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CELLULAR MECHANISMS OF DEVELOPMENT AND PLASTICITY OF THE CROSSED AND UNCROSSED CORTICORUBRAL PROJECTIONS IN THE KITTEN

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CONTENTS

This thesis is based on the following articles.

1. Presence of corssed corticorubral fibers and increase of crossed projections after unilateral lesions of the cerebral cortex of the kitten: A demonstration using anterograde transport of *Phaseolus vulgaris* leucoagglutinin. Murakami, F. and S. Higashi. Brain Res. *447:* 98-108 (1988)
2. Postnatal development of crossed and uncrossed corticorubral projections in the kitten: A PHA-L study. Higashi, S., M. Yamazaki and F. Murakami. J. Comp. Neurol. *299*:2-16 (1990)
3. Changes in axonal morphology and topographic refinement of aberrant crossed corticorubral projections following early lesions of the sensorimotor cortex in kittens. Murakami, F., S. Higashi, M. Yamazaki, and A. Tamada. Neurosci. Res. *12:* 122-139 (1991)
4. Anatomical and electrophysiological studies of aberrant corticorubral fibers induced by lesions of the cerebral cortex in kitten. Murakami, F., S. Higashi, E. Kosar, and Y. Fujito In *Post-Lesion Neural Plasticity*, H. Flohr (eds.), pp.527-536, Springer-Verlag Berlin Heidenberg 1988.
5. Formation of crossed and uncrossed projections in the central nervous system. Murakami, F., W. -J. Song, and S. Higashi. Neurosci. Res. Suppl. *13*::37-42 (1990)

Abstract

Plasticity inducing structural changes after brain lesions seems to be more remarkable in neonatal animals than in adults. In some places of the brain, aberrant projections that are never seen in adults are found after neonatal manipulation. It is not clear, however, what kind of cellular mechanism concerns in formation of those aberrant projections after neonatal operations. To examine whether axonal proliferation occurs after the lesions, I attempted to reveal morphological changes of individual axons in lesioned animals.

My study has three chapters. The crossed corticorubral projections that are never seen in adult cats are found after unilateral cortical damage at neonatal stages. First I examined whether the crossed projections already exist at the time of ablation. Although the crossed projections already existed at an age of the lesions, the amount of those was very small. This suggests that the crossed projections found in lesioned animals cannot be explained sufficiently by the blockade of axonal elimination. Besides, this also suggests the possibility that axonal proliferation occurs in pre-existing axons after lesions. Then, I studied morphological changes of individual axonal fragments during postnatal periods and developmental changes of corticorubral projections in the red nucleus. The corticorubral axons showed various kinds of developmental changes; e.g., topographic refinement occurred gradually and the number of axonal branches increased in the target region. Finally, I found that many axonal fragments having well-developed arbors appeared after the cortical lesion. These results in my study suggest that neural plasticity in neonatal animals is due to proliferation of pre-existing axons in the target areas and cellular mechanisms for axonal proliferation in lesioned animals resemble those occurring during normal nervous system development.

General Introduction

Understanding of molecular and cellular mechanisms concerning neural plasticity is one of the most important problems in the field of neuroscience. Now we know that various types of plasticity exist (Tsukahara, 1981). Among them, perturbation of neural circuit, or lesion of brain, causes unique plasticity inducing structural changes (Tsukahara, 1981). This type of plasticity has some differences between neonatal animals and adults. In some places of the brain, aberrant projections that are never seen in adults are found after neonatal manipulation (Lund and Lund, 1971; Lund, 1978; Tsukahara, 1981). For instance, by unilateral eyelid suture, the ocular dominance column from the sutured eye reduces its size in the cortex whereas it from the unsutured one expands the size (Hubel and Wiesel, 1963; Hubel et al., 1977; Wiesel and Hubel, 1963). After eye enucleation, the projections from the remaining eye spread out in the lateral geniculate nucleus (Garraghty et al., 1986; Guillery, 1972; Hickey, 1975; Robson et al., 1978; Robson, 1981) and bilateral projections are observed in the superior colliculus (Lund and Lund, 1971, 1973; Lund and Miller, 1975). After unilateral cortical ablation the crossed corticofugal projections are found (Kosar et al., 1985; Leonard and Goldbeger, 1987; Leong and Lund, 1973; Nah and Leong, 1976; Nah et al., 1981; Tsukahara et al., 1983). From these studies, neural plasticity induced by manipulation is thought to be remarkable in immature animals compared to adults.

