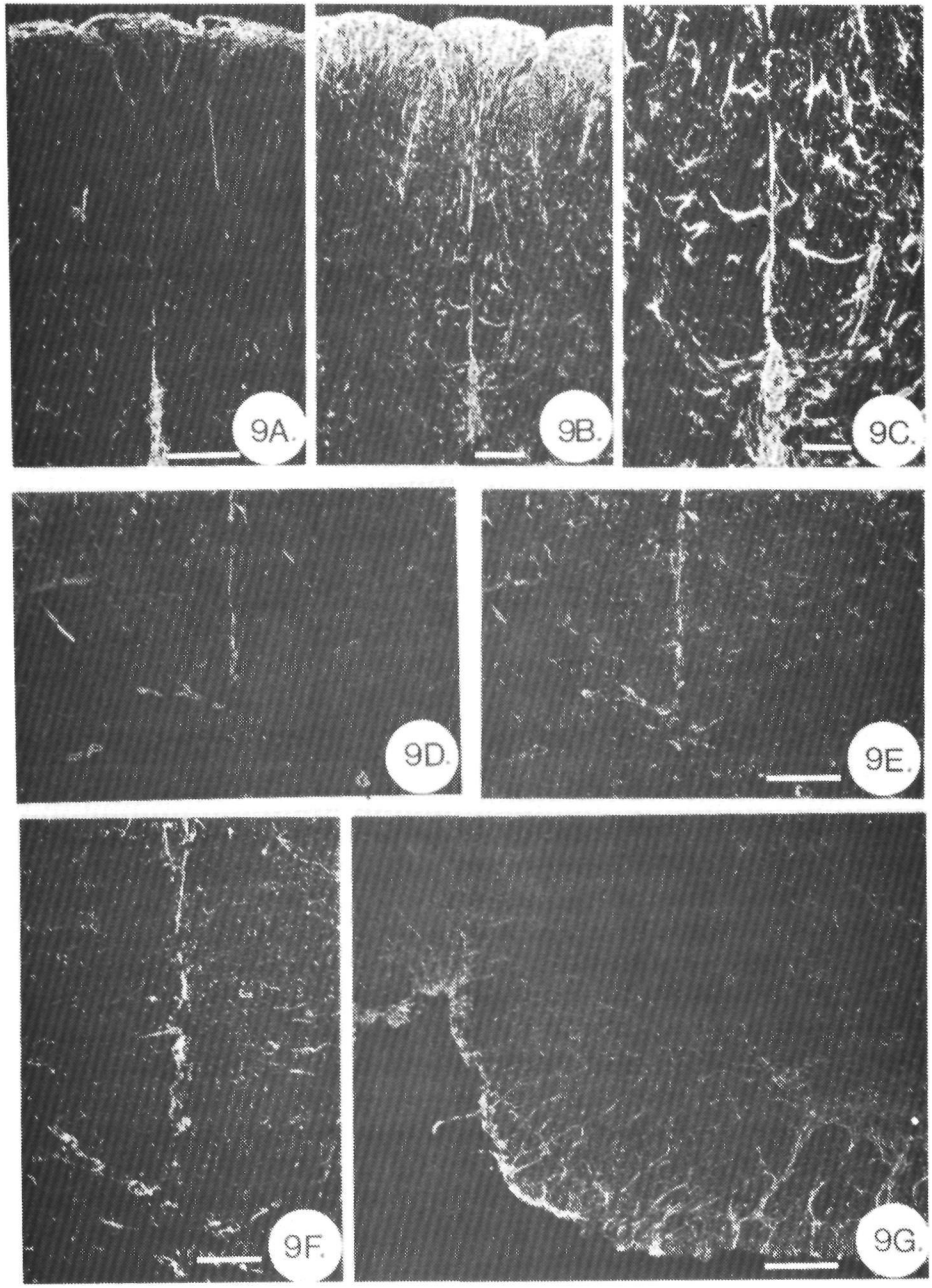

3A.
3B.
X
3C.
3D.
3E.
3F.


4A.
4B.
X
4C.
5A.
5B.
5C.
6A.
6B.
6C.


FG
FC
CST
GM
WM
D
V
100 µm
A
50 µm
R
C
B
50 µm
C


GC
GC
GC
8a.


GC
8b.


9A.
9B.
9C.
9D.
9E.
9F.
9G.

8A,8B). Based on anterograde HRP visualization (Figs.2B,2C), these growth cones represent the distal ends of the ingrowing pathfinding CST axons. Membrane specializations could not be detected between the growth cones and the VIM-ir glial processes in the CST outgrowth area. On the other hand, the growth cones frequently exhibited characteristic protrusions into the glial processes (Figs.8A,8B), suggesting a direct implication of VIM-ir glial processes in the guidance of outgrowing pioneer CST axons to more caudal spinal cord regions.

Between the 6th and the 11th postnatal day VIM-IR was considerably decreased, not only at medullary but also at spinal cord levels (Figs. 6A,9A) whereas the major VIM-ir glial structure in the midline raphe of the medulla oblongata and the median septum of the cervical and lumbar spinal cord of earlier stages is reduced to a minor one at PND11. Between PND6 and PND11 the VIM-ir glial processes within the CST area completely disappeared (e.g.Figs.6B,9A). The glial cells situated in the midline between the central canal and the dorsal funiculus as well as the radially oriented fibres in the fasciculus gracilis and the fasciculus cuneatus retained their VIM-IR during this period (Fig.9A). The number of midline VIM-ir cells between the central canal and the dorsal funiculus considerably decreased during the third and fourth postnatal weeks (Fig.9D).

GFAP-labelling further increases during the second postnatal week. At PND11 many GFAP-ir astrocytes were present in the dorsal funiculus (Fig.9B,9C), as well as in the medullary pyramids (not shown). Especially during the second postnatal week many glial cells were both GFAP- as well as VIM-ir, as demonstrated by double labelling experiments (Fig.9A,9B). At PND11 the dorsal median septum contained numerous GFAP- and only some VIM-ir glial cells. Thus the glial GFAP reactivity in late postnatal median raphe considerably differed from that in prenatal and early postnatal stages, in which it consisted of many layers of VIM-ir glial cells (Figs. 3B,4B).

No VIM-ir glial cells could be noted in the dorsal median septum in the third postnatal week (Fig.9D), whereas GFAP-ir glial cells were present up to the fifth week (Figs.9E,9F). Contrastingly, neither GFAP-IR nor VIM-IR could be noted within the midline raphe of the medulla oblongata after the second postnatal week (e.g.Fig.9G).

The VIM-ir cells in the midline between central canal and dorsal funiculus became GFAP-ir as well during the second postnatal week (as demonstrated with double labelling (Figs.9A and 9B). Between PND11 and PND22 these midline glial cells were VIM-negative and retained their GFAP-negativity up to later stages. Notably, between PND22 and PND29 GFAP-ir glial cells have vanished from the region between central canal and dorsal funiculus (Figs.9E,9F).

At four weeks of age the CST area contained many stellate shaped, GFAP-ir astrocytes, not only at medullary (Fig.9G) but also at cervical and lumbar spinal cord levels (Figs.9E,9F).

DISCUSSION

Antisera against intermediate filament proteins GFAP and VIM, specific markers for astrocytes and their precursors, respectively, were used to study the role of these glial cells in the guidance of pioneer CST axons. Outgrowing CST axons were anterogradely labelled with HRP.

General considerations

Our results demonstrate a dramatic change in labelling of glial cells during the first three postnatal weeks from the majority being VIM-positive and GFAP-negative in prenatal stages and at birth to almost all being the reverse at PND 29 at all spinal cord levels studied. With restriction to the CST outgrowth area this transition occurs mainly between E21 and PND2 at medullary levels, whereas it can be noted some days later at lumbar spinal cord levels: between PND6 and PND11 (Table 1). Double labelling experiments demonstrate a coincidence of VIM-IR and GFAP-IR within precursor-astrocytes predominantly during the second postnatal week. The expression of intermediate-sized filaments containing GFAP appears not to be correlated with the disappearance of VIM filaments indicating that the synthesis of the first type of intermediate filament protein is independent of the presence of the latter one as was suggested earlier by Schnitzer et al.(1981).

Astrocytes and CST outgrowth

Outgrowing HRP-labelled pioneer CST axons showed no preferential localization either at the periphery or at the center of the ventralmost part of the dorsal funiculus. On the contrary, they seem to be more or less randomly distributed over the presumptive CST area (Fig.5C). A quantitative analysis of the developing CST in the cervical spinal cord of the rat revealed that growth cone densities varied little and not at all statistically significant when their positional distribution over the presumptive CST area was tested (Gorgels et al.,1989[a]). CST growth cones are entering this area which consists of fascicles of unmyelinated fibres surrounded by lucent amorphous structures devoid of intermediate filaments (Figs.2A-2C). According to Schreyer and Jones (1982) these lucent structures would be large, irregularly, mainly rostrocaudally oriented glial processes with watery cytoplasm. However, Schreyer and Jones' interpretation is disputable. Both the 'watery' appearance as well as the absence of glycogen granules and free ribosomes are not in favour of interpreting them as glial processes (Peters et al.,1976; De Kort et al.,1985; Joosten et al.,1989). However, the mere absence of intermediate filaments does not necessarily implicate the absence of their particular proteins. For instance,GFAP can be present in a non-polymerized, soluble form in the cytoplasm and therefore GFAP staining may be noted even before the appearance of significant amounts of filaments (Choi,1981; Levitt et al.,1983). On account of our findings, however, that the lucent

amorphous 'watery' structures in the prospective CST area do not contain VIM-IR, whereas at these early developmental stages no GFAP staining can be detected at all, it is very unlikely that the electron-light structures in the CST outgrowth area are of glial origin. On behalf of other EM features, such as the amorphous structures containing round vesicles, mitochondria and large vacuole like structures, these profiles may represent (pre-) terminal varicosities of outgrowing axons (De Kort et al.,1985). In that case they must be interpreted as aberrant ascending fibres since they never contained any HRP labelling. Besides, no morphological relationship could ever be detected between the lucent amorphous structures and growth cones of CST pathfinding axons.
Based on transverse sections VIM-ir glial cells seem to be rather randomly distributed over the presumptive CST area (Fig.7A). However, VIM-ir glial cells are arranged in longitudinal tiers orientated parallel to the rostrocaudal axis of the developing CST (Figs.7B,7C). A comparable palisading pattern was observed not only in the adult rat spinal cord, medulla and pons (Bitner et al.,1987), but also in the fetal mouse brain (Dupouey et al.,1985). The finding of an analogous glial organization in embryonic, young postnatal and adult mouse or rat supports the idea that VIM-ir glia may be involved in various neuronal, axonal and glial aspects of the spatial organization of the mature mouse or rat spinal cord. The presence of a VIM-ir palisading architecture probably constitutes an important external constraint for the developing CST axons.
Our electron microscopic observations contain additional data for this assumption: VIM-ir glial processes adjacent to growth cones of pioneer CST axons frequently show intrusions of the latter structures (Figs.8A,8B), suggesting a direct influence of the VIM-ir glial cell processes on the outgrowth of the pathfinding CST axons. Since no membrane specializations could be detected, the contacts between the VIM-ir glial cell processes and the growth cones of the pioneer CST axons presumably are of the adhesive type. The chicken neuron-glia adhesion molecule NgCAM (Grumet et al.,1984[a]) as well as the immunologically identical cell adhesion molecules in mouse (L1) (Rathjen and Schachner, 1984) or rat (NILE) (Stallcup et al.,1985) are implicated in in vitro guidance of outgrowing axons (Rathjen,1988). It becomes an intriguing question whether these glycoproteins are involved in in vivo outgrowth of CST fibres.

The occurrence of a channel-like guidance system, consisting of glial or presumptive glial cells was substantiated in the developing optic nerve (Silver and Sidman,1980; Silver,1984; Silver and Rutishauser,1984) and corpus callosum (Silver et al.,1982) of the mouse. Although no indications are present in our material, such a channel-like glial system in the developing CST of the rat cannot be excluded completely.Serial sectioning in combination with postembedding immunocytochemical detection of VIM-ir glial cell arrangements in conjunction with visualization of HRP-labelled CST axons may provide conclusive evidence. To that end,

however, a new combination of techniques has to be developed.

Apart from the occurrence of VIM-ir processes in the presumptive CST area, a major VIM-ir glial cell accumulation is present within the midline raphe of the medulla oblongata and cervical and lumbar spinal cords in prenatal and early postnatal stages. After the first postnatal week this major VIM-ir glial septum is gradually reduced to a minor GFAP-ir one at spinal cord levels at PND 29. In the medulla oblongata neither VIM-IR nor GFAP-IR could be noted within the midline raphe beyond the end of the fourth postnatal week. The pyramidal decussation demonstrated a permanent absence of a compact VIM-ir glial cell alignment in the midline of the CST outgrowth area. The presence of such a major compact VIM-ir glial septum in the midline of both the medulla oblongata and the spinal cord as well as its absence in the decussation area during CST outgrowth suggests a decisive role of this glial barrier in the guidance of outgrowing CST axons in preventing the latter fibres from decussation. Studies on the outgrowing mouse corpus callosum and on the developing mouse optic nerve suggested a physical role for glial cells in guiding outgrowing axons (Silver,1984; Hankin and Silver,1986). Further studies on this matter may add to the present results.

The stellate GFAP expressing astrocytes that are common in adult rat fibre tracts are only present in the developing CST beyond PND8. The absence of GFAP-ir glial cells during prenatal stages and the first postnatal week demonstrates that they can not play a role in the guidance of outgrowing CST pioneer axons. A dramatic increase of GFAP-IR was noted between PND8 and PND15 (Figs.9B,9C), the same period in which myelination of CST fibres starts as was described earlier (see Chapter 4B). Therefore the formation of stellate shaped GFAP-ir astrocytes in the rat CST area may well be correlated with CST myelination, as suggested already by Dahl (1981), among others. In addition, mature stellate astrocytes are supposed to play an important role in support functions of myelinated axons (Bovolenta et al.,1984; D'Amelio et al.,1986; Berkley and Contos,1987; Bitner et al.,1987).

Besides, the onset of GFAP expression was also correlated earlier with the degeneration of an excess of axons (Bovolenta et al.,1987). Actually, transient occipital corticospinal collaterals indeed are eliminated from spinal cord levels between PND8 and PND11, as substantiated previously (Stanfield et al.,1982; Stanfield and O'Leary,1985[b];Joosten et al.,1987[b]).Therefore, some role of GFAP-ir glial cells in the entire process of axon degeneration also seems very likely.

Concludingly, the developing CST in rats is a good model to further study the positional, chemical and/or physical influences of astrocytes on axon outgrowth and -degeneration.

Acknowledgement

The authors are grateful to Dr.Frans Ramaekers for gifts of the antibodies. We also want to thank Alice Rinkens and Jos Dederen for excellent technical assistance.

CHAPTER 5C1: IMMUNOCYTOCHEMICAL LOCALIZATION OF CELL ADHESION MOLECULE L1 IN DEVELOPING RAT PYRAMIDAL TRACT.

SUMMARY

L1 is a representative of a family of carbohydrate neural cell adhesion molecules. The expression of L1 was studied during postnatal development of the rat pyramidal tract by immunohistology using polyclonal antibodies to L1 in spinal cord cervical intumescences.

On postnatal day 1 (P1), L1 immunoreactivity was present in the entire dorsal funiculus, consisting of the ascending fasciculus gracilis and fasciculus cuneatus and the descending pyramidal tract. At that time the cervical pyramidal tract contains the first outgrowing corticospinal axons. At P4 both the fasciculus gracilis and the pyramidal tract are immunoreactive whereas the fasciculus cuneatus is negative. At P10 the pyramidal tract is intensely labelled whereas both ascending bundles are negatively stained. In the period between P4 and P10 the pyramidal tract is characterized by a massive outgrowth of corticospinal axons.

During pyramidal tract myelination, between postnatal day 10 (P10) and the end of the third postnatal week (P21), L1 immunoreactivity is progressively reduced. These observations suggest that L1 may play a prominent role in outgrowth, fasciculation and the onset of myelination of rat pyramidal tract axons.

The differential L1-immunoreactivity of the pyramidal tract and the earlier developing ascending systems in rat dorsal funiculus, indicates that this polyclonal antiserum is a useful differentiating marker for outgrowing fibre tracts.

Key words :L1 cell adhesion molecule - axon outgrowth - pyramidal tract - development - rat.

INTRODUCTION

During development of the mammalian nervous system a variety of processes occurs. It is very likely that cell-cell interactions, including adhesion of cells to each other and to extracellular matrices play an important role during neuron migration, axon outgrowth and fasciculation, as well as neuron-aggregation and synapse formation.
Several glycoproteins present on cell surfaces and in extracellular matrices, operationally called "cell adhesion molecules" have been identified. They appear to be involved in adhesive interactions between neurones, glial cells and components of the extracellular matrix.
The best characterized of these glycoproteins are the neural cell adhesion molecule N-CAM (Rutishauser,1984) and L1 (Rathjen and

Schachner,1984). The latter is immunologically identical to neuron-glia CAM (Ng-CAM) and to the nerve growth factor inducible large external protein NILE (Bock et al.,1985; Friedlander et al.,1986). The glycoprotein recognized by L1 antibodies is involved in neurite fasciculation (Rathjen,1988), neuron-neuron adhesion (Keilhauer et al.,1985) and outgrowth of neurites (Rathjen,1988). Furthermore L1 is involved in the initial axon-Schwann cell interaction in developing and regenerating mouse sciatic nerve (Martini and Schachner,1986).

Because of its relatively late development, as compared with other fibre tracts, the pyramidal tract (PT) in the rat, and particularly its corticospinal component, is a very useful model to study developmental processes. The rat PT is characterized by a staggered mode of outgrowth (Schreyer and Jones,1982). Tracing studies revealed that the arrival of the first corticospinal tract (CST) fibres in the ventralmost part of the dorsal funiculus at the cervical intumescence occurs at the first postnatal day, whereas significant numbers of axons are added to the tract within the first ten days after its initial outgrowth (Gribnau et al.,1986; Joosten et al.,1989). At the cervical intumescence myelination of CST axons starts between postnatal day 10 (P10) and postnatal day 14 (P14), whereas at the end of the third postnatal week (P21) most CST axons are myelinated at this spinal cord level (see Chapter 4B).
In the study described here, the PT is examined for the presence of L1, during the period in which CST outgrowth,fasciculation and myelination at the fifth cervical segment (C5) occurs.

MATERIALS AND METHODS

Postnatal Wistar rats ranging in age from postnatal day 1 (P1) up to and including postnatal day 21 (P21) were used, and the day of birth was accounted as P0. The rats were anaesthetized by intraperitoneal injections with Nembutal (18 mg.per kg.body weight) and perfused transcardially with saline followed by 2% paraformaldehyde and 0.12% glutaraldehyde in 0.1 M. phosphate buffered saline (PBS) (pH 7.4). After perfusion the spinal cords were immediately removed and further immersed for about 1 hour in the same fixative before being transferred into cold (4°C) PBS with 5% sucrose. Then 50 µm. vibratome sections of the cervical intumescence (C5) were cut transversely on an Oxford vibratome and incubated overnight at 4°C in PBS containing 0.1% bovine serum albumin (BSA) (PBS/BSA). Thereafter the sections were treated for 10 min. with 5% DMSO in PBS and rinsed with PBS/BSA. The immunocytochemical staining was carried out according to the following protocol: The sections were incubated in 10% normal swine serum in PBS/BSA for 1 hour. Then the vibratome sections were incubated for about 2 hours with anti L1 IgG's at room temperature. Fab fragments of a rabbit antibody to mouse L1 were a generous gift of Dr.Fritz Rathjen (Max-Planck Institut Tübingen,FRG) and after specificity tests were produced as described by Rathjen and Schachner (1984). Control sections were immersed in 10% normal swine

serum in PBS/BSA. Subsequently, the sections were washed for 30 min. at room temperature in PBS/BSA and incubated with Fab fragments of swine-anti-rabbit immunoglobulins coupled with horseradish peroxidase (HRP) (DAKO), diluted 1:50 in PBS/BSA, for 1 hour at room temperature. After two ten-min. rinses with PBS/BSA HRP was visualized using the chromogen diaminobenzidine (DAB) and Nickel (Ni) as an intensifying agent (Adams,1981). Then the sections were washed in PBS and embedded in Depex. Photomicrographs were made with an automatic Zeiss-photomicroscope-II and an Agfapan-25 film.

Control sections, which were treated similarly but without the primary antiserum, invariably displayed no immunoreaction. At least three animals per age-category were examined, giving similar results.

RESULTS

The location of the descending PT as well as the ascending fasciculus gracilis (FG) and fasciculus cuneatus (FC) in the dorsal funiculus of the rat spinal cord is shown in Fig.1A.

At P1 L1-immunoreactivity (L1-ir) was found in the entire white matter of the fifth cervical spinal cord segment (Fig.1B). Both ascending FG and

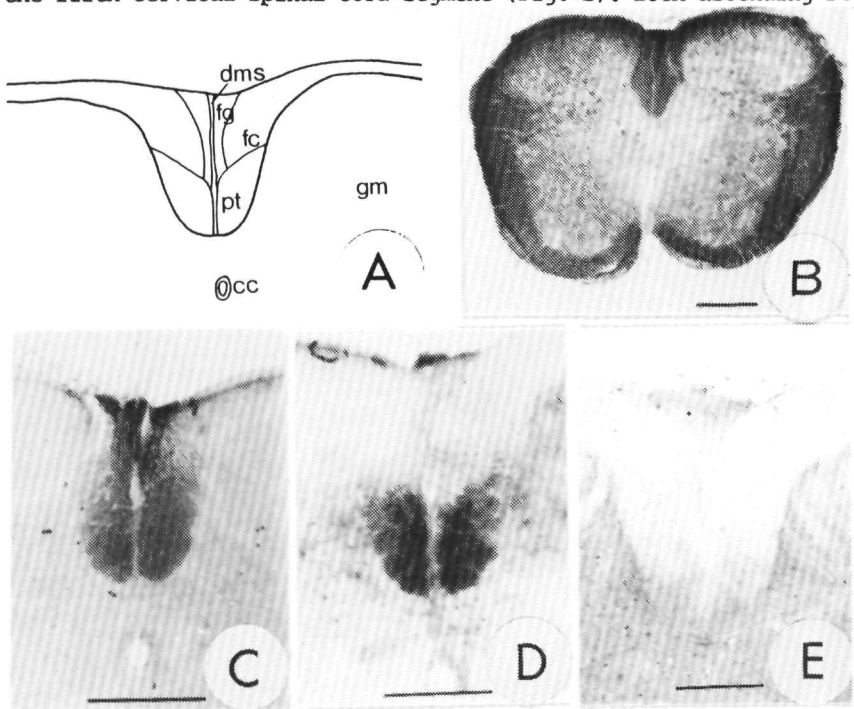


Fig.1 : **1A** :Camera lucida drawing of the dorsal funiculus of a transverse vibratome section at the cervical intumescence at postnatal day 10 (P10). cc=central canal; dms=dorsal median septum; fc=fasciculus cuneatus; fg=fasciculus gracilis; gm=grey matter; pt=pyramidal tract. **1B-1E** :Distribution of L1-immunoreactivity (L1-ir) in transverse vibratome sections of the spinal cord at the fifth cervical segment (C5) between postnatal day 1 (P1) and postnatal day 21 (P21). **Fig.B** :P1. **Fig.C** :P4: note the reduction of L1-ir in the FC. **Fig.D** :P10: the very intensely stained PT stands out against the virtually unstained ascending systems FC and FG. **Fig.E** :P21. Bar represents 100 μm.

FC as well as the descending PT were equally well stained for L1-ir. At P4, PT and FG still showed an intense immunoreaction with L1 antibodies, whereas the FC was less intensely stained (Fig.1C).
Ten days after birth the PT showed its most intense L1-ir (Fig.1D): the densely stained PT stands out against the adjacent ascending fibre tracts which are both devoid of L1-ir. Between P10 and P21 L1-ir was progressively reduced in the rat PT, although a weak L1-immunoreaction was retained up to the end of the third postnatal week (Fig.1E). During the period studied the dorsal median septum was marked by the absence of L1-ir (Fig.1B-1E).

DISCUSSION

With the use of polyclonal anti-L1 IgG's (Rathjen and Schachner,1984) a relation between L1-ir and the developmental state of the PT in the rat was demonstrated.

A clear L1-ir was noted during the entrance of pioneer CST axons into the fifth cervical segment at P1. These pioneer axons, with growth cones at their distal ends, penetrate an area consisting of astroglia, fascicles of unmyelinated axons and lucent amorphous structures (Joosten et al.,1989). Neuron-glia interaction has been postulated to underlie the outgrowth of pioneer axons in developing fibre tracts. The chicken neuron-neuron and neuron-glia adhesion molecule Ng-CAM, closely related to mouse L1 (Friedlander et al.,1986), is involved in neuron-glia adhesion (Grumet et al.,1984[a],1984[b]). However, polyclonal L1 antibodies interfere with neuron-neuron but not with neuron-astrocyte or astrocyte-astrocyte adhesion (Keilhauer et al.,1985). It is therefore likely that the polyclonal L1 and Ng-CAM antibodies recognize different sets of functional sites on the L1 antigen. The molecular domains of the L1 antigen involved in neuron-glia interaction are still undetected.
At the same time outgrowing CST pioneer axons might be guided by means of axon-axon interactions mediated by L1 antigens located on the fascicles of unmyelinated ascending axons in the presumptive CST area (Joosten et al.,1989). The very intense L1-ir at P4 and P10, i.e. after the initial outgrowth when significant numbers of axons are added to the tract (Gribnau et al.,1986), suggests that L1 may be instrumental in the fasciculation of PT axons. Together with other studies (Beasley and Stallcup,1987; Godfraind et al.,1988) our results indicate that L1 may be important in the guidance of axons to their targets by means of axon fasciculation.

The differential L1-ir of the PT vs. the ascending fibre systems in the dorsal funiculus after the first postnatal day can be explained by the fact that FG and FC develop several days before birth (Altman and Bayer,1984). Whereas PT myelination starts between P10 and P14 (Joosten et al.,1989), FG myelinates at P4 and FC even at the time of birth (Matthews and Duncan,1971). The subsequent reduction of L1-ir in the

fibre tracts located in the dorsal funiculus may be related to their respective myelination periods. Although myelination of rat CST axons is mainly completed at P21, yet a slight L1 immunoreaction could be noted in the PT area (Fig.1E). The presence of detectable L1 antigen in PT of P21 rats may well be explained by the presence of numerous unmyelinated CST axons within this tract (see Chapter 4C). The clear L1-ir during the onset-period of CST myelination (P10-P14) indicates that L1 might also be involved in the initial axon-oligodendrocyte interaction. Using immunoelectronmicroscopy the involvement of L1 in the initial axon-Schwann cell interaction was demonstrated previously in the developing mouse sciatic nerve (Martini and Schachner,1986).

Summarizing, our observations suggest that L1 may play a prominent role in the outgrowth, the fasciculation and the onset of myelination of rat pyramidal tract. Further research has to be carried out on the ultrastructural localization of L1-ir as related to the developmental state of the rat pyramidal tract.

Acknowledgements

The authors thank Jos Dederen for expert technical assistance, Theo Gorgels and Gert Joosten for critical reading of the manuscript and Fritz Rathjen for his generous gift of the L1 antiserum.

CHAPTER 5C2 : IMMUNOELECTRONMICROSCOPICAL LOCALIZATION OF CELL ADHESION MOLECULE L1 IN DEVELOPING RAT PYRAMIDAL TRACT

SUMMARY

The glycoprotein L1 is a cell adhesion molecule that has been proposed to function in the PNS in axon fasciculation and onset of myelination. In this report we localized L1 during the development of a major central pathway: the pyramidal tract (PT). The (sub)-cellular localization of L1 was determined both by pre-embedding staining on vibratome sections and by immunogoldlabelling on ultracryosections in developing rat PT at C5. On arrival at C5, i.e. at postnatal day 1 (P1), growth cones of pioneer fibres did not exhibit L1-immunoreactivity (L1-IR). In the contactzone between PT growth cones and glial processes no L1-IR was observed. A distinct L1-IR was noted on small unmyelinated ascending axons situated in the entrance area of the PT growth cones. Also on later arriving, i.e. between P2 and P10, small unmyelinated fasciculating PT axons L1 was present. It is our impression that L1 is localized in an irregular patchy way on the outer side of the axonal membrane. During the onset of PT myelination, i.e. between P10 and P14, L1 could not be detected on axons ensheathed by oligodendrocytic processes. When myelination is largely completed, i.e. at P21, the L1 antigen could be localized within the axoplasma of both unmyelinated and myelinated PT axons. Furthermore, L1 could be observed occasionally on small unmyelinated PT axons. Whereas compact myelin was always L1-negative, L1 was noted periaxonally between the axolemma and compact myelin and at (para)-nodal regions at the contact zone between axolemma and oligodendrocytic processes. From these results it may be deduced that: 1. L1 is involved in fasciculation of outgrowing later arriving PT fibres, 2. in contrast to developing PNS L1 is not involved in the onset of myelination in this central tract, 3. L1 might play an additional adhesive role in myelinated rat PT. The data are discussed in the view of the considerable developmental plasticity of this central tract.

Key words: adhesion molecule L1 - guidance - pyramidal tract - immuno-electronmicroscopy - myelination - plasticity - rat

INTRODUCTION:

The formation of adequate functional nerve connections is based upon the correct sequence of cell-cell interactions during the development of the central nervous system. Among these distinct, yet coordinated, events such as neuron migration, axon outgrowth and guidance, synapse formation

as well as myelination play an important role. Several glycoproteins present on cell surfaces and/or extracellular matrices appear to be involved in the chemical, adhesive interaction among neurons as well as between neurons and glial cells or components of the extracellular matrix.

In addition to the more general adhesive molecules (neural cell adhesion molecule (NCAM), Laminin and N-Cadherin (Rogers et al.,1983; Rutishauser,1984; Edelman,1988)) local adhesive glycoproteins are described (for reviews see Dodd and Jessell,1988; Rutishauser and Jessell, 1988). Among the local adhesive molecules the glycoprotein L1 is the best characterized (Rathjen and Schachner,1984). The cell surface molecule L1,which is involved in Ca^{++}-independent adhesion and aggregation of cell bodies (Schachner et al.,1985), is immunologically identical to the rat nerve growth factor-inducible large external protein (NILE) (Bock et al.,1985) or 69A1 (Piggott and Kelly,1986) and to chicken neuron-glia CAM (Ng-CAM) (Friedlander et al.,1986).

L1 is synthethized as an integral membrane glycoprotein with a relative molecular weight (M_r) of 200.000 (Faissner et al.,1985; Linneman et al.,1988).Besides the membrane bound L1, a soluble form of this protein, with a slightly lower M_r, exists (Bock et al.,1985). Biochemical studies reveal that although the total amount of L1 varies with age showing a peak value in early postnatal life of rat, still a considerable amount remains present after postnatal day 15 (Linneman et al.,1988). Based on in vitro experiments L1 has been found to mediate neuron-to-neuron but not neuron-to-astrocyte interaction (Keilhauer,1985), to be involved in neurite fasciculation in the PNS (Fischer et al.,1986; Rathjen,1988), in the outgrowth of neurites (Chang et al.,1987) as well as the migration of cerebellar granule cells from the external to the internal granular layer (Lindner et al.,1983).

In the early postnatal mouse cerebellum L1 has been recognized on neurons but not on glia and fibroblasts or fibroblast-like cells (Rathjen and Schachner,1984). In vivo the (sub)-cellular localization of the CAM L1 was determined in developing and regenerating mouse sciatic nerve (Nieke and Schachner,1985; Martini and Schachner,1986,1988): with the use of various immunoelectronmicroscopical staining techniques L1 was localized on small unmyelinated fasciculating axons and axons ensheathed by non-myelinating Schwann cells (Martini and Schachner,1986). Furthermore Schwann cells were positive to L1 when the Schwann cell process had turned 1.5 loops; thereafter neither axon nor Schwann cell could be detected to express the L1 antigen. From the in vitro and in vivo experiments it may be deduced that L1 is involved in axon fasciculation, initial axon-Schwann cell interaction and the onset of myelination in the mouse peripheral nervous system (PNS) (Martini and Schachner,1986, 1988).

The aim of the present study was to localize L1 during the development of a major central motor pathway in the rat; the pyramidal tract (PT).

The PT was chosen because of its relatively late development as compared with other fibre tracts (Schreyer and Jones,1982; Gribnau et al.,1986; Joosten et al.,1989). Furthermore, the developing PT is a CNS tract capable of a considerable developmental plasticity (Kalil and Reh,1982; Bregman and Goldberger,1982,1983; Bernstein and Stelzner,1983; Bregman et al.,1989). PT axons are able to take an aberrant route through adjacent undamaged CNS tissue to reach normal targets (Bregman et al.,1989). Besides, the occurrence of numerous transient corticospinal projections (see Chapter 2B,2C;Stanfield et al.,1982) may be indicative for the plasticity of this particular CNS tract. With respect to this plasticity it is interesting to compare the (sub)-cellular localization of L1 in this CNS tract and the (regenerating) sciatic nerve as a representative of the PNS (Martini and Schachner, 1986,1988).

Light microscopical observations on the presence of L1 during rat PT development at the fifth cervical segment suggest that L1 may play a prominent role in the outgrowth, fasciculation and onset of myelination of PT axons (see Chapter 5C1). Thus, an EM analysis of the (sub)-cellular localization of L1 in developing rat PT may add to our understanding of not only developmental processes in the CNS but also the different regenerative capacities of CNS versus PNS.

MATERIALS AND METHODS

Animals and tissue processing

Wistar rats varying in age between postnatal day 1 (P1) and postnatal day 21 (P21) were used. The day of birth was accounted as P0.

The rats were anaesthetized by intraperitoneal injection of an aqueous solution of nembutal (18 mg per kg body weight) and perfused through the heart with 10-40 ml 2% paraformaldehyde and 0.12% glutaraldehyde in 0.1 M phosphate buffered-saline (PBS), pH 7.4. Besides with this fixative rats were also perfused with 2% paraformaldehyde and 2% glutaraldehyde in PBS (pH 7.4).

After perfusion the brains and spinal cords were resected immediately from the skulls and vertebral columns, respectively, and then postfixed in the same fixative for about 1 hr before being transferred into cold (4°C) PBS with 5% sucrose. Immersion for at least 16 hr. does neither affect the tissue intregity nor the antigenicity.

Samples of the cervical intumescence (fifth or sixth cervical segment, C5/C6) were cut tranversely on a vibratome (Oxford Instruments) and incubated overnight at 4°C in PBS containing 0.1% bovine serum albumine (BSA).

Antibodies

To localize the L1 antigen F_{ab} fragments of a rabbit antibody to mouse L1 were used. This polyclonal antiserum was a generous gift of Dr.Fritz

Rathjen (Dept.Mol.Neurobiologie,Hamburg,FRG). The L1 antiserum was produced and described by Rathjen and Schachner(1984). For pre-embedding staining polyclonal antibodies were visualized by F_{ab} fragments of swine-anti-rabbit immunoglobulins coupled with horseradish-peroxidase (HRP) (DAKO) at a dilution of 1:50 in 0.1 M PB,pH 7.4. For immunogold labelling on ultracryosections goat-anti-rabbit IgG's adsorbed to colloidal gold (15 nm diameter, Janssen Pharmaceutica) were used at dilutions of 1:25.

Pre-embedding staining

Vibratome sections were treated for 10 min with 5% DMSO in PBS (Fushiki and Schachner,1986) and rinsed with PBS/BSA. Thereafter the sections were incubated in 10% normal swine serum in PBS/BSA for 1 hr at room temperature. Then the vibratome sections were incubated for 2 hr with anti L1 IgG's at room temperature. Control sections were immersed with 0.1 M PBS or 10% normal swine serum in PBS/BSA. After rinsing 30 min in 0.1 M PBS/BSA, pH 7.4, the sections were incubated with F_{ab} fragments of swine-anti-rabbit IgG's coupled with HRP for 1 hr at room temp. After washing for 20 min (twice 10 min) with PBS/BSA HRP was visualized using the chromogen diaminobenzidine hydro-chloride (DAB; Sigma Chemical GmbH) and Nickel (Ni) as an intensifying agent (Adams,1981). Then the sections were washed in PBS and embedded in Depex for light microscopical immunocytochemical localization. For electronmicroscopy sections were washed in PBS (pH 7.4) and then postfixed in 1% OsO_4 in 0.1 M PB (pH 7.4). Subsequent dehydration in an ascending series of ethanol was followed by embedding in Epon 812 on repelcoated slides. Ultrathin sections were cut on a Reichert OM-4 ultramicrotome and mounted on 75 mesh formvar (0.8%)-coated copper grids. All sections were counterstained with uranylacetate (20 min) and lead citrate (10 min). The sections were examined in a Philips EM-300 electron micoscope at an accelerating voltage of 60 kV.

Immunogold-labelling on ultracryosections

Very thick vibratome sections (300 um) or hand cut segments of the cervical intumescence were immersed in a 5% gelatin solution in 0.1 M PBS, pH 7.4 at 37°C for 1 hour. After the gelatin was allowed to solidify as a thin slab at 4°C, the embedded tissue blocks were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS for 30 min (Geuze and Slot,1980; Gorgels et al.,1989[b]; van Lookeren-Campagne et al.,1989). Then the dorsal funiculus was cut out of the tissue blocks and further processed. The small tissue blocks (max.1 mm^3) were infused in an ascending series (three steps) of a mixture of polyvinylpyrrolidone (PVP) sucrose in 0.1 M PBS, pH 7.4 up to a final concentration of 20% PVP and 2.0 M sucrose, for at least 3 hours (Tokuyasu,1986). The tissue blocks were then mounted on specimen stubs and frozen in liquid nitrogen.

Ultrathin frozen sections (< 100 nm) were cut at approximately -100°C on a Reichert OM-4 ultramicrotome equipped with an ultracryodevice. The

sections were picked up by a wireloop filled with 2.3 M sucrose solution in PBS; and transferred to formvar- and carbon coated 300 mesh nickel grids. The grids were maintained, sections facing down on 2% gelatin in PBS at 4°C for 2 hr, followed by floating on PBS (Slot and Geuze,1983; Tokuyasu,1978,1986).

The sections were immunostained essentially according to published procedures (Tokuyasu,1980; Griffiths et al.,1983; Slot and Geuze,1983). Briefly: to quench free aldehyde groups the grids were floated on drops of a 0.02 M glycine solution in PBS,pH 7.4 (3 x 10 min). The immunostaining was carried out by sequentially floating the grids on drops of the following solutions: primary antibodies in 0.1 M PBG (overnight at 4°C); PBS-glycine (3 x 10 min); colloidal-gold labelled GAR IgG's in PBG for 60 min; PBS-glycine (3 x 10 min); aqua bidest (3 x 10 min). Staining and stabilization of the membrane structures was carried out with neutral uranyl acetate-oxalate (pH 7.0) (Tokuyasu,1980). Finally, the grids were embedded in uranyl-acetate containing methylcellulose (Methocell, Fluka AG) during 10 min on ice. After air-drying the sections were viewed in a Philips EM-300 at 80 kV.

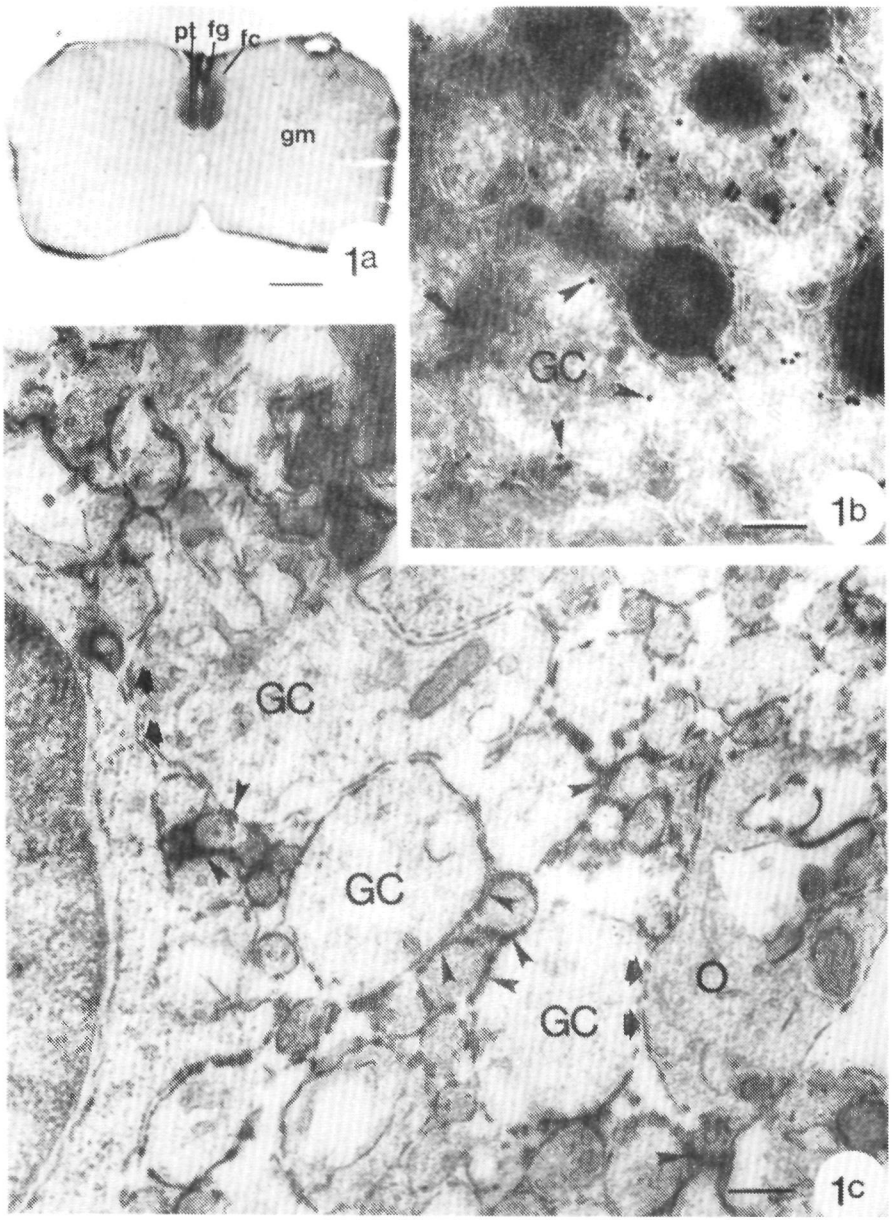
RESULTS

The (sub)-cellular localization of L1 was determined both by pre-embedding staining on vibratome-sections and by immunogold labelling on ultracryosections in developing rat PT at C5. For this purpose pyramidal tracts were taken of 1-, 4-, 10-, 14- and 21-d. old Wistar rats.