Although neural plasticity, following structural changes, is found in many places of the brain, it is not clear whether cellular mechanisms concern in observation of those aberrant projections after neonatal operations. Two possibilities have been proposed. The fact that aberrant projections are not observed in adults (Tsukahara, 1981; Lund and Lund, 1971; Nah et al., 1981) suggest the first possibility that axonal proliferation occurs and then structural

changes of neural connection are induced. The second one is that although aberrant projections seem to be formed by axonal proliferation, it is due to a blockade of the elimination process (Panneton, 1986; Hubel et al., 1977; Land and Lund, 1979; Leonard and Goldberger, 1987). The reason for this is that neural connections have redundancy at the developmental stage; extraneous projections are formed first and then these projections are eliminated as development proceeds (Cowan et al., 1984; Innocenti, 1981; Ivy and Killackey, 1982). Besides the two possibilities, however, we could consider the third possibility that pre-existing axons cause proliferation after manipulation. In this case, it is necessary for transient projections at the age of manipulation to exist to have axonal proliferation. This idea may contain characteristics common to the previously mentioned possibilities.

To reveal cellular mechanisms for appearance of the aberrant projection after the neonatal manipulation, and to guess the molecular process constructing cellular mechanisms, it is very useful to reveal morphological changes of individual axon occurring in parallel to plastic changes of neural connections. Unfortunately, there has been no study clearly defining which is the most reasonable in these three possibilities. Therefore it is still unclear what kinds of cellular mechanisms concern in neural plasticity at neonatal stages. In my research, I propose to study fine morphology of corticorubral axons following unilateral cortical ablation and compare this with morphological changes observed at normal development.

My study has three chapters. In chapter 1, since it is well known that the crossed corticuorubral projections observed after unilateral cortical lesion at neonatal stages of the kitten (Kosar et al., 1985; Leonard and Goldberger, 1987; Tsukahara et al., 1983; Villablanca et al., 1982, 1988), I examined whether the crossed projections already existed at the time of ablation to know whether it is possible to explain the appearance of the crossed corticorubral projections by either of the first or second possibilities. The

results showed that the crossed projections have already existed at the age of lesion. The number of projections, however, was very small. This result suggests that the crossed projections observed in lesioned animals cannot be explained by either the first or the second possibilities. Besides, these results also suggest the possibility that axonal proliferation occurs after lesion. Then, in chapter 2, I first studied morphological changes of individual axonal fragments and developmental changes of corticorubral projections in the red nucleus to test the third possibility. The results in this chapter showed that as corticorubral projections grew during postnatal development, topographic refinement also occurred gradually and the number of axonal branches increased in the target region. Finally, in chapter 3, I tried to figure out whether any kind of change in axons could be found in the crossed projections. In this chapter I showed that many axonal fragments having well-developed their arbors appeared after the cortical lesion, though these fragments cannot be seen before the lesion. Also, the results showed that the projections formed after lesion are similar to those at the point of axonal branching and the refinement of topographic order in normal animals.

These results in my study suggest that neural plasticity in neonatal animals is due to proliferation of pre-existing axons in the target areas and cellular mechanisms for axonal proliferation in lesioned animals are similar to those occurring during normal nervous system development.

Chapter 1.

PRESENCE OF CROSSED CORTICORUBRAL FIBERS AND INCREASE OF CROSSED PROJECTIONS AFTER UNILATERAL LESIONS OF THE CEREBRAL CORTEX OF THE KITTEN: A DEMONSTRATION USING ANTEROGRADE TRANSPORT OF *PHASEOLUS VULGARIS* LEUCOAGGLUTININ

Abstract

Brain lesions made during early developmental stages produce more prominent remodeling of synaptic organization than those made in adults. This difference in the extent of neuronal or synaptic plasticity between immature and mature animals may be due to difference in capacity for axonal elongation. Alternatively, it could be due to the prevention of retraction of exuberant projections present only in the early developmental stages. Aberrant crossed corticorubral projections seen after neonatal hemispherectomy have been ascribed to collateral sprouting. To determine whether these results from prevention of retraction of crossed fibers, we studied the corticorubral pathway in normal kittens and compared it with that observed after unilateral cortical lesion, using the plant lectin *Phaseolus vulgaris* leucoagglutinin (PHA-L). One to two weeks after injection of PHA-L, many immunocytochemically labeled fibers were observed in the red nucleus (RN) ipsilateral to the cortical injection. Although very few, latbeled fibers were also seen in the RN contralateral to the injection in normal kittens. By contrast, many labeled fibers were seen in the RN contralateral to the injection in lesioned animals. Many growth-cone like axonal endings were also observed. The abundant crossed corticorubral fibers seen in lesioned may be ascribed to the increase in the number of fibers crossing the midline towards the contralateral RN or they could be due to increase branching of preexisting fibers.

INTRODUCTION

Brain lesion made during early development have been thought to produce greater axonal sprouting and synaptogenesis than do lesions made later in life (Cotman and Nadler, 1978; Spear, 1984; Tsukahara, 1981). Early brain damage is often associated with abnormal projections, which are not observed after adult brain damage. Age differences in the effects of brain damage could be related to the growth capacity of the neurons or ro the enviroment allowing growth of neuronal processes. Alternatively, they could be related to exuberant projections, which are exclusively seen during early development (Cotman et al., 1984; Innocenti et al., 1977; O'Leary et al., 1981; Stanfield and O'Leary, 1985; Stanfield et al., 1982; Tolbert and Panneton, 1983;), and the prevention of their retraction by brain damage (Panneton, 1986).

Aberrant crossed corticorubral projections have been observed after unilateral neonatal cortical damage in the kitten (Kosar et al., 1985; Tsukahara et al., 1981; Tsukahara et al., 1983; Villablanca et al., 1982) and rat (Nah and Leong, 1976; 1976; Nah et al., 1980). This has been ascribed to collateral sprouting of corticorubral fibers. However, Leonard and Goldberger reported that they found crossed corticorubral projection in 1-day-old kittens (Leonard and Goldberger, 1987). Based on their findings, they raised the possibility that the bilateral projection resulted from the maintenance of a preexisting exuberant pathway which should have been retracted during normal development. Kosar et al. failed to find crossed corticorubral projection in 7-30-day-old kittens (Kosar et al., 1985). However, it is often difficult to discriminate small number of labeled terminals from non-specific precipitates when tetramethylbenzidine (TMB) method is utilized to react horseradish peroxidase (HRP) (Mesulam, 1982).

Plant lectin from red kidney bean, *Phaseolus vulgaris* leucoagglutinin (PHA-L), is known to be an excellent anterograde tracer (Gerfen and

Sawchenko, 1984). PHA-L, when delivered iontophoretivcallyinto the CNS with fine-tipped micropipettes and localized immunohistochemically, discetely labeled call bodies and their dendrites in a small area near the site of injection, along with their axons and terminal specializations. Hence, PHA-L has been successfully applied to various loci in the central nervous system as anterograde tracer (Caelsen and Heimer, 1986; Keller et al., 1985; Watts et al., 1987).

With this method we demonstrate the presence of a crossed corticorubral projection in the kitten and the increase in the density of the projection after neonatal cortical lesion.

MATERIALS AND METHODS

PHA-L (2.5%, Vector Labs.) was injected iontophoretically into the left pericruciate cortex of two groups of animals: 7 intact control kittens 2-7 weeks of age and 6 kittens which had previously received unilateral ablation of the sensorimotor cortex. The ablations were performed by suction 3-4.5 weeks after birth and animals were allowed to survive for an additional 1-6 weeks. Age of some animals were estimated from the time of teeth eruption and their body weight (Masuda and Suzuki, 1981). All subsequent procedures were identical for both group of animals. The animals were anaesthetized with Nebutal (17.5 mg/kg i.p.) supplemented with ketamine and the sensorimotor cortex ablated.