Although the use of 2% paraformaldehyde and 2% glutaraldehyde in PBS as a fixative resulted in a better ultrastructural preservation both after pre-embedding procedure as well as ultracryomicrotomy the use of 2% paraformaldehyde and 0.12% glutaraldehyde was preferred because of the higher amount of immunolabelling (both procedures).

Because of identical results obtained after pre-embedding on vibratome sectioned tissue as compared with immunogold labelling on ultracryo-sectioned material misinterpretations due to the diffusion of the

Fig.1: Localization of the L1 antigen in developing rat pyramidal tract at C5 during the entrance of the growth cones of pioneer axons (P1).
1A: Distribution of L1-IR in a transverse vibratome section. Pyramidal tract (PT) and Fasciculus Gracilis (FG) are L1-ir, whereas Fasciculus Cuneatus (FC) and Grey Matter (GM) are L1-neg. Bar= 100 µm. **1B:** Immunogoldlabelling on ultracryosections of transverse sectioned PT of 1-d. old rat. The labelling is localized at the unmyelinated axons, which probably belong to ascending fibre tracts situated in the dorsal funiculus. No uniform labelling at the unmyelinated axons is obtained. At the contact zone between growth cone (GC) and unmyelinated axons L1-IR can be observed (arrowheads). Bar=0.2 µm. **1C:** Pre-embedding immunostaining on transverse vibratome section of 1-d. old rat PT. Small unmyelinated axons show L1-IR (arrowheads). Note the variation in labelling intensities between axons. At the contact zone between growth cones (GC) (arrows) and between growth cones and glial processes (O=oligodendrocytic)(arrows) no L1-IR can be noted.Bar= 0.2 µm.


pt
fg
fc
gm
1a
GC
1b
GC
GC
GC
O
1c

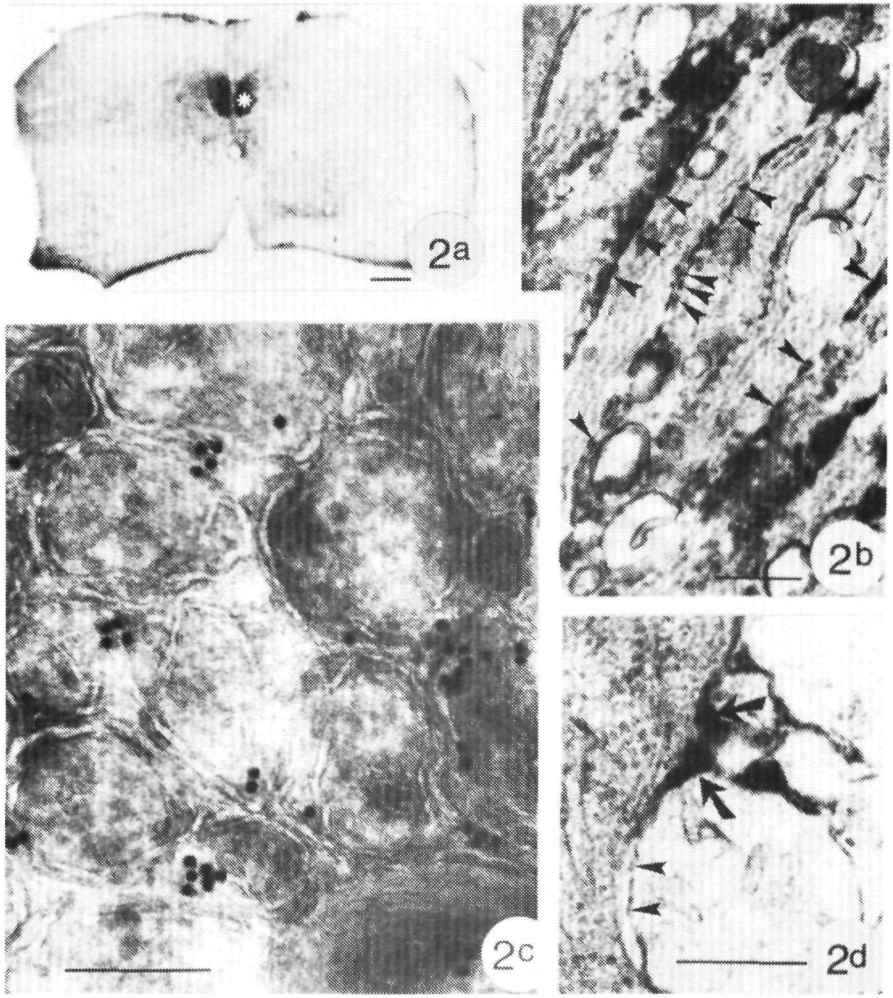


Fig.2: Immunocytochemical staining of the CAM L1 in the developing rat PT during the fasciculation phase of outgrowth (P2-P10).
2A: In the transverse spinal cord section of a 10-d old rat the L1-IR PT stands out against the virtually unstained adjacent fibre tracts and grey matter (GM). Bar= 100 µm. 2B: L1-immunolabelling performed by pre-embedding staining procedures on horizontally sectioned PT. The L1 antigen is localized at the axonal membrane of the small unmyelinated fasciculating PT axons (arrowheads). Note the patchy distribution of DAB-reaction product (arrowheads) Bar= 0.2 µm. 2C: Immunogold labelling on ultracryosectioned PT of 10-d. old rat. Note the typical accumulation of goldlabelling on the unmyelinated PT axons. Bar= 0.2 µm. 2D: At the contact zone between unmyelinated PT axons and oligodendrocytic processes L1-IR can be observed (arrows), probably due to the presence of the L1 antigen on the small axon. L1-IR is absent between larger unmyelinated axons and the same glial process (arrowheads). Bar= 0.2 µm.

peroxidase reaction product can be excluded.

Control sections of both procedures were devoid of L1-IR (for the ultracryomicrotomy-procedure see Fig.4B).

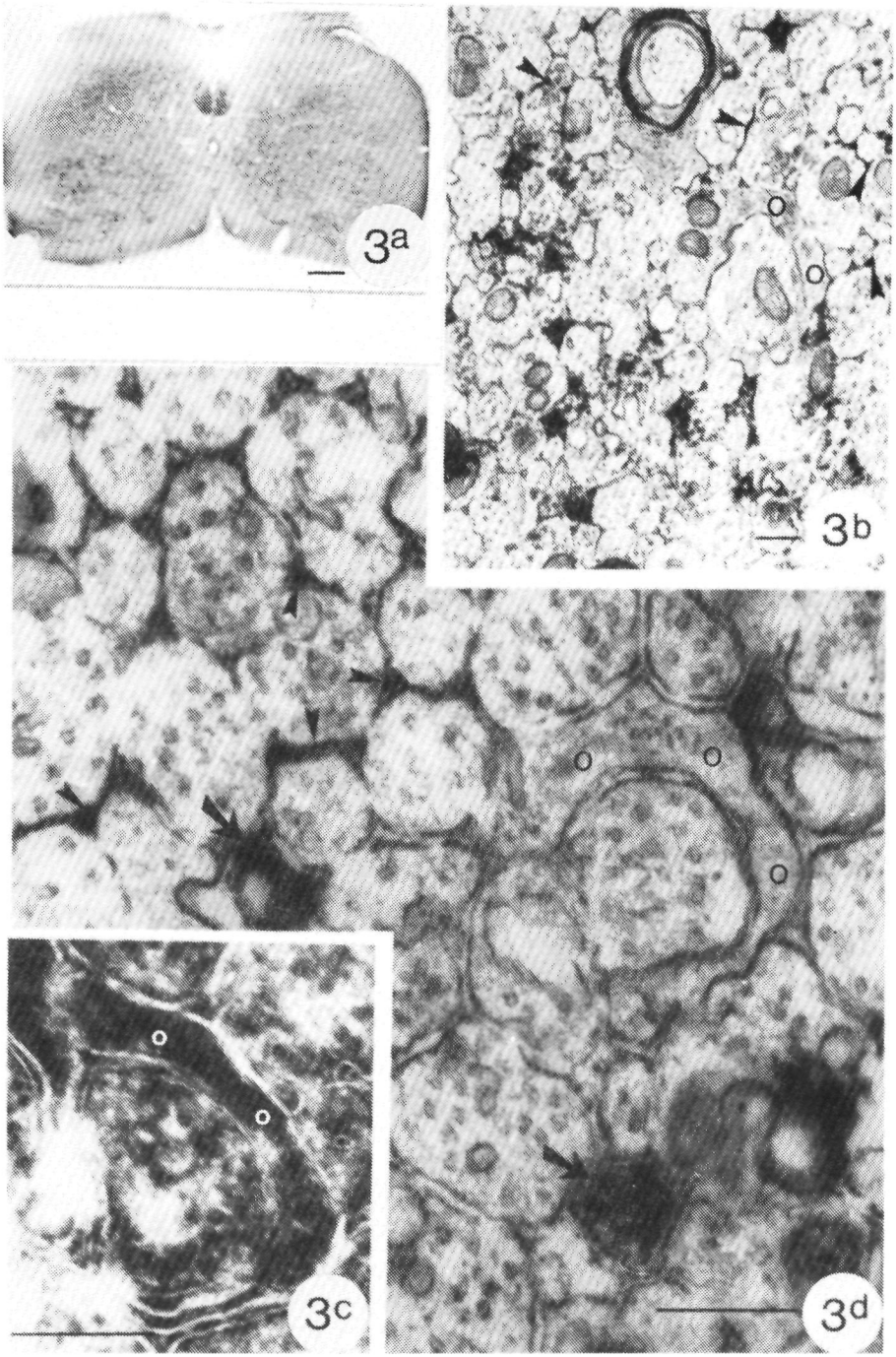
During the entrance of PT growth cones of pioneer fibres at C5, i.e. at P1 (Joosten et al.,1989) a clear L1-IR was noted in the ventralmost part of the dorsal funiculus (Fig.1A). Detectable levels of L1 antigens were seen on small, unmyelinated axons (Figs.1B,1C). These unmyelinated axons in the presumptive PT area are not of corticospinal origin but probably belong to ascending systems situated in the dorsal funiculus (see Discussion). Growth cones of PT pioneer axons did not exhibit L1-IR (Fig.1C), but L1-IR was noted when they were situated adjacent to other small unmyelinated axons (Figs.1B,1C). At the interface between two PT growth cones no L1-IR could be observed (Fig.1C). Also in the contact zone between glial processes (astroglial or oligodendroglial) and PT growth cones L1-IR could not be detected (Fig.1C).

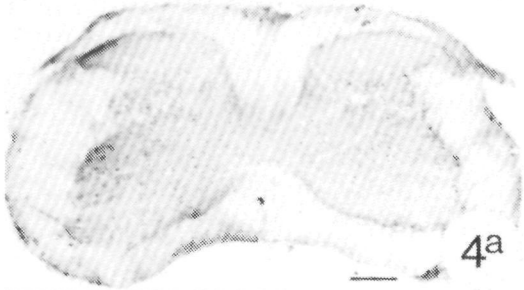
During the period in which the bulk of later arriving unmyelinated PT axons enter the ventralmost part of the dorsal funiculus, i.e. between P2 and P10, a very strong L1-IR could be observed (Fig.2A). Both in transversely and horizontally sectioned material L1-IR was noted on small, unmyelinated fasciculating PT axons (Figs.2B-2D). Because of local accumulations of DAB-reaction product on vibratome sectioned material (Fig.2B) as well as the local distribution of immunogold label on ultracryosections (Fig.2C) it is our impression that L1 is localized in an irregular patchy way on the outer side of the axonal membrane. When unmyelinated PT axons were situated adjacent to glial processes (Fig.2D) a distinct L1-IR could be noted. This immunoreactivity is, however, probably not due to any kind of adhesive interactions between the oligodendrocyte and the PT axon (see Discussion).

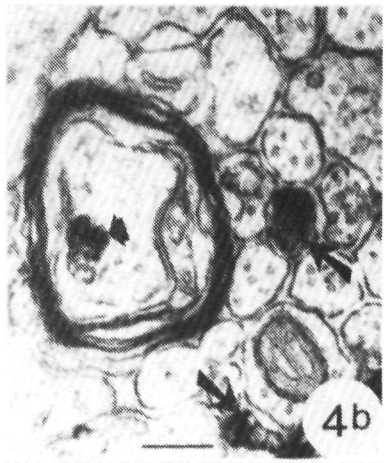
A clear reduction of L1-IR could be observed in the PT area between P10 and P14, i.e. during the onset of myelination (Fig.3A), as compared with the fasciculation phase of outgrowth (Fig.2A). Nevertheless, at the EM level L1-IR was noted in between the unmyelinated PT axons occasionally (Figs.3B,3D). Furthermore, L1-IR was noted within the axoplasm of some small unmyelinated profiles (Figs.3B,3D). PT axons enveloped by early oligodendrocytic processes (or mesaxon) did not express the L1 antigen (Figs.3B-3D). All glial processes observed were L1-negative (Figs.3B-3D).

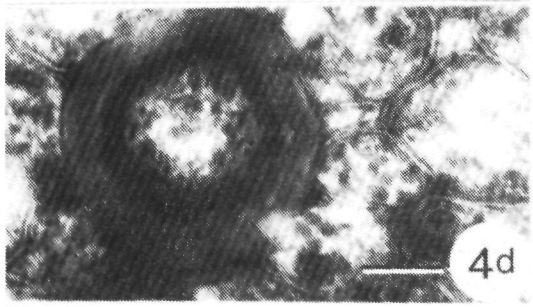
Although myelination of rat CST axons is mainly completed at P21 yet a slight L1-IR could be noted in the PT area (Fig.4A). Both pre-embedding

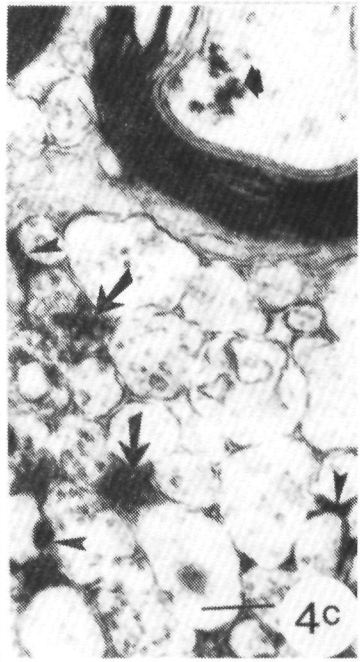
Fig.3: Localization of the L1 antigen in developing rat PT during onset of myelination (P10-P14).
3A: Distribution of L1-IR in a transverse vibratome section at P14. Still a considerable amount of L1-IR can be noted in the PT area. Bar=100 µm.
3B, 3D: pre-embedding technique on transverse vibratome sections of rat PT at P14. Still at some places L1-IR can be observed in between unmyelinated PT axons (arrowheads). No DAB reaction product can be located during the onset of myelination between oligodendrocytic processes or mesaxons (O) and PT axon. Strong L1-ir is observed in the axoplasma of some small unmyelinated PT axons (arrows). Bar= 0.2 µm.
3C: Immunogoldlabelling on transverse ultracryosectioned PT at P14: No label is observed between oligodendrocytic mesaxon (O) and PT axon during initiation of myelination. Bar= 0.2 µm.

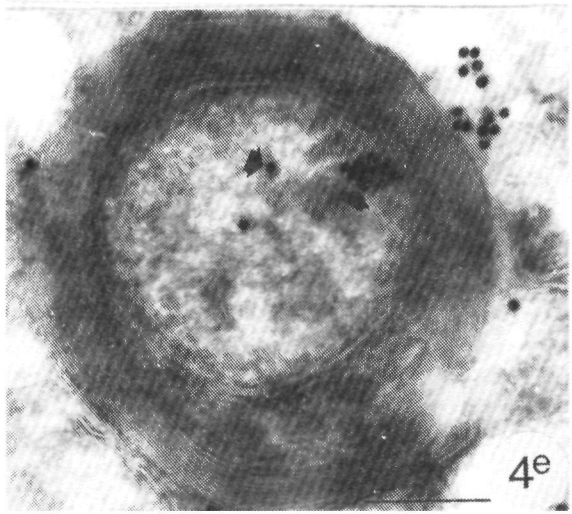

3a
3b
3c
3d


4a


4b


4d


4c


4e

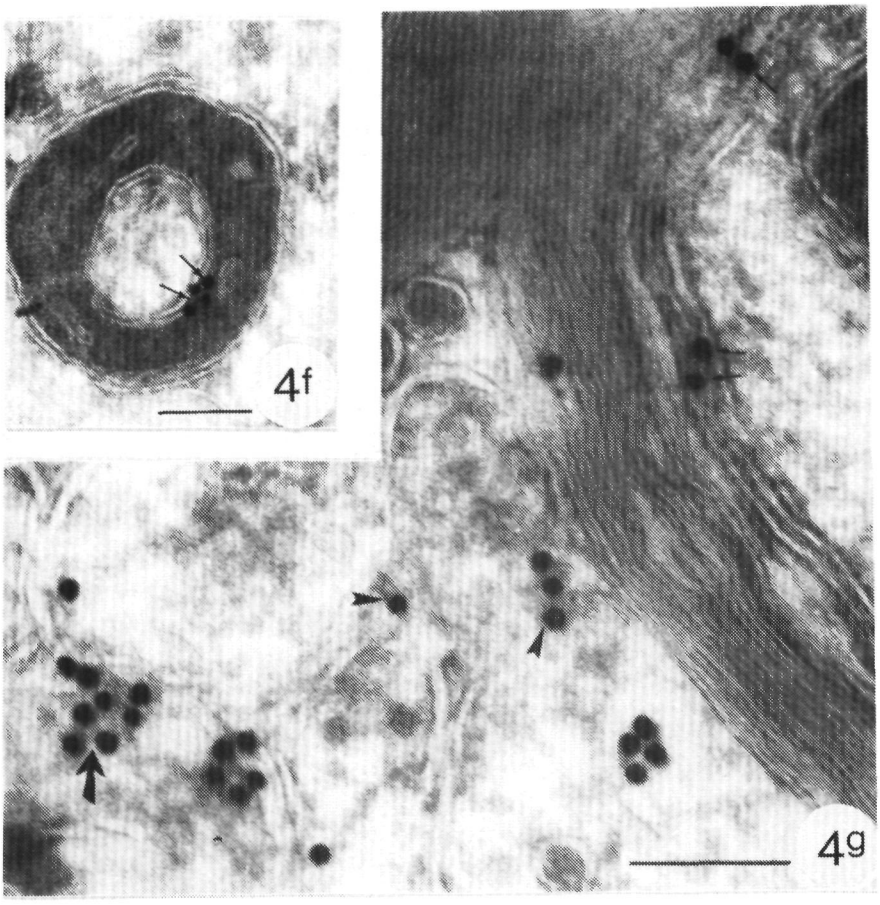


Fig.4: L1-immunoreactivity in the PT of a 3 week old rat at C5 when myelination of this tract is mainly completed. **4A:** A weak L1-ir in the PT area was retained up to the end of the third postnatal week (star). **4B, 4C:** Immunolabelling was performed by pre-embedding staining procedures. Note the presence of DAB reaction product in the axoplasma of myelinated (small arrows) as well as unmyelinated axons (large arrows). Occasionally L1-ir can be noted between small unmyelinated PT axons (arrowheads). Bar= 0.2 µm. **4D:** Immunoelectronmicroscopy of a ultracryocontrol section treated with antibodies to normal swine serum. Bar= 0.2 µm.
4E-4G: Immmunogoldlabelling on ultracryosections.
4E: L1-ir is noted in the axoplasma of the myelinated PT axon (thick arrow). Bar= 0.2 µm **4F:** At the paranodal region L1 is located at the contact zone between PT axon and oligodendrocytic process (small arrow) Bar= 0.2 µm. **4G:** Note the immunogold labelling periaxonally between the axolemma and compact myelin (small arrow). Compact myelin does not contain L1.
Furthermore L1-ir can be observed in between unmyelinated PT axons (arrowheads) but also in the axoplasma (large arrow).Bar= 0.2 µm.

procedures as well as immunogold labelling on ultracryosections revealed the presence of the L1-antigen within the axoplasm of some myelinated (Figs.4B-4D) as well as unmyelinated PT axons (Figs. 4B,4C,4G).Occasionally, L1-IR could be observed in between the unmyelinated PT axons (Fig.4C,4G). Using ultracryosectioned tissue immunogoldlabelling could be noted within the axoplasma of myelinated PT axons (Fig.4E) but also periaxonally between the axolemma and compact myelin (Figs.4E and 4G). Compact myelin did not contain the L1 antigen. At the paranodal region immunogold label could be located at the contact zone between axolemma and the oligodendrocytic process (Fig.4F).

DISCUSSION

In the present investigation the in situ expression of the cell adhesion molecule L1 in the developing rat PT is described. The subcellular localization of L1 was determined both by pre-embedding staining on vibratome sections and by immunogold labelling on ultracryosections. Our findings confirm and extend previous immunocytochemical studies on the localization of L1 in mouse sciatic nerve and cerebellum (Rathjen and Schachner,1984; Fushiki and Schachner,1986; Mirsky et al.,1986; Martini and Schachner,1986,1988; Pehrsohn and Schachner,1987; Godfraind et al.,1988).

In the spinal cord the outgrowth of the major component of the rat PT, the corticospinal tract (CST), primarily occurs during the first ten postnatal days (Schreyer and Jones,1982; Gribnau et al.,1986). Our results demonstrate that L1 is present on unmyelinated fibres in the ventralmost part of the dorsal funiculus during ingrowth of the first CST axons. Tracer studies have indicated that at that time these unmyelinated fibres are not of cortical origin (Joosten et al.,1989), but probably belong to the ascending systems located in the dorsal funiculus, i.e. the fasciculus cuneatus and the fasciculus gracilis (Dunkerley and Duncan, 1969; Matthews and Duncan,1971).

Furthermore growth cones of pioneer CST axons are devoid of L1 as can be deduced from the negative L1-IR on their apposition (Fig.1C). L1-IR, however, is noted between growth cones and adjacent ascending unmyelinated axons (Figs.1B,1C). Although the presence of L1 at the particular region of the pioneer growth cone contacting the unmyelinated axons can not be excluded by our morphological data, the latter L1-IR probably can be attributed to the exclusive presence of L1 on the ascending unmyelinated axons. Whereas the involvement of L1 in the guidance of pioneer CST axons appears to be unlikely, contact-inhibition experiments by injection of the L1 antiserum prior to the arrival of the first CST axons may add to this conclusion.

L1-IR was absent between CST growth cones and glial cells (Fig.1C), as was also noted in embryonic mouse cortex (Godfraind et al.,1988). In vitro studies revealed that polyclonal L1 antibodies do not interfere

with neuron-astrocyte or astrocyte-astrocyte adhesion (Keilhauer et al.,1985). Nevertheless, neuron-astrocyte interaction has been postulated to mediate the guidance of outgrowing pioneer axons in developing CST, since growth cones of pioneer CST axons frequently exhibit protrusions into vimentin-immunoreactive precursor astrocyte processes, suggesting an adhesive type of contact (Joosten and Gibnau,1989[b]). Since the chicken neuron-glia adhesion molecule NgCAM, which is immunologically closely related to mouse L1 (Friedlander et al.,1986) is involved in neuron-glia adhesion (Grumet et al.,1984[a],1984[b]) it is conceivable that L1 and NgCAM might have different sets of functional sites. Thus, the possibility that a functional site of the L1 antigen, which is not recognized by polyclonal L1 antibodies, is implicated in neuron-astrocyte interaction and subsequent guidance of CST pioneer fibres can not be excluded. Besides, the adhesive type of contact between outgrowing pioneer axons and (precursor)- astrocytes may be mediated by another adhesion molecule, as for instance J1 (Godfraind et al.,1988).

Our findings provide conclusive evidence for L1 to be implicated in the fasciculation of the later arriving bulk of CST fibres. In vitro studies (Chang et al.,1987; Keilhauer et al.,1985; Lagenauer and Lemmon,1987) and in vivo observations (Rathjen and Schachner,1984; Beasley and Stallcup; Martini and Schachner,1986,1988; Godfraind et al.,1988; Joosten et al.,1989) already indicated that L1 is instrumental in the fasciculation of outgrowing axons within the cortex as well as in other pathways.

It is intriguing that the intensity of the L1-immunostaining varies along the unmyelinated PT axons (Fig.2B). In developing mouse sciatic nerve a similar non-uniform labelling of L1-immunoperoxidase reaction product was noted, although that phenomenon was not affirmed by the relatively low sensitive post-embedding technique on lowicryl-embedded tissue (Bendayan et al.,1987; Martini and Schachner,1986, 1988). Since accumulations of immunogold labelling were also found after ultracryomicrotomy (Fig.2C), artefacts due to, for instance, the diffusion of DAB-reaction product can be excluded. It is therefore our impression that L1 is localized in a patchy way on the axonal membrane.

Axons ensheathed by oligodendrocytic processes do not express the L1 antigen (Figs.3B-3D). This observation strongly suggests that L1 is not implicated in the mediation of the initial contact between PT axons and oligodendrocytes. The presence of L1 at the axon-Schwann cell interface after the latter cell had turned approximately 1.5 loops indicates that L1 may be implicated in the initial axon-Schwann cell interaction and onset of myelination in developing mouse sciatic nerve (Martini and Schachner,1986). Furthermore, L1 antibodies have been shown to interfere with adhesion of neurons to Schwann cells in vitro (Kleitman et al.,1988). It is , however, still not known whether or not such a molecular signal (L1) leads an axon-Schwann cell interaction to myelinate in one case and not to myelinate in the other.

The discrepancy between the absence of L1 during the initial PT axon myelination on the one hand and the presence during initial sciatic nerve

axon-Schwann cell interaction on the other, might be significant in the understanding of different regenerative capacities of CNS versus PNS.

L1-IR was also noted in the PT-area after completion of the myelination phase. This might be an interesting finding in view of the fact that the developing PT is a central tract with a considerable prolonged capacity of plasticity upon lesions (Kalil and Reh,1982; Bregman and Goldberger, 1982,1983; Bernstein and Stelzner,1983; Bregman et al.,1989). Although the transient expression of the membrane bound L1 during development coincides with a decreased capacity for axonal sprouting in the latter, the presence of the L1 antigen on some unmyelinated axons during the later stages of PT development suggests that L1 might play a decisive role in the capacity of regrowth as well as re-routing of PT axons (Schreyer and Jones,1987; Bregman et al.,1989). The latter capacity, however, may not only be related to an intrinsic change in the neuron's growth potential but also to an alteration in the environmental factors in which the axons grow (Bregman et al.,1989).

In addition to the localization of L1-IR on the axonal membrane of unmyelinated axons, we were able to demonstrate the presence of L1 within the axoplasma of some unmyelinated as well as myelinated axons (Figs.3D,4B,4C,4G). Of course, this phenomenon can hardly be related with the process of axon guidance. Biochemical studies pointed out that various modifications of L1 occur during development (Linneman et al.,1988). The significance of these modifications for the functioning of L1 remains to be investigated. The presence of a soluble form of the L1 protein (Bock et al.,1985), as well as the fact that a considerable amount of L1 remains present in rat cortex explant cultures beyond P15 (Linneman et al.,1988) suggest that this protein not only plays a role in adhesion of axons during outgrowth, but certainly will also have other still unknown functions. Probably, the use of a polyclonal antiserum against L1 resulted in the recognition of the varying biochemical forms of this CAM and therefore the localization of L1 within the axoplasma of unmyelinated as well as myelinated PT axons.

The question of the function of the L1-containing subclass of PT axons still remains to be answered. Quantitative research on the development of rat PT at the third cervical segment revealed a considerable decrease of the total number of axons after the first postnatal week (Gorgels et al.,1989[a]). The period of axon loss comprises a phase of rapid axon loss which coincides with the retraction of transient corticospinal projections (see Chapter 2B,2C; Stanfield et al.,1982). Possibly, the transient corticospinal projections may account for the subclass of L1-containing PT axons during the second postnatal week. Since transient corticospinal projections neither from medial prefrontal nor from occipital parts of the cortex (Joosten et al.,1987[b]; Joosten and Van Eden,1989) do reach lumbar spinal cord levels, the determination of L1-ir in developing rat PT at the lumbar intumescence would be an interesting objective of future research.

The discriminative immunogold labelling technique on ultracryosections

allowed us to visualize the L1 antigen periaxonally between the axolemma and compact myelin as well as at the nodal and paranodal region in the contact zone between axolemma and glial processes (Figs.4E-4G).
The presence of L1 between axolemma and compact myelin is somewhat surprising. L1 ceases to be expressed on both the axon and the myelinating Schwann cell in mouse sciatic nerve, when the Schwann cell processes have turned for 1.5 loops around the axons, as was demonstrated with the use of postembedding immunoelectron-microscopy. Although our results implicate distinct localizations of L1 in CNS versus PNS, the use of different EM techniques (with varying sensitivities) might account for this phenomenon. The LM study of Mirsky et al.(1986) who, using polyclonal L1 antibodies, demonstrated a faint L1 immunofluorescence near myelinated fibres in a teased nerve preparation from rat sciatic nerve, favours our observations. Although L1 might play a role in additional adhesive mechanisms between axolemma and compact myelin, the function of L1 in myelinated peripheral as well as central nervous tissue remains unknown. As suggested by Mirsky et al.(1986) L1 might operate in the adult rat PNS in the linkage between neurons and glia. The presence of L1 on nodal and paranodal regions supports this hypothesis, because of the close apposition and interaction of the myelinating oligodendrocyte and the axon at the nodal and paranodal region (Peters et al.,1976; Wiggins et al.,1988). It is reasonable to assume that molecular mechanisms of cell adhesion play a central role at these sites. Light microscopically a distinct L1-staining was noted at the nodes of Ranvier in rat sciatic nerve (Mirsky et al.,1986). Furthermore, ultrastructural immunogold techniques indicated that Ng-CAM, which is closely related to L1 (Friedlander et al.,1986), is enriched in the nodal axolemma of myelinated as well as within the nodal regions of the myelinating Schwann cell (Rieger et al.,1986).

The present study describes the ultrastructural localization of the CAM L1 in developing rat PT at the fifth cervical segment. Our findings demonstrate that L1 mediates axon fasciculation during PT outgrowth. They also suggest that L1 is not implicated in initial PT myelination in contradistinction to myelination of sciatic nerve axons. Further research may contribute to our understanding of the development of central versus peripheral fibre tracts. Especially an analysis of the relation between the regrowth of PT axons and the localization, c.q. the participation of L1 in that process could help to suggest ways by which injured central fibre tracts can be restored.

Acknowledgements: The authors thank Dr.Fritz Rathjen for his generous gift of the L1-antiserum. This work would not have been possible without the use of the ultracryounit at the Dept.of Pathology (Prof.dr.D.Ruiter) and the Philips EM-300 electronmicroscope at the Dept. of Cell Biology (Prof.dr.A.Stadhouders).

Chapter 6

General Discussion and Summary

CHAPTER 6: GENERAL DISCUSSION AND SUMMARY

This thesis focuses on the postnatal development of the corticospinal tract (CST) of the rat. The development of this long descending pathway was analysed using a number of light- and electron-microscopical techniques. As pointed out in the introduction (Chapter 1.3) the following three questions were of main interest in this investigation:

- **How does the corticospinal component of the rat pyramidal tract develop?**

- **Is collateral elimination confined to the occipital cortex or is it a general phenomenon in the development of the corticospinal tract?**

- **Which factors do play a role in the outgrowth and guidance of corticospinal axons in the rat?**

With the results of the experiments as described in Chapters 2-5 these questions will be addressed hereafter. Furthermore attention will be payed to some new questions which evolved from our findings.

The rat CST development is characterized by a staggered mode of outgrowth (Schreyer and Jones,1982; Gribnau et al.,1986). Our electronmicroscopic observations on anterogradely labelled outgrowing CST axons demonstrate that the main wave of unmyelinated labelled axons is preceded by a number of pathfinding axons, which are characterized by dilatations at their distal ends: the growth cones (Chapter 4B). By contrast, the later arriving unmyelinated labelled axons do not exhibit such large thickenings at their distal ends (Chapter 4B). Rat CST outgrowth is further characterized by a waiting period of about 2 days between the arrival of the CST pioneer axons at the dorsal funiculus of a given spinal cord segment, and the first extension into the respective spinal grey matter (Wise et al.,1979[a]; Schreyer and Jones,1982; Gribnau et al.,1986). Massive CST termination is noted in the spinal grey dorsal horn and intermediate regions after another period of two days.

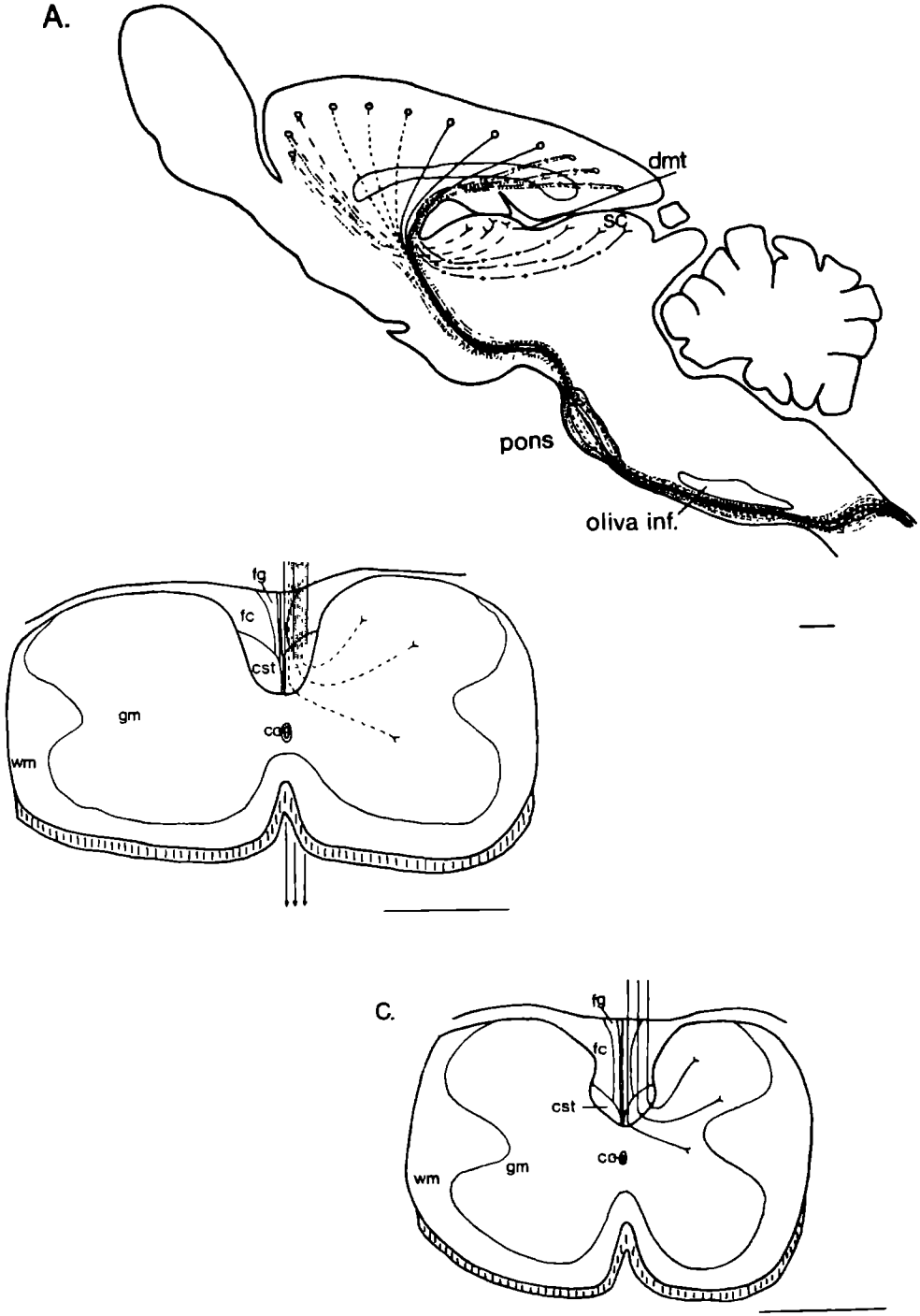
During the first postnatal week the developing rat CST contains three components (as summarized in Fig.1). Two maintaining sets of fibres: the first originating from a group of corticospinal (CS) neurons situated more anteriorly in the sensorimotor cortex and projecting to the cervical spinal grey, and the second with their parent neurons situated more posteriorly in the sensorimotor cortex and terminating in the lumbar grey. In addition, a third transient component is present, emanating from neurons in the occipital as well as the medial prefrontal part of the cortex. Although the somatotopical arrangement of the two maintaining components of the developing CST shows similarities with those in the

adult rat (Wise et al.,1979[a]; Leong,1983; Miller,1987) the extent of this resemblance remains to be established. It is very unlikely that the exact adult somatotopical arrangement of CS neurons in the cortex is already achieved during early postnatal development. Retrograde neuroanatomical tracer studies have revealed that reorganization or focussing of cortical neurons is a common feature in developing fibre systems (Stanfield et al.,1982; Adams et al.,1983[a]; Mihailoff et al.,1984; Stanfield and O'Leary,1985[a],1985[b]; Chalupa and Killacky,1989; Reinoso and Castro, 1989). In addition, these studies prove that this kind of focussation is accomplished by collateral elimination. Collateral elimination, however, implicates that the parent neurons must have established a permanent projection to another structure. Indeed, at least some of the occipital neurons which send transitory projections to the spinal cord (Chapter 2B) later project to the superior colliculus (O'Leary and Stanfield,1985). Similarly, cortical neurons in the medial prefrontal cortex, which give rise to transient spinal projections (Chapter 2C) can later be found to project to the medial dorsal thalamus (van Eden and Kros,1989) (see Fig.1).

Our anterograde tracing experiments (Chapters 2B,2C), in conjunction with the latter retrograde tracing studies demonstrate the extent of the phenomenon of collateral elimination during the refinement of CS projecting neurons in the cortex. In conclusion, collateral elimination is probably a general phenomenon in the development of the entire rat cortex and its emanating fibre systems (Fig.1).

The disappearance of transient CS projections during the second postnatal week coincides with a reduction of the total number of axons present during the development of the rat PT at the third cervical segment as was electronmicroscopically demonstrated by Gorgels et al. (1989[a]). Although a number of axonal ultrastructural features often related to axon degeneration (such as electron-dense bodies, large vacuoles, lamellated bodies) can be observed at the time, the question whether or not transient axons undergo degenerative morphological changes during their elimination, remains to be answered. In the developing corpus callosum of the cat the number of accumulations of vesicles and swollen mitochondria increases during the phase of rapid axonal elimination (Berbel and Innocenti,1988), suggesting axonal degeneration. Besides EM-analysis and quantification of developing tracts in normal tissue the use of anterograde tracing experiments combined with electron

Fig.1.: The rat corticospinal tract originates in the sensorimotor cortex (A) and projects to the spinal grey via the medullary pyramids. During the first postnatal week the developing CST contains three components. First, neurons situated more anteriorly in the sensorimotor cortex projecting to the cervical spinal grey (- - - -) (B) ;secondly, neurons situated more posteriorly in the sensorimotor cortex terminating in the lumbar spinal grey (————) (C) ;and a third, transient component emanating from neurons in the occipital ( ) as well as the medial prefrontal ( ) cortex. Neurons in the occipital cortex eventually project to the superior colliculus (-·-·-)(sc) whereas neurons in the medial prefrontal cortex (– – – –) terminate in the mediodorsal nucleus of the thalamus (dmt). cc=central canal; cst=corticospinal tract; fc=fasciculus cuneatus; fg=fasciculus gracilis; gm=grey matter; wm=white matter. Bar= 1mm.


A.
dmt
sc
pons
oliva inf.
fg
fc
cst
gm
wm
C.
fg
fc
cst
gm
wm

microscopic detection (see Chapter 4A) may be a useful tool to attack the problem of retraction or degeneration of transient axons. Preliminary observations on HRP-labelled occipitospinal axons reveal the presence of the typical crystalline HRP-TMB-(AHM) reaction product in close proximity to large electron-dense bodies. Phagocytosis of transient axons by glial cells, however, may reduce the number of degenerative phenomena. In the mouse visual pathway the rise of stellate shaped GFAP-ir astrocytes coincides with the diminution of excess of axons (Bovolenta and Mason,1987). Similarly, in the developing rat CST a dramatic increase of GFAP-IR is noted during the second postnatal week (Chapter 5B), i.e. the same period during retraction of transient CS axons with their parent neurons in the occipital (Chapter 2B) or the medial prefrontal cortex (Chapter 2C).

The key events involved in the elimination of transient axonal projections most likely occur at the terminal site. Transplantation experiments in the developing rat and hamster cortex are in favour of this hypothesis (Schneider and Jhavieri,1974; Innocenti,1986; Porter et al.,1987). Parts of fetal occipital cortex transplanted in the prospective sensorimotor cortex subsequently develop into a cortical locale with maintaining CST axons up to adulthood (Schneider and Jhavieri,1974). This phenomenon suggests that all layer V neurons have similar projectional capacities and that positional differences are not pre-disposed by genomic qualities (Tolbert,1987). In other words, transitory projections do not seem to be eliminated because they are intrinsically wrong. Up till now, the cellular processes and mechanisms underlying collateral elimination are still a matter of dispute. In view of the role that transient projections may have as a substrate for developmental brain plasticity (see Chapter 2A) it is essential to understand the key events involved in processes concerning the elimination as well as the maintaining of axonal projections. With respect to a better undrstanding of these processes a clear picture of the mode of CST outgrowth may be instrumental (Chapters 2,3,4). Furthermore, factors involved in the outgrowth and guidance of the maintaining CST axonal projections probably also play a role in processes concerning the disappearance of transient CST axons.

The most plausible explanation of the pathfinding of axons during their outgrowth is that a number of guidance cues act together. Our results provide evidence that at least two different factors are involved in CST guidance in spinal cord white matter (tract area) (Fig.2).
The characteristic arrangement of vimentin-immunoreactive (VIM-ir) precursor astrocytes strongly suggests a mechanical way of routing the outgrowing CST pioneer fibres (Fig.2). A prominent VIM-ir glial septum was noted during corticospinal outgrowth in the midline raphe of the medulla oblongata and spinal cord whereas it is absent in the decussation area of the corticospinal tract fibres. Besides having a positional role, (Singer et al.,1979; Vanselow et al.,1989) VIM-ir glial cells probably

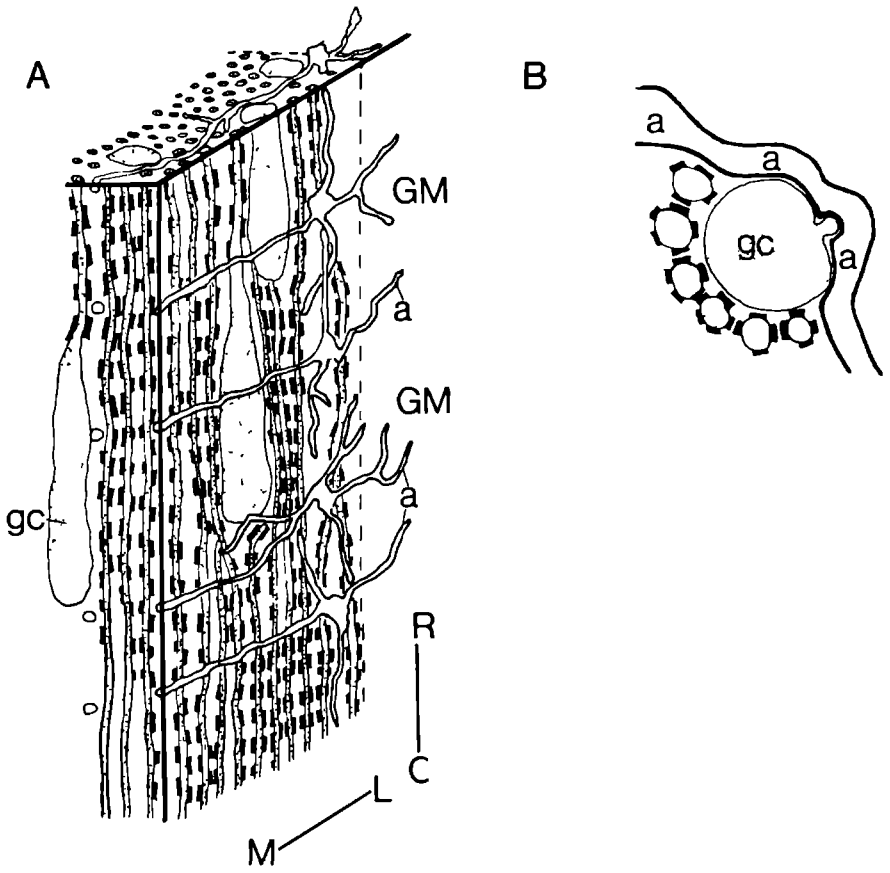


Fig.2.: Outgrowth and guidance of CST pioneer fibres in spinal cord white matter (A) .VIM-ir astrocytes (a) are situated in longitudinal tiers with their processes orientated perpendicular to the outgrowing CST pioneer fibres with their growth cones (GC), suggesting a mechanical way of routing (stereotropism). CST growth cones (GC) show protrusions into the VIM-ir astrocytes indicating an adhesive way of interaction (B) .During the entrance of pioneer CST fibres, the cell adhesion molecule L1 is absent on their growth cones but present on unmyelinated axons, which probably belong to ascending systems situated in the dorsal funiculus. C=Caudal; GM=Grey Matter; L=Lateral; M=Medial; R=Rostral.

are also involved in both a chemotropic and an adhesive way of interacting with outgrowing pioneer CST axons (Fig.2). In addition, the differential adhesiveness among axons induced by the cell adhesion molecule L1 plays an important role in the fasciculation of the later arriving CST axons (Fig.2; Chapter 5C1; 5C2). Although L1 is not located on the growth cones of the pioneer axons it is present on the axonal membranes of these CST fibres, and therefore might play a role in their maintenance (Fig.2).

The rather consistent guidance conditions in which outgrowing pioneer CST axons proceed in the spinal cord white matter may account for the

rather smooth and lengthy shape of CST pioneer growth cones (Fig.3; Chapter 3). On the other hand, pioneer growth cones of CST axons intruding into the grey matter (target area) exhibit a very complex and spiny morphology (Fig.3; Chapter 3). This change in CST growth cone morphology from tract area to target area not only seems to reflect distinct interactions along altered micro-environments but possibly also indicates changing predominant guidance cues. Assumingly, the mechanisms and factors leading outgrowing CST axons towards their target area (VIM-ir glial cells; L1 cell adhesion molecule,among others) differ from those involved in the correct guidance of CST axons within the target area. Our observations, however, do not exclude the involvement of the precursor-astrocytes or the L1 cell adhesion molecule in CST axon guidance within the target area (spinal grey matter). The mechanisms and guidance factors having an essential impact on the guidance of CST axons within the target area remain to be established.

First, the signal that causes the CST axons to enter the spinal grey might occur at the level of their parent cell bodies, i.e. the layer V pyramidal cells in the sensorimotor cortex. Because thalamocortical fibres appear to form synapses as soon as they enter the cortex during the first postnatal week (Wise and Jones,1978), their synapse formation on layer V pyramidal cells might be the trigger. Therefore, it would be interesting to know whether there is a correlation between CST spinal cord outgrowth and the maturation of the originating neurons in the cortex. Besides a possible role of the developmental state of the parent neurons on the entering of their CST pioneer axons into the spinal grey, the totally different growth cone morphology of the latter axons as compared with CST growth cones in the tract area suggests that guidance cues operating at the terminal site may as well be important. It is conceivable that chemotropic factors (local signals of diffusable agents arising from the target cells) serve as local attractants to axons that enter the target area (Purves and Lichtman,1985; Dodd and Jessell,1988). A molecule for which a chemotropic role has been postulated is nerve growth factor (NGF) (Levi-Montalcini and Booker,1960; Mensini-Chen et al.,1978). However, this chemotropic factor could not be demonstrated within the target region of the developing CST. Nevertheless, other chemotropic factors may as well guide CST axons in the target area. For example, in the embryonic rat spinal cord, floor plate cells secrete a diffusable factor that evokes the outgrowth of commissural axons from spinal cord explants and in addition orients these axons (Dodd and Jessell,1988). Other central fibre systems also exhibit a directed growth in such a way that they probably are guided by chemotropism (Bonhoeffer and Huf,1982; Dodd and Jessell,1988). In addition to the more adhesive molecules acting in the guidance of growth cones cell surface molecules located on oligodendrocytes inhibit axon extension and therefore contribute to the local selection of axonal pathways (Caroni and Schwab,1988[a],1988[b]; Schwab and Schnell,1989).

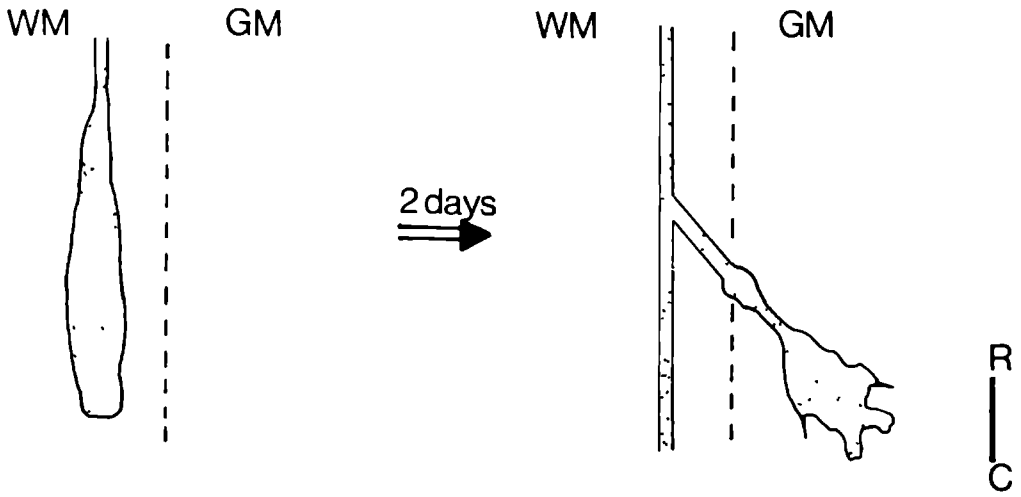


Fig.3.: Growth cone morphology of CST pioneer fibres at the lumbar intumescence of rat spinal cord. The outgrowing pioneer CST axons in the tract area (white matter WM) at P5 are characterized by their rather smooth and lengthy shape (A), whereas CST axons entering the target area (grey matter GM) exhibit a complex and spiny morphology (B). C=Caudal; R=Rostral.

With respect to research on the developing rat CST a major future research goal might be the isolation and subsequent identification of chemotropic factors situated in the spinal cord grey matter. In my opinion the isolation of chemotropic factors located in the cervical spinal grey on the one hand and in the lumbar grey matter on the other is strongly advocated. This suggestion emerges from the fact that differences can be observed between arrival and outgrowth of CST axons at the cervical intumescence after labelling the anterior or the intermediate cortical area (Chapter 2B): CST axons with their cells of origin in the anterior part of the sensorimotor cortex enter the spinal grey about 2 days after their arrival in the dorsal funiculus, whereas axons of intermediate CS neurons do not show any outgrowth at cervical spinal cord levels. Therefore, a chemotropic factor located in the cervical CST target area might guide and attract CST axons from anteriorly situated CS neurons, but not the outgrowth of those CST axons with their cells of origin in the intermediate cortical part. Besides,the cervical spinal grey might contain other chemical factors repulsing 'lumbar' CST axons and vice versa. Such inhibitors were located in the tectum upon the formation of the retinotectal map (Stuermer,1988). The latter CST axons may be guided into the lumbar target area by another chemotropic factor arising from the target cells at the respective site.

As expressed earlier, our major goal to understand CST outgrowth and

guidance must not be restricted to the knowledge of the maintaining components but also has to be addressed at the transient CST projections. Our observation that transient CST projections extend up to the fifth thoracic segment (Chapters 2B,2C) but never exhibit any outgrowth into the adjacent spinal grey indicates that these axons are responsive to guidance factors in the tract (precursor-astrocytes, cell adhesion molecule L1 among others) but probably are not susceptive of those cues (chemotropic) which lead to colonization of the target.

Research on guidance cues in the outgrowing CST may as well contribute to the understanding of the scarce regenerative capacities of the adult mammalian CNS. In the rat the CST not only provides an excellent model to study in vivo outgrowth and development of a central tract but also to analyse their potential regeneration, especially because of the great experimental accessibility of the CST. Lesions of the prospective CST pathway combined with anterograde tract tracing demonstrated the aberrant rerouting of developing CST axons around the lesion site through adjacent undamaged tissue (Kalil and Reh,1982; Bernstein and Stelzner,1983; Schreyer and Jones,1983,1987; Bregman and Goldberger,1983). Since this developmental plasticity becomes restricted at 5-6 days of age (Bregman et al.,1989) it is a major research goal to understand the factors involved in outgrowth and guidance of regenerating rat CST axons.

As pointed out earlier, VIM-ir precursor-astrocytes as well as the CAM L1 are two guidance cues involved in normal CST guidance and therefore may be important in regeneration processes. With respect to the CAM L1 it should be noted that this molecule is involved both in CNS and in PNS fasciculation processes during axon-outgrowth (Rathjen,1988; Chapter 5C) as well as in the onset of myelination in mouse sciatic nerve (Martini and Schachner,1986,1988). Especially the latter observation is intriguing in view of the different regenerative capacities of rat CNS versus PNS, since our results demonstrate that L1 is not involved in the onset of CST myelination (Chapter 5C2).

Although outgrowth, fasciculation and myelination processes certainly are important aspects in rat CST regeneration it should be noted that the presence of unmyelinated CST axons in adult rat PT (Chapter 4C) and the presence of L1 in between (Chapter 5C2) may also account for some regenerative capacities lateron.

In summary, the presert investigation may initiate further research in the broad field of the complex mechanisms involved in the outgrowth, guidance and regrowth of fibre tracts in the central nervous system of mammals.

Samenvatting

In dit proefschrift wordt de ontwikkeling van de corticospinale baan in de rat bestudeerd met behulp van licht- en electronenmicroscopische technieken. De experimenten worden beschreven in de hoofdstukken 2,3,4 en 5. Drie van deze hoofdstukken (2,3 en 4) hebben betrekking op de bestudering van de ontwikkeling van de corticospinale baan in de rat, terwijl in hoofdstuk 5 de rol van enkele mogeljke geleidingsfactoren op de uitgroeiende corticospinale vezels wordt bekeken. In Hoofdstuk 6 worden de resultaten van de vier voorafgaande hoofdstukken besproken en met elkaar in verband gebracht.

De ontwikkeling van de corticospinale baan bij de rat is reeds aan bod gekomen in een aantal studies (o.a. Donatelle,1977; Schreyer and Jones,1982 en Gribnau et al.,1986). Het gebruik van een aantal recente experimentele en/of electronenmicroscopische technieken levert echter een aanzienlijke uitbreiding van onze kennis ten aanzien van het dynamische proces van de ontwikkeling en uitgroei van de corticospinale baan in het bizonder,en van het centrale zenuwstelsel van de rat in het algemeen, op.

Hoofdstuk 2 beschrijft de relatie tussen de injectieplaats in de cortex, daar waar de corticospinale oorsprongscellen liggen, en het labellingspatroon van de zich ontwikkelende corticospinale baan in het ruggemerg van de rat gedurende de eerste twee weken na de geboorte. Met behulp van lichtmicroscopische kleuringen kan de anterograde tracer WGA-HRP gevisualiseerd en gekwantificeerd worden. De belangrijkste conclusie van de experimenten is dat de zich ontwikkelende corticospinale baan al gedurende de eerste twee postnatale weken in drie componenten onderverdeeld kan worden. Van één component liggen de oorsprongscelllen in het anterior of voorste gedeelte van de sensomotorische cortex en het bijbehorende projectieveld in de grijze stof van de cervicale intumescentie. Daarnaast is er een component met z'n oorsprongscelllen in het intermediaire (middelste) gedeelte van de sensomotorische schors en het bijbehorende projectieveld in de grijze stof van de lumbale intumescentie. De derde component is een tijdelijke: cellichamen gesitueerd in de occipitale (Hoofdstuk 2B) dan wel de mediale prefrontale (Hoofdstuk 2C) cortex projecteren naar het ruggemerg gedurende de eerste postnatale week. Door eliminatie van hun corticospinale collateraal in de tweede postnatale week, en niet door het afsterven van de cellichamen, verdwijnt de tijdelijke projectie. Doordat collateraleneliminatie kenmerkend is voor zowel mediale prefrontale- als occipitale cortex-neuronen, is daarmee aannemelijk gemaakt dat dit proces een algemeen verschijnsel is in de cortexontwikkeling van de rat.

In Hoofdstuk 3 komt de morfologie van anterograad HRP gelabelde distale verdikte uiteinden, of groeiconussen, van de eerste corticospinale axonen in het lumbale ruggemerg aan bod. Met behulp van een nieuwe combinatie van HRP-kleuring en intensiverings is het mogelijk de groeiconusmorfolo-

gie lichtmicroscopisch te bestuderen. Het blijkt dat de groeiconussen van de corticospinale pionieraxonen in de witte stof (tract area) gekenmerkt zijn door hun langgerekte en relatief eenvoudige morfologie. De eerste corticospinale groeiconussen in de lumbale grijze stof (target area) zijn kleiner en complexer (naast vinger- en lamelachtige ook vele doornachtige uitstulpingen). Deze verandering in groeiconusmorfologie van de corticospinale pionieraxonen gedurende hun uitgroei wijst op veranderende interacties van deze structuren met hun omgeving.

In Hoofdstuk 4 wordt de postnatale ontwikkeling van de corticospinale baan in de rat op electronenmicroscopisch nivo bestudeerd. Om het anterograad getransporteerde HRP in zich ontwikkelend neuronaal weefsel zichtbaar te maken werd gebruik gemaakt van een nieuw ontwikkelde combinatie van het uiterst gevoelige tetramethylbenzidine (TMB) en de stabilizator ammoniumheptamolybdate (AHM) (Hoofdstuk 4A). Hiermee is het mogelijk het kristallijne HRP-TMB-(AHM) reactieproduct in uitgroeiende corticospinale vezels van de rat electronenmicroscopisch zichtbaar te maken. Met behulp van deze techniek wordt duidelijk dat de uitgroei van gelabelde ongemyeliniseerde corticospinale axonen in het ruggemerg van de rat hoofdzakelijk plaatsvindt gedurende de eerste tien postnatale dagen (Hoofdstuk 4B). Een van de belangrijkste bevindingen in deze studie is wel dat gedurende de trapsgewijze uitgroei en bundelvorming van de corticospinale baan de grote golf van uitgroeiende axonen wordt vooraf gegaan door een aantal pioniervezels dat gekarakteriseerd wordt door verdikkingen aan hun uiteinden: de groeiconussen. De later arriverende ongemyeliniseerde corticospinale axonen vertonen niet zulke grote verdikkingen aan hun uiteinden. Een andere bevinding betreft de timing en voortgang van de myelinisatie van de corticospinale baan: het myelinisatie proces start rostraal (cervicaal) rond dag 14 en ontwikkelt zich naar caudaal gedurende de derde en vierde postnatale week. De aanwezigheid in de volwassen pyramidebaan van gelabelde ongemyeliniseerde naast gemyeliniseerde axonen geeft aan dat in ieder geval een aantal van de ongemyeliniseerde profielen hun oorsprong hebben in de cortex en dus corticospinaal zijn (Hoofdstuk 4C). Dit kan belangrijke functionele implicaties tot gevolg hebben.

In Hoofdstuk 5 wordt de rol van astrocyten (Hoofdstuk 5B) en het celadhesie molecuul L1 (Hoofdstuk 5C1,5C2) in de geleiding van uitgroeiende corticospinale baan axonen onderzocht.
De locatie van precursor-astrocyten tijdens de aankomst van de eerste corticospinale axonen (pioniervezels) wordt bepaald met behulp van verscheidene licht- en electronenmicroscopische immunocytochemische technieken.

Gedurende de ingroei van corticospinale axonen is er sprake van een zeer duidelijk aanwezig vimentine-immunoreactief precursor-astrocyten septum in de mediane raphe van medulla oblongata en ruggemerg, terwijl deze glia-barriere afwezig is in de decussatie, daar waar de corticospinale vezels kruisen. Verder blijkt dat de uitlopers van precursor-astrocyten in longitudinale rijen parallel aan de rostrocaudale as, maar

loodrecht op de uitgroeiende corticospinale axonen, zijn gerangschikt. Interessant is verder het feit dat de groeiconussen van de corticospinale pioniervezels vaak instulpingen vertonen in die vimentine immunoreactieve precursor-astrocyt uitlopers. Precursor-astrocyten lijken dan ook naast een positionele rol gebaseerd op chemische interacties, waarschijnlijk tevens een fysische rol, gebaseerd op de vorming van een barrière, te spelen in de geleiding van uitgroeiende corticospinale axonen.

De expressie van het celadhesie molecuul L1 is bestudeerd gedurende de ontwikkeling van de piramidebaan van de rat door middel van immunohistologie (Hoofdstuk 5C1) en op ultrastructureel nivo met behulp van de pre-embedding techniek op vibratoom coupes als wel de immunogoudlabelling op ultracryocoupes (Hoofdstuk 5C2).
De belangrijkste conclusies uit de waarnemingen zijn dat: 1. het L1 voorkomt op dunne ongemyeliniseerde uitgroeiende piramidebaan axonen en dus zeer waarschijnlijk een belangrijke rol speelt in de bundelvorming van de axonen, 2. het L1 niet voorkomt op corticospinale groeiconussen en dus geen rol van betekenis zal spelen in de geleiding van deze pioniervezels, 3. omdat het L1 niet gevisualiseerd kan worden in de axon-oligodendrocyt interactie zal het niet betrokken zijn bij de aanvang van het myelinisatieproces van de corticospinale baan bij de rat.

In Hoofdstuk 6 wordt gepoogd de resultaten en conclusies van de vier voorafgaande hoofdstukken zoveel mogelijk met elkaar in verband te brengen en te integreren opdat een beter inzicht verkregen wordt in het begrijpen van processen die plaatsvinden in de uitgroeiende en zich ontwikkelende corticospinale baan in de rat. Één ding is duidelijk: naast het feit dat het onderzoek, zoals beschreven in dit proefschrift, enkele vragen probeert te beantwoorden heeft het wellicht nog meer vragen opgeworpen. Toch is de corticospinale baan bij de rat een goed modelsysteem om processen als uitgroei, geleiding, collateraleneliminatie en in de toekomst mogelijk regeneratie in het centrale zenuwstelsel te bestuderen. Naast de morfologische bevindingen zoals gerapporteerd in dit proefschrift is het voor het verkrijgen van een volledig inzicht in de biologische functie van bovengenoemde processen noodzakelijk gegevens vanuit vele andere biologische disciplines te verzamelen en te integreren.

References

Adams,J.C. (1981) Heavy metal intensification of DAB-based HRP reaction product. J.Histochem.Cytochem. 29: 775.

Adams,C.E.,Mihailoff,G.A.,and D.J.Woodward (1983[a]) A transient component of the developing corticospinal tract. Neurosci.Lett.36: 243-248.

Adams,C.E.,Woodward,D.J.,and G.A.Mihailoff (1983[b]) Development of sensorimotor and visual corticopontine projections in the rat. In: Developing and regenerating vertebrate nervous systems. Coates,P.W., Markwald,R.R.,and A.D.Kenny,eds.,pp.211-222,A.R.Liss, New York.

Altman,J.,and S.A.Bayer (1984) The development of the rat spinal cord. In: Advances in anatomy,embryology,and cell biology, Beck,F., Hild,W.,van Limborh,J.,Ortmann,R.,Pauly,J.E.,and T.H.Schiebler,eds., pp.1-166, Springer Berlin.

Angevine,J.B.(1986) The nervous tissue. In: Textbook of histology, Bloom,F.E.,and D.W.Fawcett,eds.,pp.311-366. Philadelphia: WB Saunders Co.

Armand,J. (1982) The origin,course and terminations of corticospinal fibers in various mammals. Prog.Brain Res.57: 329-360.

Bastiani,M.J.,and C.S.Goodman (1984) Neuronal growth cones: specific interactions mediated by filopodial insertion and induction of coated vesicles. Proc.Natl.Acad.Sci.USA 81: 1849-1853.

Bates,C.A.,and H.P.Killacky (1984) The emergence of a discretely distributed pattern of corticospinal projecting neurons. Dev.Brain Res.13: 265-273.

Baumal,R.,Kahn,H.J.,Bailey,B.,Philips,M.J.,and W.Hanna (1980) The value of immunohistochemistry in increasing diagnostic precision of undifferentiated tumours by the surgical pathologist. Histochem. J. 16: 1061-1078.

Beasley,L.,and W.B.Stallcup (1987) The nerve growth factor-inducible large external (NILE) glycoprotein and neural cell adhesion molecule (N-CAM) have distinct patterns of expression in the developing rat central nervous system. J.Neurosci. 7: 708-715.

Beck,C.H.,and W.W.Chambers (1970) Speed,accuracy and strenght of forelimb movement after unilateral pyramidotomy in rhesus monkeys. J.Comp.Physiol.Psychol.70: 1-22.

Beckstead,R.M. (1979) An autoradiographic examination of corticocortical- and subcortical projections of the mediodorsal projection (prefrontal) cortex in the rat. J.Comp.Neurol.184: 43-62.

Bendayan,M.,Nanci,A.,and F.W.K.Kan (1987) Effect of tissue processing on colloidal gold chemistry. J.Histochem.Cytochem.35: 983-996.

Bentley,D.,and M.Caudy (1983) Pioneer axons lose directed growth after selective killing of guidepost cells. Nature 304: 62-65.

Bentley,D.,and H.Keshishian (1982) Pioneer neurons and pathways in insect appendages. Trends Neurosci.5: 354-358.

Berbel,P.,and G.M.Innocenti (1988) The development of the corpus callosum in cats: A light and electronmicroscopic study. J Comp.Neurol. 276: 132-156.

Berg,D.K. (1982) Cell death in neuronal development. Regulation by trophic factors. In: Neuronal development,Spitzer,N.C.,ed., pp.297-331, Plenum Press, New York.

Berkley,K.J.,and N.Contos (1987) A glial-neuronal-glial communication system in the mammalian central nervous system.Brain Res.414: 49-67.

Berlot,J.,and C.S.Goodman (1984) Guidance of peripheral pioneer neurons in the grasshopper: adhesive hierarchy of epithelial and neuronal surfaces. Science 223: 493-496.

Bernstein,D.R.,and W.Stelzner (1983) Developmental plasticity of the corticospinal tract (CST) following mid-thoracic "over-hemi-section" in the neonatal rat. J.Comp.Neurol. 221: 371-385.

Biedenbach,M.A.,DeVito,J.L.,and A.C.Brown (1986) Pyramidal tract of the cat: axon size and morphology. Exp.Brain Res.61: 303-310.

Bignami,A.,and D.Dahl (1973) Differentiation of astrocytes in the cerebellar cortex and pyramidal tracts of the newborn rats. An immunofluorescence study with antibodies to a protein specific to astrocytes. Brain Res.49: 393-402.

Bignami,A.,and D.Dahl (1974) Astrocyte-specific protein and neuro-glial differentation. An immunofluorescence study with antibodies to the glial fibrillary acidic protein. J.Comp.Neurol.153: 27-38.

Bignami,A.,Eng,L.F.,Dahl,D.,and C.T.Uyeda (1972) Localization of the glial fibrillary acidic protein in astrocytes by immuno-fluorescence. Brain Res.43: 429-435.

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

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

Bock,E., Richter-Landsberg,C., Faissner,A.,and M.Schachner (1985) Demonstration of immunochemical identity between the nerve growth factor-inducible large external (NILE) glycoprotein and the cell adhesion molecule L1. EMBO J. 4: 2765-2768.

Bonhoeffer,F.,and J.Huf (1982) In vitro experiments on axon guidance demonstrating an anterior-posterior gradient in the tectum. EMBO J. 1: 427-431.

Bovolenta,P.,Liem,R.K.H.,and C.A.Mason (1984) Development of cere-bellar astroglia: transitions in form and cytoskeletal content. Dev.Biol.102: 248-259.

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

Bovolenta,P.,and C.Mason (1987) Growth cone morphology varies with position in the developing mouse visual pathway from retina to first targets. J.Neurosci.7: 1447-1460.

Bray,D. (1979) Mechanical tension produced by nerve cells in tissue culture. J.Cell Sci.37: 391-410.

Bray,D.,Bunge,M.B.,and K.Chapman (1987) Geometry of isolated sensory neurons in culture: effects of embryonic age and culture substratum. Exp.Cell Res. 168: 127-137.

Bregman,B.,and M.E.Goldberger (1982) Anatomical plasticity and sparing of function after spinal cord damage in neonatal cats. Science 217: 553-555.

Bregman,B.,and M.E.Goldberger (1983) Infant lesion effect: III Anatomical correlates of sparing and recovery of function after spinal cord damage in newborn and adult cats. Dev.Brain Res. 9: 137-154.

Bregman,B.,Kunkel-Bagden,E.,McAttee,and A.O'Neill (1989) Extension of the critical period for developmental plasticity of the corticospinal pathway. J.Comp.Neurol.282: 355-370.

Brown,L.T.,Jr.(1971) Projections and terminations of the cortico-spinal tract in rodents. Exp.Brain Res.13: 432-450.

Burmeister,D.W.,and D.J.Goldberg (1988) Micropruning: the mechanism of turning of Aplysia growth cones at substrate borders in vitro. J.Neurosci.8: 3151-3159.

Caroni,P.,and M.E.Schwab (1988[a]) Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. J.Cell Biol.106: 1281-1288.

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

Carson,K.A.,and M.-M.Mesulam (1982) Electron microscopic demonstration of neural connections using horseradish peroxidase: a comparision of the tetramethylbenzidine procedure with seven other histochemical methods. J.Histochem.Cytochem.30: 425-435.

Castro,A.J. (1972) Motor performance in rats. The effects of pyramidal tract section. Brain Res.44: 313-323.

Caudy,M.,and D.Bentley (1986) Pioneer growth cone steering along a series of neuronal and non-neuronal cues of different affinities. J.Neurosci.6: 1781-1795.

Chalupa,L.M.,and H.P.Killackey (1989) Process elimination underlies ontogenetic change in the distribution of callosal projection neurons in the postcentral gyrus of fetal rhesus monkey. Proc.Natl. Acad.Sci.USA 86: 1076-1079.

Chang,S.,Rathjen,F.G.,and J.Raper (1987) Extension of neurites on axons is impaired by antibodies against specific neural cell surface glycoproteins. J.Cell Biol.104: 355-362.

Chiba,A.,Sheperd,D.,and R.K.Murphy (1988) Synaptic rearrangement during postembryonic development in the cricket. Science 240: 901-905.

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

Chung,K.,and R.E.Coggeshall (1987) Postnatal development of the rat dorsal funiculus. J.Neurosci 7: 972-977.

Cowan,W.M.,Fawcett,J.W.,O'Leary,D.D.M.,and B.B.Stanfield (1984) Regressive events in neurogenesis. Science 225: 1258-1265.

Crandall,J.E.,Whitcomb,J.M.,and V.S.Caviness Jr. (1985) Development of the spino-medullary projection from the mouse barrel field. J.Comp.Neurol.239: 205-215.

Dahl,D. (1981) The Vimentin-GFA protein transition in rat cytoskeleton occurs at the time of myelination. J.Neurosci.Res.6: 741-748.

Dahl,D.,Rueger,D.C.,and A.Bignami (1981) Vimentin, the 57,000 molecular weight protein of fibroblast filaments, is the major cytoskeletal component in immature glia. Europ.J.Cell Biol.24: 191-196.

D'Amato,C.J.,and S.P.Hicks (1978) Normal development and post-traumatic plasticity of corticospinal neurons in rats. Exp.Neurol.60: 557-569.

D'Amato,C.J.,and S.P.Hicks (1980) Development of the motor system: effects of radiation on developing corticospinal neurons and locomotor function. Exp.Neurol.70: 1-23.

D'Amelio,F.E.,Gibbs,M.A.,Mehler,W.R.,Philpott,D.E.,and W.Savage (1986) Axoglial contacts in the area postrema of the cat: An ultrastructural study. The Anat.Rec.215: 407-412.

Dederen,P.J.W.C.,and E.A.J.Joosten (1989) A triple labelling method: HRP anterograde tract tracing combined with double immunofluorescent cell staining in developing neural tissue of the rat. J.Neurosci.Meth. in press.

De Kort,E.J.M.,and Van Aanholt (1983) On the development of the pyramidal tract in the rat. Neurosci.Lett. Suppl.14: 85.

De Kort,E.J.M., Gribnau A.A.M., Van Aanholt H.T.H.,and R.Nieuwenhuys (1985) On the development of the pyramidal tract in the rat I The morphology of the growth zone. Anat.Embryol.172: 195-204.

DeMyer,W. (1967) Ontogenesis of the rat corticospinal tract. Normal events and effects of intra-uterine neurosurgical lesions. Arch.Neurol. 175: 207-232.

Deschenes,M,Labelle,A.,and P.Landry (1979) Morphological characteristics of slow and fast PT-cells in the cat. Brain Res.178: 251-274.

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

Dodd,J.,Morton,S.B.,Karagogeos,D.,Yamamoto,M.,and T.M.Jessell (1988) Spatial regulation of axonal glycoprotein on subsets of embryonic spinal neurons. Neuron 1: 105-116.

Donatelle,J.M.(1977) Growth of the corticospinal tract and the development of placing reactions in the postnatal rat. J.Comp.Neurol.175: 207-232.

Donoghue,J.P.,and S.P.Wise (1982) The motor cortex of the rat: cytoarchitecture and microstimulation mapping. J.Comp.Neurol.212: 76-88.

Dunkerley,G.B.,and D.Duncan (1969) A light and electronmiroscopic study of the normal and degenerating corticospinal tract in the rat. J.Comp. Neurol. 137: 155-184.

Dupouey,P.,Benjelloun,S.,and D.Gomes (1985) Immunohistochemical demonstration of an organized cytoarchitecture of the radial glia in the CNS of the embryonic mouse.Dev.Neurosci.7: 81-93.

Edelman,G.M. (1984) Cell adhesion molecules: a molecular basis for animal form. Sci.Am. 250: 118-129.

Edelman,G.M. (1985) Cell adhesion and the molecular processes of morphogenesis. Ann.Rev.Biochem.54: 135-169.

Edelman,G.M. (1988) Morphoregulatory molecules. Biochemistry 27: 3533-3543.

Edwards,J.S.,Chen,S.-W.,and M.W.Berns (1981) Cercal sensory development following microlesions of embryonic apical cells in Acheta domesticus. J.Neurosci.1: 250-258.

Edwards,M.A.,and M.Murray (1985) Selective retinal reinnervation of a surgically created tectal island in goldfish II Electron microscopic analysis. J.Comp.Neurol.232: 386-400.

Elger,C.E.,Janszen,R.W.C.,Speckmann,E.-J.,and H.Caspers (1977) Cortico-spinal connections in the rat I Monosynaptic and polysynaptic responses of cervical motoneurons to epicortical stimulation. Exp.Brain Res.28: 385-404.

Elger,C.E.,Janszen,R.W.C.,Speckmann,E.-J.,and H.Caspers (1978) Influence of the corticospinal system on cervical and lumbar motoneurons in the rat. J.Physiol.74: 203-209.

Faissner,A.,Kruse,J.,Goridis,C.,Bock,E.,and M.Schachner (1984) The neural cell adhesion molecule L1 is distinct from the N-CAM related group of surface antigens BSP-2 and D-2. EMBO J. 3: 733-737.

Fischer,G.,Kunemund,V.,and M.Schachner (1986) Neurite outgrowth patterns in cerebellar microexplant cultures are affected by antibodies to the cell surface glycoprotein L1. J.Neurosci.6: 605-612.

Friedlander,D.R., Grumet,M.,and G.M.Edelman (1986) Nerve-growth factor enhances expression of neuron-glia cell adhesion molecule in PC12 cells, J.Cell Biol. 102: 413-419.

Frost,D.O. (1984) Axonal growth and target selection during development: retinal projections to the ventrobasal complex and other "nonvisual" structures in the neonatal Syrian hamsters. J.Comp.Neurol.230: 576-592.

Frost,D.O.,and G.E.Schneider (1979) Postnatal development of retinal projections in Syrian hamsters: A study using autoradiographic and anterograde degeneration techniques. Neurosci.4: 1649-1677.

Fujisawa,H. (1987) Mode of growth of retinal axons within the tectum of Xenopus tadpoles, and implications in the ordered neuronal connection between the retina and the tectum. J.Comp.Neurol.260: 127-139.

Fushiki,S.,and M.Schachner (1986) Immunocytological localization of cell adhesion molecules L1 and N-CAM and the shared carbohydrate epitope L2 during development of the mouse neocortex. Dev.Brain Res.24: 153-167.

Geuze,H.J.,and J.W.Slot (1980) Disproportional immunostaining patterns of two secretory proteins in guinea pig and rat exocrine pancreatic cells. An immunoferritin and fluorescence study. Eur.J.Cell Biol. 21: 93-100.

Godfraind,C., Schachner,M.,and A.M.Goffinet (1988) Immunohistological localization of cell adhesion molecules L1, J1, N-CAM and their common carbohydrate L2 in the embryonic cortex of normal and reeler mice. Dev.Brain Res. 42: 99-111.

Goodman,C.S.,Bastiani,M.J.,Doe,C.Q.,du Lac,S.,Helfand,S.L.,Kuwada,J.Y.,and S.B.Thomas (1984) Cell recognition during neuronal development. Science 225: 1271-1279.

Goodman,C.S.,and M.Bate (1981) Neuronal development in the grasshopper. TINS.4: 163-169.

Goodman,D.C.,Jarrad,L.E.,and J.F.Nelson (1966) Corticospinal pathways and their termination in the albino rat. Anat.Rec.154: 462.

Gorgels,T.G.M.F.,De Kort,E.J.M,Van Aanholt,H.T.H.,and R.Nieuwenhuys (1989a) A quantitative analysis of the development of the pyramidal tract in the cervical spinal cord in the rat. Anat.Embryol. 179: 377-385.

Gorgels,T.G.M.F., Van Lookeren-Campagne,M,Oestreicher,A.B.,Gribnau,A.A.M., and W.H.Gispen (1989b) B50/GAP43 is localized at the cytoplasmic side of the plasma membrane in developing and adult rat pyramidal tract. J.Neurosci. in press.

Gribnau,A.A.M., De Kort,E.J.M., Dederen,P.J.W.C.,and R.Nieuwenhuys (1986) On the development of the pyramidal tract in the rat II An anterograde tracer study of the outgrowth of the corticospinal fibers.Anat.Embryol. 175: 101-110.

Griffin,G.,Watkins,L.R.,and D.J.Mayer (1979) HRP pellets and slow-release gels: two techniques for greater localization and sensitivity. Brain Res.168: 595-601.

Griffiths,G.,Simons,K.,Warren,G.,and K.T.Tokuyasu (1983) Immunoelectron microscopy using thin frozen sections: application to studies of the intracellular transport of semilike forest virus spike glycoproteins. Methods Enzymol. 96: 466-483.

Grumet,M., Hoffman,S.,and G.M.Edelman (1984a) Two antigenetically related cell adhesion molecules of different specificities mediate neuron-neuron and neuron-glia adhesion. Proc.Natl.Acad.Sci.USA, 80: 267-271.

Grumet,M., Hoffman,S., Chuong,C.-M.,and G.M.Edelman (1984b) Polypeptide components and binding functions of neuron-glia cell adhesion molecules. Proc.Natl.Acad.Sci.USA, 81:1789-1793.

Gundersen,G.W.,and J.N.Barrett (1979) Neuronal chemotaxis: chick dorsal root axons turn toward high concentrations of nerve growth factor. Science 206: 1079-1080.

Hall,R.D.,and E.P.Lindholm (1974) Organization of motor and somatosensory neocortex in the albino rat. Brain Res.66: 23-38.

Hamburger,V.,and R.W.Oppenheim (1982) Naturally occuring neuronal death in vertebrates. Neurosci Comm. 1: 39-55.

Hankin,M.H.,and J.Silver (1986) Mechanisms of axonal guidance. The problem of intersecting fiber systems. In:Developmental Biology, Browder,L.W.,ed.,Vol.2,pp.565-603, Plenum Publ.Corp..

Hardy,H.,and L.Heimer(1977) A safer and more sensitive substitute for diaminobenzidine in the light microscopic demonstration of retrograde and anterograde axonal transport of HRP. Neurosci.Lett 5: 235-240.

Harris,W.A.,Holt,C.E.,and F.Bonhoeffer (1987) Retinal axons with and without their somata, growing to and arborizing in the tectum of Xenopus embryos: a time-lapse video study of single fibers in vivo. Development 101: 123-133.

Harris,W.A.,Holt,C.E.,Smith,T.A.,and N.Gallenson (1985) Growth cones of developing retinal cells in vivo, on culture surfaces, and in collagen matrices. J.Neurosci.Res. 13: 101-122.

Haydon,P.G.,McCobb,D.P.,and S.B.Kater (1984) Serotonin inhibits growth cone motility and synaptogenesis of specific identified neurons. Science 226: 561-564.

Haydon,P.G.,Cohan,C.S.,McCobb,D.P.,Miller,H.R.,and S.B.Kater (1985) Neuron-specific growth cone properties as seen in identified neurons of Heliosoma. J.Neurosci.Res.13: 135-147.

Henrikson,C.K.,and J.E. Vaughn (1974) Fine structural relationships between neurites and radial glial processes in developing mouse spinal cord. J.Neurocytol. 3: 659-675.

Henry,M.A.,Westrum,L.E.,and L.R.Johnson (1985a) Ultrastructure of transganglionic HRP transport in cat trigeminal system. Brain Res.334: 255-266.

Henry,M.A.,Westrum,L.E.,and L.R.Johnson (1985b) Enhanced ultrastructural visualization of the horseradish peroxidase-tetramethylbenzidine reaction product. J.Histochem.Cytochem.33: 1256-1259.

Hepp-Reymond,M.C.M.,Wiesendanger,M.,Brumert,A.,Mackel,A.,Unger,R.,and J.Wespi (1970) Effects of unilateral pyramidotomy on conditioned finger movement in the monkey. Brain Res.24: 544-551.

Heumann,D.,Leuba,G.,and T.Rabinowicz (1978) Postnatal development of the mouse cerebral neocortex II Evolution of the cortical volume, of the population of neurons and glia cells. J.Hirnforsch.19: 385-393.

Hicks,S.P.,and C.J. D'Amato (1975) Motor-sensory cortex-corticospinal system and developing locomotion and placing in rats. Am.J.Anat.143: 1-42.

Hicks,S.P.,and C.J. D'Amato (1980) Development of the motor system: Hopping rats produced by prenatal irradiation. Exp.Neurol.70: 24-39.

Hildebrand,C.,and S.G.Waxman (1984) Postnatal differentiation of rat optic nerve fibers: electronmicroscopic observations on the development of the nodes of Ranvier and axoglial relations. J.Comp.Neurol.224:25-37.

Ho,R.K.,and C.S.Goodman (1982) Peripheral pathways are pioneered by an array of central and peripheral neurones in grasshopper embryos. Nature 279: 404-406.

Holley,J.A.,and J.Silver (1987) Growth pattern of pioneering chick spinal cord axons.Dev.Biol.123: 375-388.

Horder,T.J. (1978) Functional adaptability and morphogenetic opportunism, the only rules for limb development? In: Formshaping movements in neurogenesis, Jacobson,C.,and T.Ebendahl,eds.,pp.181-192, Amlqvist and Wiksell Stockholm.

Humphrey,T. (1960) The development of the pyramidal tracts in human fetuses correlated with cortical differentiation. In: Structure and function of the cerebral cortex, Tower,Schade,eds.,pp 93-103, Proc. of the second Int.Meeting of Neurobiologists, Elsevier,Amsterdam.

Humphrey,D.R.,and W.S.Corrie (1978) Properties of the pyramidal tract neuron system within a functionally defined subregion of the primate motor cortex. J.Neurophysiol.41: 216-243.

Huttenlocher,P.R. (1970) Myelination and the development of functions in the immature pyramidal tract. Exp.Neurol.29: 405-415.

Hynes,R.O. (1987) Integrins: a family of cell surface receptors. Cell 48: 549-554.

Innocenti,G.M. (1981) Growth and reshaping of axons in the establishment of visual callosal connections. Science 212: 824-827.

Innocenti,G.M. (1986) General organization of callosal connections in the cerebral cortex. In: The cerebral cortex, Jones,E.G.,and A.Peters,eds., pp.291-353, Plenum Press.

Innocenti,G.M. (1988) Loss of axonal projections in the development of the mammalian brain. In: The making of the nervous system; Parnavelas,J.G., Stern,C.D.,and R.V.Stirling,eds., pp.319-339, Oxford University press.

Innocenti,G.M.,Fiore,L.,and R.Caminiti (1977) Exuberant projection into the corpus callosum from the visual cortex of newborn cats. Neurosci. Lett.4:237-242.

Itoh,K.,Konishi,A.,Nomura,S.,Mizuno,N.,Nakamura,Y.,and T.Sugimoto (1979) Application of coupled oxidation reaction to electronmicroscopic demonstration of horseradish peroxidase: cobalt glucose oxidase method. Brain Res.175: 341-346.

Ivy,G.O.,Akers,R.M.,and H.P.Killacky (1979) Differential distribution of callosal projection neurons in the neonatal and adult rat.Brain Res.173: 532-537.

Ivy,G.O.,and H.P.Killacky (1981) The ontogeny of the distribution of callosal projection neurons in the rat parietal cortex. J.Comp.Neurol. 195: 367-389.

Ivy,G.O.,and H.P.Killacky (1982) Ontogenetic changes in the projections of neocortical neurons. J.Neurosci.2: 735-743.

Jaffe,L.F. (1981) The role of ionic currents in established developmental patterns. Philos.Trans.R.Soc. Lon. (Biol) 295: 553-566.

Jaffe,L.F.,and M.-M. Poo (1979) Neurites grow faster towards the cathode than the anode in a steady field. J.Exp.Zool.209: 115-128.

Janszen,R.W.C.,Speckmann,E.J.,Caspers,H.,and C.E.Elger (1977) Corticospinal connections in the rat II Oligosynaptic and polysynaptic responses of lumbar motoneurons to epicortical stimulation. Exp.Brain Res.28: 405-420.

Johnston,R.N,and N.K.Wessels (1980) Regulation of the elongating nerve fibers. Curr.Top.Dev.Biol.16: 165-206.

Jones,E.G.,Schreyer,D.J.,and S.P.Wise (1982) Growth and maturation of the rat corticospinal tract. Progr. Brain Res.57: 361-379.

Joosten,E.A.J.,and A.A.M.Gribnau (1988) Unmyelinated corticospinal axons in adult rat pyramidal tract. An electron microscopic tracer study. Brain Res.459: 173-177.

Joosten,E.A.J.,and A.A.M.Gribnau (1989[a]) Immunocytochemical localization of cell adhesion molecule L1 in developing rat pyramidal tract. Neurosci.Lett.,100: 94-98.

Joosten,E.A.J.,and A.A.M.Gribnau (1989[b]) Astrocytes and guidance of outgrowing corticospinal tract in the rat. An immunocytochemical study using anti-vimentin and anti-glial fibrillary acidic protein. Neurosci.,in press.

Joosten,E.A.J.,Gribnau,A.A.M.,and P.J.W.C.Dederen (1987[a]) Ultrastructural visualization of anterogradely transported horseradish peroxidase in the developing corticospinal tract of rat.J.Histochem.Cytochem.35: 623-626.

Joosten,E.A.J.,Gribnau,A.A.M.,and P.J.W.C.Dederen (1987[b]) An anterograde tracer study of the developing corticospinal tract in the rat: three components. Dev.Brain Res. 36: 121-130.

Joosten,E.A.J.,Gribnau,A.A.M.,and P.J.W.C.Dederen (1987[c]) Postnatal development of the corticospinal tract in the rat II An electron microscopic anterograde HRP study. Neurosci.(Suppl) 22: S210.

Joosten,E.A.J.,Gribnau,A.A.M.,and P.J.W.C.Dederen (1989) Postnatal development of the corticospinal tract in the rat. An ultrastructural anterograde tracer HRP study. Anat.Embryol.179: 449-456.

Joosten,E.A.J.,and C.G.Van Eden (1989) An anterograde tracer study on the development of corticospinal projections from the medial prefrontal cortex in the rat.Dev.Brain Res. 45: 313-319.

Kalil,K. (1984) Development and regrowth of the rodent pyramidal tract. TINS 7: 394-398.
Kalil,K. (1988) Growth and guidance of axons in two pathways from the mammalian cerebral cortex. In: From message to mind, Easter,S.S.Jr., Barald,K.F.,and B.M.Carlson, eds., pp.134-150, Sinauer Assoc.
Kalil,K.,and C.Norris (1985) Rearrangement of axon fascicles in the decussation of the pyramidal tract. Soc.Neurosci.11: 584(abstr).
Kalil,K,and T.Reh (1982) A light- and electronmicroscopic study of regrowing pyramidal tract fibers. J.Comp.Neurol. 211: 265-275.
Kalil,K.,and T.Reh (1979) Redirection of pyramidal tract axons in neonatal hamsters: some mechanisms of axonal guidance during development. Soc.Neurosci.5: 629 (abstr.)
Kalil,K.,and G.E.Schneider (1975) Motor performance following unilateral pyramidal tract lesions in the hamster. Brain Res.100: 170-174.
Kapfhammer,J.P.,and J.A.Raper (1987) Collapse of growth cone structure on contact with specific neurites in culture. J.Neurosci.7: 201-212.
Keilhauer,G., Faissner,A.,and M.Schachner (1985) Differential inhibition of neurone-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion of L1,L2 and N-CAM antibodies. Nature (Lond.) 316: 728-730.
Killacky,H.P.,and L.M.Chalupa (1986) Ontogenetic change in the distribution of callosal projection neurons in the postcentral gyrus of the fetal rhesus monkey. J.Comp.Neurol.244: 331-348.
Kleitman,N.,Simon,D.K.,Schachner,M., and R.P.Bunge (1988) Growth of embryonic retinal neurites elicited by contact with Schwann cell surfaces is blocked by antibodies to L1. Exp.Neurol. 102: 298-306.
Krayanek,S.,and S.Goldberg (1981) Oriented extracellular channels and axonal guidance in the embryonic chick retina. Dev.Biol.84: 41-50.
Krettek,J.E.,and J.L.Price (1977) The cortical projections of the mediodorsal nucleus and adjacent thalamic nuclei in the rat. J.Comp.Neurol.171: 157-192.
Krieg,W.J.S. (1946) Connections of the cerebral cortex. I The albino rat. A topography of cortical areas. J.Comp. Neurol.84: 221-275.
Kucera,P.,and M.Wiesendanger (1985) Do ipsilateral corticospinal fibers participate in the functional recovery following unilateral pyramidal lesions in monkeys? Brain Res.348: 297-303.
Kuypers,H.G.J.M. (1981) Anatomy of descending pathways. In: Brookhart,J.,and V.Mountcastle,eds., Handbook of physiology- the nervous system II.,pp.597-666, American Pysiol.Soc. Bethesda MD.
Lagenaur,C.,and V.Lemmon (1987) An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. Proc.Natl.Acad.Sci. 84: 7753-7757.
Landis,S.C. (1983) Neuronal growth cones. Ann.Rev.Physiol.45: 567-580.
Landry,F.,Wilson,C.J.,and S.T.Kitai (1984) Morphological and electrophysiological characteristics of pyramidal tract neurons in the rat. Exp.Brain Res.57: 177-190.
Langford,L.A.,and R.E.Coggeshall (1981) Unmyelinated axons in the posterior funiculi. Science 211: 176-177.
Lasek,R.J.,and M.J.Katz (1987) Mechanisms at the axon tip regulate metabolic processes critical to axonal elongation. Prog. Brain Res.71: 49-60.
Lawrence,D.G.,and H.G.J.M.Kuypers (1968) The functional organization of the motor system in the monkey. I The effects of bilateral pyramidal lesions. Brain 91: 1-14.
Leenen,L.P.H.,Meek,J.,and R.Nieuwenhuys (1982) Unmyelinated fibres in the pyramidal tract of the rat: a new view. Brain Res.246: 297-301.
Leenen,L.P.H.,Meek,J.,Postuma,P.R.,and R.Nieuwenhuys (1985) A detailed morphometrical analysis of the pyramidal tract of the rat. Brain Res. 359: 65-80.
Leong,S.K. (1983) Localizing the corticospinal neurons in neonatal, developing and mature albino rat. Brain Res.265: 1-9.

Leong,S.K.,Shieh,J.Y.,and W.C.Wong (1984[a]) Localizing spinal cord-projecting neurons in adult albino rats. J.Comp.Neurol.228: 1-17.

Leong,S.K.,Shieh,J.Y.,and W.C.Wong (1984[b]) Localizing spinal cord-projecting neurons in neonatal and immature albino rats. J.Comp. Neurol.228: 18-23.

Letourneau,P.C. (1975) Cell-to-substratum adhesion and guidance of axonal elongation. Dev.Biol.44: 92-101.

Letourneau,P.C. (1979) Cell-substratum adhesion of neurite growth cones, and its role in neurite elongation. Exp.Cell Res.124: 127-138.

Letourneau,P.C. (1982) Nerve fiber growth and its regulation by extrinsic factors. In: Neuronal development, Spitzer,N.C.,ed., Plenum Press,New York.

Letourneau,P.C. (1985) Axonal growth and guidance. In: Molecular bases of neural development, Edelman,G.M.,ed.,pp.269-294.

Levi-Montalcini,R.,and P.U.Angletti (1963) Essential role of the nerve growth factor in the survival and maintenance of dissociated sensory and sympathetic embryonic nerve cells in vitro. Dev.Biol.7: 653-659.

Levi-Montalcini,R.,and B.Booker (1960) Destruction of sympathetic ganglia in mammals by an antiserum to a nerve growth protein. Proc.Natl.Acad. Sci.USA 46: 384-391.

Levitt,P.,Cooper,M.L.,and P.Rakic (1983) Early divergence and changing proportions of neuronal and glial precursor cells in the primate cerebral ventricular zone. Dev.Biol.96: 472-484.

Lindner,J.,Rathjen,F.G.,and M.Schachner (1983) L1 mono- and polyclonal antibodies modify cell migration in early postnatal mouse cerebellum. Nature 305: 427-430.

Linneman,D.,Evardsen,K.,and E.Bock (1988) Developmental study of the cell adhesion molecule L1. Dev.Neurosci. 10: 34-42.

Lockerbie,R.O. (1987) The neuronal growth cone: a review of its locomotory, navigational and target recognition capabilities. Neurosci.20: 719-729.

Ludwin,S.K.,Kosek,J.C.,and L.F.Eng (1976) The topographical distribution of S-100 and GFA proteins in the adult rat brain. An immunohistochemical study using horseradish peroxidase-labeled antibodies. J.Comp.Neurol.165: 197-208.

Marfurt,C.F.,Zaleski,E.M.,Adams,C.E.,and C.L.Welther (1986) Sympathetic nerve fibres in rat orofacial and cerebral tissues as revealed by the WGA-HRP tracing technique: a light and electron microscopic study. Brain Res. 366: 373-378.

Martin,G.F.,Cabana,T.,Culberson,J.L.,Curry,J.J.,and J.Tschismedia (1980) The early development of corticobulbar and corticospinal systems. Studies using the North American opossum. Anat.Embryol.161: 157-213.

Martini,R.,and M.Schachner (1986) Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and MAG) and their shared carbohydrate epitope and myelin basic protein in developing sciatic nerve. J.Cell Biol.103: 2439-2448.

Martini,R.,and M.Schachner (1988) Immunoelectron microscopic localization of neural cell adhesion molecules (L1,N-CAM and myelin-associated glyco-protein) in regenerating adult mouse sciatic nerve. J.Cell Biol. 106: 1735-1746.

Mason,C.A. (1982) Development of the terminal arbors of retino-geniculate axons in the kitten I Light microscopic observations. Neurosci.7: 541-559.

Mason,C.A. (1985) Growing tips of embryonic cerebellar axons in vivo. J.Neurosci.Res.13: 55-73.

Matthews,M.A.,and D.Duncan (1971) A quantitative study of morphological changes accompanying the initiation and progress of myelin production in the dorsal funiculus of the rat spinal cord. J.Comp.Neurol.142: 1-22.

McGeorge,A.J.,and R.L.M.Faull (1989) The organization of the projection from the cerebral cortex to the striatum in the rat. Neurosci.29:503-537.

McLean,J.W.,and P.K.Nakane (1974) Periodate-lysine-paraformaldehyde.A new fixative for immunoelectronmicroscopy.J.Histochem.Cytochem. 22: 1077-1085.

Mediratta,N.K.,and J.A.R.Nicoll (1983) Conduction velocities of corticospinal axons in the rat studied by recording cortical antidromic responses. J.Physiol.336: 545-561.

Mensini-Chen,M.G.,Chen,J.S.,and R.Levi-Montalcini (1978) Sympathetic nerve fibers ingrowth in the central nervous system of neonatal rodent upon intracerebral NGF injection. Arch.Ital.Biol.116: 53-84.

Mesulam,M.-M. (1978) Tetramethylbenzidine for horseradish peroxidase neurochemistry: a non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. J.Histochem.Cytochem.26: 106-117.

Mesulam,M.-M., and E.J.Mufson (1980) The rapid anterograde transport of horseradish peroxidase. Neurosci.5: 1277-1286.

Mesulam,M.-M.,and D.L.Rosene (1979) Sensitivity in horseradish peroxidase neurochemistry: a comparative and quantitative study of nine methods. J.Histochem.Cytochem.27: 763-773.

Mihailoff,G.A.,Adams,C.E.,and D.J.Woodward (1984) An autoradiographic study of the postnatal development of sensorimotor and visual components of the corticopontine system. J.Comp.Neurol.222: 116-127.

Miller,M.W.(1987) The origin of corticospinal projection neurons in the rat. Exp.Brain Res.67: 339-351.

Mirsky,R.,Jessen,K.R.,Schachner,M.,and C.Goridis (1986) Distribution of the adhesion molecules N-CAM and L1 on peripheral neurons and glia in adult rats. J.Neurocytol. 15: 799-815.

Murray,E.A.,and J.D.Coulter (1981) Organization of corticospinal neurons in the monkey. J.Comp.Neurol.195: 339-365.

Murray,M.,and M.A.Edwards (1982) A quantitative study of the reinnervation of the goldfish optic tectum following optic nerve crush. J.Comp.Neurol.209: 363-373.

Nardi,J.B. (1983) Neuronal pathfinding in developing wings of the moth Manduca sexta. Dev.Biol.95: 163-174.

Naus,C.G.,Flumerfeldt,B.A.,and A.W.Hrycyshyn (1985) An HRP-TMB ultrastructural study of rubral afferents in the rat.J.Comp.Neurol.239: 453-465.

Neafsey,E.J.,Bold,E.L.,Haas,G.,Hurley-Gins,K.M.,Quirck,G.,Sievert,C.F., and R.R.Terreberry (1986) The organization of the rat motor cortex: a microstimulation mapping study. Brain Res.Rev. 11: 77-96.

Nieke,J.,and M.Schachner (1985) Expression of the neural cell adhesion molecules L1 and N-CAM and their common carbohydrate epitope L2/HNK-1 during development and after transection of the mouse sciatic nerve. Different. 30: 141-151.

Nordlander,R.H. (1987) Axonal growth cones in the developing amphibian spinal cord. J.Comp.Neurol. 263: 485-496.

Nordlander,R.H.,and M.Singer (1982) Morphology and position of growth cones in the developing Xenopus spinal cord. Dev.Brain Res.4: 181-193.

Oldfield,B.J.,Hou-Yu,A.,and A.-J.Silverman (1985) A combined electron microscopic HRP and immunocytochemical study of the limbic projections to rat hypothalamic nucleï containing vasopressin and oxytocin neurons. J.Comp.Neurol.231: 221-231.

O'Leary,D.D.M. (1987) The remodeling of early axonal projections through the selective loss of neurons and axon collaterals. In: Selective neuronal death, O'Connor,ed.,pp.113-142, Ciba foundation symposium no.126, Wiley, Cichester, U.K.

O'Leary,D.D.M.,Fawcett,J.W.,and W.M.Cowan (1986) Topgraphic targeting errors in the retinocollicular projection and their elimination by selective ganglion cell death. J.Neurosci.6: 3692-3705.

O'Leary,D.D.M.,and B.B.Stanfield (1985) Occipital cortical neurons with transient pyramidal tract axons extend and maintain collaterals to subcortical but not intracortical targets. Brain Res.336: 326-333.

O'Leary,D.D.M.,and B.B.Stanfield (1986) A transient pyramidal tract projection from the visual cortex in the hamster and its removal by selective collateral elimination. Dev.Brain Res. 27: 87-99.

O'Leary,D.D.M.,Stanfield,B.B.,and W.M.Cowan (1981) Evidence that the early postnatal restriction of the callosal projection is due to the elimination of axon collaterals rather than the death of neurons. Dev.Brain Res.1: 607-617.

O'Leary,D.D.M.,and T.Terashima (1988) Cortical axons branch to multiple subcortical targets by interstitial axon budding: implications for target recognition and "waiting periods". Neuron 1: 901-910.

O'Leary,D.D.M.,and S.Thanos (1985) A transient retinofugal pathway in the chick embryo and its possible role in the guidance of axons to the retina. Anat.Rec.211: 142A.

Olucha,F.,Martinez-Garcia,F.,and C.Lopéz-Garcia (1985) A new stabilizing agent for the tetramethylbenzidine (TMB) reaction product in the histochemical detection of horseradish peroxidase (HRP). J.Neurosci.Meth.13: 131-138.

Oppenheim,R.W. (1986) The absence of significant postnatal motoneuron death in the brachial and lumbar spinal cord of the rat. J.Comp.Neurol. 246: 281-286.

Osborn,M.,Debus,E.,and K.Weber (1984) Monoclonal antibodies specific for vimentin. Eur.J.Cell Biol.34: 137-143.

Patterson,P.H. (1988) On the importance of being inhibited or saying no to growth cones. Neuron 1: 263-267.

Persohn,E.,and M.Schachner (1987) Immunoelectronmicoscopic localization of the neural cell adhesion molecules L1 and N-CAM during postnatal development of the mouse cerebellum. J.Cell Biol.105: 596-576.

Peters,A.,Palay,S.L.,and HdeF.Webster (1976) The fine structure of the nervous system. WB Saunders Comp. Philadelphia-London-Toronto.

Phelps,C.H. (1972) The development of glio-vascular relationships in the rat spinal cord. An electron microscopic study. Z.Zellforsch.128: 555-563.

Pigott,R,and J.S.Kelly (1986) Immunocytochemical and biochemical studies with the monoclonal antibody 69A1: similarities of the antigen with the cell adhesion molecules L1, NILE and Ng-CAM. Dev.Brain Res. 29: 111-122.

Pixley,S.K.R.,and J.DeVellis (1984) Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. Dev.Brain Res.15: 201-209.

Porter,L.L.,Cedarbaum,J.M.,O'Leary,D.D.M.,Stanfield,B.B.,and H.Asanuma (1987) The physiological identification of pyramidal tract neurons within transplants in the rostral cortex taken from the occipital cortex during development. Brain Res. 436: 136-142.

Provis,J.M.,and P.L.Penfold (1988) Cell death and the elimination of retinal axons during development. Progr. in Neurobiol.31: 331-347.

Purves,D.,and J.W.Lichtman (1985) Principles of neural development. Sunderland,Sinauer.

Rakic,P. (1971) Guidance of neurons migrating to fetal monkey neocortex. Brain Res.33: 471-476.

Rakic,P.,Stensaas,L.J.,Sayre,E.P.,and R.L.Sidman (1974) Computer-aided three-dimensional reconstruction and quantitative analysis of cells from serial electron microscopic montages of foetal monkey brain. Nature 250: 31-34.

Ralston,D.D.,Milroy,A.M.,and H.J.Ralston III (1987) Non-myelinated axons are rare in the medullary pyramids of the macaque monkey. Neurosci. Lett.73: 215-219.

Ramaekers,F.C.S.,Puts,J.J.G.,Moesker,O.J.,Jap,P.H.K.,and G.P.Vooijs (1981) Use of antibodies to intermediate filaments in the characterization of human tumors. Cold Spring Harbor Symp.Quant. Biol.46: 331-339.

Ramaekers,F.C.S.,Puts,J.J.G.,Moesker,O.J.,Kant,A.,Huysmans,A.,Haag, D.,Jap,P.H.K.,Herman,C.,and C.P.Vooijs (1983) Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours: an overview. Histochem.J.15: 691-713.

Ramon y Cajal (1890) A quelle époque apparaissent les expensions des cellules nerveuses de la moëlle épinière du poulet? Anat.Anz.5: 609-613.

Raper,J.A.,Bastiani,M.,and C.S.Goodman (1983) Pathfinding by neuronal growth cones in grasshopper embryos II Selective fasciculation onto specific axonal pathways. J.Neurosci.3: 31-41.

Rathjen,F.G. (1988) A neurite outgrowth-promoting molecule in developing fiber tracts. TINS 11: 183-184.

Rathjen,F.G.,and M.Schachner (1984) Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. EMBO J. 3: 1-10.

Rathjen,F.G.,Wolff,J.M.,Chang,S.,Bonhoeffer,F.,and J.A.Raper (1987[a]) A novel chick cell surface glycoprotein which is involved in neurofascin-neurite interactions. Cell 51: 841-849.

Rathjen,F.G.,Wolff,J.M.,Frank,R.,Bonhoeffer,F.,and U.Rutishauser (1987[b]) Membrane glycoproteins involved in neurite fasciculation. J.Cell Biol. 104: 343-353.

Reh,T.A.,and M.Constantine-Patton (1985) Growth cone target interactions in the frog retinotectal pathway. J.Neurosci.Res.13: 89-100.

Reh,T.,and K.Kalil (1981) Development of the pyramidal tract in the hamster I A light microscopical study. J.Comp.Neurol.200: 55-67.

Reh,T.,and K.Kalil (1982) Development of the pyramidal tract in the hamster II An electron microscopic study. J.Comp.Neurol.205: 77-88.

Reinoso,B.S.,and A.J.Castro (1989) A study of corticospinal remodelling using retrograde fluorescent tracers in rats. Exp.Brain Res. 74: 387-394.

Rieger,F.,Daniloff,J.K.,Pincon-Raymond,M.,Crossin,K.L.,Grumet,M.,and G.M.Edelman (1986) Neuronal cell adhesion molecules and cytotactin are colocalized at the node of Ranvier. J.Cell Biol. 103: 379-391.

Roberts,A.,and J.S.H.Taylor (1983) A study of the growth cones of developing embryonic sensory neurites. J.Embryol.Exp.Morphol.75: 31-47.

Rogers,S.L.,Letourneau,P.C.,Palm,S.L.,McCarthy,J.,and L.T.Furcht (1983) Neurite extension by pheripheral central nervous system neurons in response to sustratum bound fibronectin and laminin. Dev.Biol. 98: 212-220.

Rutishauser,U. (1984) Developmental biology of a neural cell adhesion molecule. Nature 310: 549-554.

Rutishauser,U.,and T.M.Jessell (1988) Cell adhesion molecules in vertebrate neural development. Nature 310: 549-554.

Rutishauser,U.,Thiery,J.-P.,Brackenbury,R.,Sela,B.A.,and G.M.Edelman (1976) Mechanisms of adhesion among cells from neural tissues of the chick embryo. Proc.Natl.Acad. Sci USA 73: 577-581.

Rye,D.B.,Saper,C.B.,and B.H.Wainer (1984) Stabilization of the tetramethylbenzidine (TMB) reaction product: Application for retrograde and anterograde tracing, and combination with immunohistochemistry. J.Histochem.Cytochem.32: 1145-1153.

Sakumoto,T.,Nagai,T.,Kimura,M.,and T.Maeda (1980) Electronmicroscopic visualization of tetramethylbenzidine reaction product of horseradish peroxidase neurochemistry. Cell Mol.Biol.26: 211-216.

Schachner,M.,Faissner,A.,Fischer,G.,Keilhauer,G.,Kruse,J.,Kunemund,V., Lindner,J.,and H.Wernecke (1985) Functional and structural aspects on the cell surface in mammalian nervous system development. In: The cell in contact, Edelman G.M.,and J.-P. Thiery,eds.,pp.257-275, John Wiley and Sons Inc.,New York.

Schneider,G.E.,and S.R.Jhaveri (1974) Neuroanatomical correlates of spared or altered function after early brain lesions in the newborn hamster. In: Plasticity and recovery of function in the central nervous system, Stein,D.G.,Rosen,J.J.,and N.Butter,eds.,pp.85-109, Academic Press New York,NY.

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

Schönitzer,K.,and H.Hollander (1981) Anterograde tracing of horseradish peroxidase (HRP) with the electron microscope using tetramethylbenzidine reaction. J.Neurosci.Met.4: 373-383.

Schreyer,D.J.,and E.G.Jones (1982) Growth and target finding by axons of the corticospinal tract in prenatal and postnatal rats. Neuroscience 7: 1837-1853.

Schreyer,D.J.,and E.G.Jones (1983) Growing corticospinal axons by-pass lesions of neonatal rat spinal cord. Neurosci.9: 31-40.

Schreyer,D.J.,and E.G.Jones (1987) Growth of corticospinal axons on prosthetic substrates into the spinal cord of neonatal rats. Dev.Brain Res. 35: 291-299.

Schreyer,D.J.,and E.G.Jones (1988[a]) Topographic sequence of outgrowth of corticospinal axons in the rat: a study using retrograde axonal labelling with Fast blue. Dev.Brain Res.38: 89-101.

Schreyer,D.J.,and E.G.Jones (1988[b]) Axon elimination in the developing corticospinal tract of the rat. Dev.Brain Res.38: 103-119.

Schwab,M.E.,and L.Schnell (1989) Region-specific appearance of myelin constituents in the developing rat spinal cord. J.Neurocytol.18: 161-169.

Shankland,M. (1984) Positional determination of supernumerary blast cell death in the leech embryo. Nature 307: 541-543.

Shaw,G.,and D.Bray (1977) Movement and extension of isolated growth cones. Exp.Cell Res.104: 55-62.

Shaw,G.,Osborn,M.,and K.Weber (1981) An immunofluorescence microscopical study of the neurofilament triplet proteins,vimentin and glial fibrillary acidic protein within the adult rat brain. Eur.J.Cell Biol.26: 68-82.

Silver,J. (1984) Studies on the factors that govern directionality of axonal growth in the embryonic optic nerve and at the chiasm of mice. J.Comp.Neurol.223: 238-251.

Silver,J.,Lorenz,S.E.,Wahlsten,D.,and J.Coughlin (1982) Axonal guidance during development of the great commissures: descriptive and experimental studies in vivo, on the role of preformed glial pathways. J.Comp.Neurol. 210: 10-29.

Silver,J.,and M.Robb (1979) Studies on the development of the eye cup and optic nerve in normal mice and in mutants with congenital optic nerve aplasia. Dev.Biol.68: 175-190.

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

Silver,J.,and R.L.Sidman (1980) A mechanism for the guidance and topographic patterning of retinal ganglion cell axons.J.Comp.Neurol. 189: 101-111.

Singer,M.,Nordlander,R.H.,and M.Edgar (1979) Axonal guidance during neurogenesis and regeneration in the spinal cord of the newt. The blueprint hypothesis of neuronal pathway patterning. J.Comp.Neurol. 185: 1-22.

Slot,J.W.,and H.J.Geuze (1984) Gold markers for single and double immunolabelling of ultrathin cryosections. In: Immunolabelling for electronmicroscopy, J.M.Polak, and J.M.Varndell,eds., pp.129-142, Elsevier Science Publ.,Amsterdam.

Sommer,J.,and M.Schachner (1981) Monoclonal antibodies (01 to 04) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. Dev.Biol.83: 311-327.

Sperry,R.W. (1963) Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proc.Natl.Acad.Sci.USA 50: 703-710.

Stallcup,W.B.,and L.L.Beasley (1985) Involvement of the nerve growth factor inducible large external glycoprotein (NILE) in neurite fasciculation in primary cultures of rat brain. Proc.Natl.Acad.Sci.USA 82: 1276-1280.

Stallcup,W.B.,Beasley,L.L.,and J.M.Levine (1985) Antibody against nerve growth-factor inducible large external (NILE) glycoprotein labels nerve fiber tracts in the developing rat nervous system. J.Neurosci.5: 1090-1101.

Stanfield,B.B. (1984) Postnatal reorganization of cortical projections: the role of collateral elimination. TINS 7:31-40.

Stanfield,B.B.,and D.D.M.O'Leary (1985a) Fetal occipital cortical neurones transplanted to the rostral cortex can extend and maintain a pyramidal tract axon. Nature 313: 135-137.

Stanfield,B.B.,and D.D.M.O'Leary (1985b) The transient corticospinal projection from the occipital cortex during the postnatal development of the rat.J.Comp.Neurol.238: 236-248.

Stanfield,B.B., O'Leary,D.D.M.,and C.Fricks (1982) Selective collateral elimination in early postnatal development restricts cortical distribution of rat pyramidal tract neurones. Nature 298: 371-373.

Stein,B.E.,McHaffie,J.G.,Harting,J.K.,Huerta,M.F.,and T.Hashikawa (1985) Transient tectogeniculate projections in neonatal kittens: an autoradiographic study. J.Comp.Neurol.239: 402-412.

Stelzner,D.J. (1971) The normal postnatal development of synaptic endfeet in the lumbosacral spinal cord and responses in the hindlimbs of the albino rat. Exp.Neurol.31: 337-357.

Stuermer,C.A.O. (1988) Retinotopic organization of the developing retinotectal projection in the zebrafish embryo. J.Neurosci.8: 4513-4530.

Takahashi,K.(1965) Slow and fast groups of pyramidal tract cells and their respective membrane properties. J.Neurophysiol.28: 908-924.

Takeichi,M. (1987) Cadherins: a molecular family essential for selective cell-cell adhesion and animal morphogenesis. Trends Genet.3: 213-217.

Tascos,N.A.,Parr,J.,and N.K.Gonatas (1982) Immunocytochemical study of the glial fibrillary acidic protein in human neoplasms of the central nervous system. Human Pathology 13: 454-458.

Terashima,T.,Inouee,K.Inouee,Y.,Mikoshiba,K.,and Y.Tsukada (1983) Distribution and morphology of corticospinal tract neurons in reeler mouse cortex by the retrograde HRP method. J.Comp.Neurol.218: 314-326.

Thomas,A.P.(1985) An ultrastructural and morphometric study of unmyelinated axons in the pyramidal tract of monkeys. Soc.Neurosci. Abstr.373: 18.

Thomas,A.,Westrum,L.E.,DeVito,J.L.,and M.A.Biedenbach (1984) Unmyelinated axons in the pyramidal tract of the cat. Brain Res.301: 162-165.

Tokuyasu,K.T. (1978) A study of positive staining of ultrathin frozen sections. J.Ultrastruc.Res. 63: 287-307.

Tokuyasu,K.T. (1980) Immunochemistry on ultrathin frozen sections. Histochem.J. 12: 381-403.

Tokuyasu,K.T. (1986) Cryosections for immunohistochemistry. Proc.XIth Int. Congress EM, pp.42-43.

Tolbert,D.L. (1987) Intrinsically directed pruning as a mechanism regulating the elimination of transient collateral pathways. Dev.Brain Res.33: 11-21.

Tosney,K.W.,and L.T.Landmesser (1985) Growth cone morphology and trajectory in the lumbosacral region of the chick embryo. J.Neurosci.5: 2345-2358.

Valentino,K.L.,and E.G.Jones (1982) The early formation of the corpus callosum. A light and electron microscopic study of foetal and neonatal rats. J.Neurocytol.11: 583-609.

Valentino,K.L.,Jones,E.G.,and S.A.Kane (1983) Expression of GFAP immunoreactivity during development of long fiber tracts in the rat CNS. Dev.Brain Res.9: 317-336.

Van Eden,C.G. (1985) Postnatal development of rat prefrontal cortex. PhD-thesis, University of Amsterdam.

Van Eden,C.G.,and J.M.Kros (1989) Transient corticospinal projections from the (pre-) frontal cortex in the rat. Exp.Brain Res.,in press.

Van Eden,C.G.,and H.B.M.Uylings (1985) Cytoarchitectonic development of the prefrontal cortex in the rat. J.Comp.Neurol.241: 253-267.

Van Eden,C.G.,and H.B.M.Uylings (1986) Addendum to: stereotaxic operations in the neonatal rat: a novel and simple method. J.Neurosci.Methods 15: 352.

Van Lookeren-Campagne,M.,Oestreicher,A.B.,Van Bergen-Henegouwen,P.M.P., and W.H.Gispen (1989) Ultrastructural immunocytochemical localization of B50/GAP43, a protein kinase C substrate, in isolated presynaptic nerve terminals and neuronal growth cones. J.Neurocytol. in press.

Van Mier,P.,and H.J.Ten Donkelaar (1989) Structural and functional properties of reticulospinal neurons in early-swimming stage Xenopus embryos. J.Neurosci.9: 25-37.

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

Westman,J.,Danckwardt-Lilliestrom,N.,Dietrichs,E.,Svensson,B.A.,and F.Walberg (1986) ULtrastructure of spinal efferents to the lateral reticular necleus: an EM study using anterograde transport of WGA-HRP complex. J.Comp.Neurol.246: 301-311.

Wiesendanger,M.(1981) The pyramidal tract: its structure and function. Handbook of behavioral neurobiology, Vol.5, motor coordination, Plenum, New York, pp.401-492.

Wiggins,R.C.,Chonjie,G.,Delaney,C.,and T.Samorajski (1988) Development of axonal-oligodendroglial relationships and junctions during myelination of the optic nerve. Int.J.Dev.Neurosci. 6: 233-243.

Williams,R.W.,Bastiani,M.J.,Lia,B.,and L.M.Chalupa (1986) Growth cones, dying axons, and developmental fluctuations in the fiber population of the cat's optic nerve. J.Comp.Neurol.246: 32-69.

Williams,R.W.,and L.M.Chalupa (1982) Prenatal development of retinocollicular projections in the cat: an anterograde tracer transport study. J.Neurosci.2: 604-622.

Wise,S.P.,and J.P.Donoghue (1986) Motor cortex of rodents. In: Cerebral cortex, Vol.5, Jones,E.G.,and A.Peters,eds., pp.243-270, Plenum Publishing Coorp.

Wise,S.P.,and E.G.Jones (1978) Developmental studies of thalamocortical and commissural connections in the rat somatic sensory cortex. J.Comp. Neurol. 178: 187-208.

Wise,S.P.,Murray,E.A.,and J.D.Coulter (1979[a]) Somatotopic organization of corticospinal and corticotrigeminal neurons in the rat. Neurosci.4: 65-78.

Wise,S.P.,Fleshman,J.W.,and E.G.Jones (1979[b]) Maturation of pyramidal cell form in relation to developing afferent and efferent connections of rat somatic sensory cortex. Neurosci.4: 1275-1297.

Woolsey,C.N. (1958) Organization of the somatic sensory and motor areas of the cerebral cortex. In: Biological and biochemical basis of behavior, Harlow,H.F.,and C.N.Woolsey,eds.,pp.63-81, The University of Wisconsin press.

Wozniak,W.,and R.O'Rahilly (1982) An electronmicroscopic study of myelination of pyramidal fibers at the level of the pyramidal decussation in the human fetus. J.Hirnf.23: 331-342.

The results of this thesis have been published or are in preparation for publication:
CHAPTER 2:

2A: **Joosten,E.A.J.,Gribnau,A.A.M.,and P.J.W.C.Dederen (1987)** An anterograde tracer study of the developing corticospinal tract in the rat: three components. Dev.Brain Res.36: 121-130.
2B: **Joosten,E.A.J.,and C.G.Van Eden (1989)** An anterograde tracer study on the development of corticospinal projections from the medial prefrontal cortex in the rat. Dev.Brain Res.45: 313-319.

CHAPTER 3:

3: **Joosten,E.A.J.** Corticospinal growth cones in developing rat lumbar spinal cord. Submitted.

CHAPTER 4:

4A: **Joosten,E.A.J.,Gribnau,A.A.M.,and P.J.W.C.Dederen (1987)** Ultrastructural visualization of anterogradely transported horseradish peroxidase in the developing corticospinal tract of rat.J.Histochem. Cytochem.35: 623-626.
4B: **Joosten,E.A.J.,Gribnau,A.A.M.,and P.J.W.C.Dederen (1989)** Postnatal development of the corticospinal tract in the rat. An ultrastructural anterograde tracer study. Anat.Embryol.179: 449-456.
4C: **Joosten,E.A.J.,and A.A.M.Gribnau (1988)** Unmyelinated corticospinal axons in adult pyramidal tract. An electron microscopic tracer study. Brain Res.459: 173-177.

CHAPTER 5:

5B: **Joosten,E.A.J.,and A.A.M.Gribnau (1989)** Astrocytes and guidance of outgrowing corticospinal tract in the rat. An immunocytochemical study using anti-vimentin and anti-glial fibrillary acidic protein. Neurosci. in press.
5C1: **Joosten,E.A.J.,and A.A.M.Gribnau (1989)** Immunocytochemical localization of cell adhesion molecule L1 in developing rat pyramidal tract. Neurosci.Lett.100: 94-98.
5C2: **Joosten,E.A.J.,Gribnau,A.A.M.,and T.G.M.F.Gorgels (1989)** Immunoelectronmicroscopical localization of cell adhesion molecule L1 in developing rat pyramidal tract. Submitted.

Related Articles:

Dederen,P.J.W.C.,and E.A.J.Joosten (1989) A triple labelling method: HRP anterograde tract-tracing combined with double immunofluorescent cell staining in developing neural tissue of the rat.J.Neurosci Meth.in press.

Gorgels,T.G.M.F.,Joosten,E.A.J.,and A.A.M.Gribnau (1989) Synapses between pyramidal tract growth cones and immature oligodendrocytes.Submitted.

CURRICULUM VITAE

De auteur werd geboren op 2 augustus 1958 te Horn (Limburg).

1970-1976: Atheneum B opleiding, Bisschoppelijk College te Roermond.

1976-1979: HBO-B analytische opleiding, STOVA Wageningen.

1979-1980: militaire dienstplicht.

1980-1985: studie Biologie, Katholieke Universiteit Nijmegen.
Hoofdvak: Dierfysiologie (Prof.dr.A.Van Overbeeke)
Bijvakken: Genetica (Prof.dr.W.Hennig)
Cyto-Histologie (Prof.dr.C.Jeruzalem)

1986-1989: wetenschappelijk assistent op de vakgroep Anatomie en Embryologie, Faculteit der Geneeskunde en Tandheelkunde, Katholieke Universiteit Nijmegen. Participatie aan het Medisch-biologisch en Neuroanatomisch onderwijs van de vakgroep.

30 januari 1988: "Bolk-prijs 1987", toegekend door de Nederlandse Anatomen Vereniging voor de voordracht: "Een electronenmicroscopische analyse van de postnatale ontwikkeling van de corticospinale baan bij de rat".

Vanaf 1 september 1989 is hij werkzaam als Universitair Docent bij de vakgroep Anatomie en Embryologie, KU Nijmegen.

Stellingen behorende bij het proefschrift:
"Axonal growth and guidance during formation of the pyramidal tract in the rat".

1

De eliminatie van corticospinale collateralen is een algemeen optredend fenomeen tijdens de ontwikkeling van de cortex bij de rat.
'dit proefschrift'

2

De volwassen pyramidebaan bij de rat bevat ongemyeliniseerde axonen waarvan de oorsprongscellen zich in de cortex bevinden.
'dit proefschrift'

3

De zich ontwikkelende pyramidebaan bij de rat wordt gekarakteriseerd door een geleidelijke uitgroei van zijn vezels.
Gorgels T. et al. (1989) Anat.Embryol.179: 377-385
'dit proefschrift'

4

De afwezigheid van het cel adhesie molecuul L1 tijdens het begin van de myelinisatie vormt één van de mogelijke verklaringen voor het geringe regeneratievermogen van het centrale zenuwstelsel bij zoogdieren.
Martini R., Schachner M. (1986) J.Cell Biol.103: 2439-2448
Kleitman N. et al. (1988) Exp.Neurol.102: 298-306
'dit proefschrift'

5

De groeiconus symboliseert de dynamiek tijdens de ontogenese van het zenuwstelsel.

6

De vormkenmerken van de gewrichtsvlakken en de gewrichtsbanden van het eerste carpometacarpale gewricht zijn illustratief voor de essentiele rol van spieren bij de stabilisatie van dit gewricht.

7

Van het immuun systeem bij de mens is nog zo weinig bekend dat de huidige klinische behandelingsstrategie bij een chronische trombocytopenische aandoening veelal berust op 'trial and error' methoden.

8

Marathonlopers dienen gediskwalificeerd te worden indien tussen start en finish een moment voorkomt waarop zij twee voeten gelijktijdig aan de grond hebben.

9

Tegen de tijd dat de mens, machines intelligentie heeft bijgebracht is hij meer kwijt dan rijk.

10

Onderzoek aan een zenuwbaan is soms een zenuwenbaan.

Nijmegen, 11 oktober 1989 **E.A.J.Joosten**