Injection of PHA-L were placed in 1-4 different lociof the sensorimotor cortex at 1.5 mm depth. Avoiding the medial supplementary cortical areas which have been reported to project sparsely to the contralateral red nucleus (RN), we injected PHA-L into the pericruciate cortex. PHA-L, 2.5 % in 0.05 M Tris-buffered saline (TBS), pH 7.6, was injected iontophoretically through glass micropippetes with a tip of 20-140 um. A positive pulsed current of 5 uA (7.5 sec on and 7.5 sec off) was applied for 15 min. After survival times of 7-14 days the animals were deeply anaesthetized and perfused transcardially with phosphate-buffered saline (PBS), at room temperature, followed by a mixture of 2.5 % gultaraldehyde and 2% paraformaldehyde in 0.12 M phosphate buffer, pH 7.4. The brains were postfixed for 2 hours and the brainstem containing with the RN was dissected out and cut oronally with a Microslicer (Dosaka EM, Kyto) or a freezing microtome at 50 um. Sections from some of animals were incubated 30 min in 1 % sodium borohydride (NaBH4, Wako) in PBS at room temperature () and rised several times in PBS for 1-1.5 h at 4 C. The sections were then washed 3 times in TBS with 0.05-0.5 % Triton X-100, pH 8.6, in 1% normal rabbit serum (TBS-T), incubated for 12-14 h at room temperature in affinity-purified anti-PHA (L+E) serum

(Vector Labs., raised in goat), diluted 1/2000 in TBS-T, pH 8.6, and then processed for immunohistochemistry following the avidin-biotin complex (ABC) method (Hsu et al., 1981). The ABC complexes were finally reacted with diaminobenzidine tetrahydrochloride. All the immunohistochemical steps following the ABC method were carried out in TBS-T, pH 7.6, in 1% normal rabbit serum. Most of the sections were counterstained with Methylen blue. Part of the sections were postfixed in 2% OsO4, stained en bloc and flat-embedded in Epon.

The sensorimotor cortex was cut serially in the sagittal plane at 100 um and processed identically, although no cortical section was embedded in Epon. The injection site was examined later under a light microscope.

Labeled fibers were drawn with a drawing tube attached to a light microscope or photographed using Kodak Wratten gelatin filter No. 45A.

RESULTS

Lesion of the sensorimotor area

The ablation were centered on area 4 and included area 4, 6, 3a, 3b, 1, 2 and 5. Although we intended to ablate the whole sensorimotor area, part of the above-mentioned areas were spared. Fig. 1 shows an example of lesioned cortex. In this particular axample areas 4 and 3 seem to be completely ablated but area 6, 5, 1 and 2 are partly spared. About half of the animals showed hydrocephalus and the intact cortex exhibited protrution of gyri into the damaged hemisphere.

Injection site

Fig. 2 shows a photomicrograph of injection sites seen in a parasagittal section of the sensorimotor cortex. Two different injection sites are visible in this section. Many darkly labeled cells with fine details of their dendrites were found in the injection sites snd it was usually possible to trace their axons down to the white matter (arrow). In the example shown here, the labeled neurons are localized in layer V and the diameter of the injection site as represented by darkly stained cells was about 350 um x 700 um. Injection sites ranged 100-600 um in width by 300 -900 um in depth.

Red nucleus - normal animals

As demonstraed by silver impregnation methods (Niimi et al., 1963; Rinvik, 1965; Rinvik and Walberg, 1963; Sadun, 1975) corticorubral fibers leave the cerebral peduncle at the level of the RN and rostral to the RN as well (Fig. 3). Some of the fibers are directed to the ipsilateral RN, nucleus Darkschewitsch and pretectal region, where they make extensive branchings and varicosities. The pattern of labelings is very similar to that reported in adults cats using autoradiography.

In the RN ipsilateral to injection, the labeled fibers are generally seen throughout the rostrocaudal extent of the RN in most of the animals. The fibers

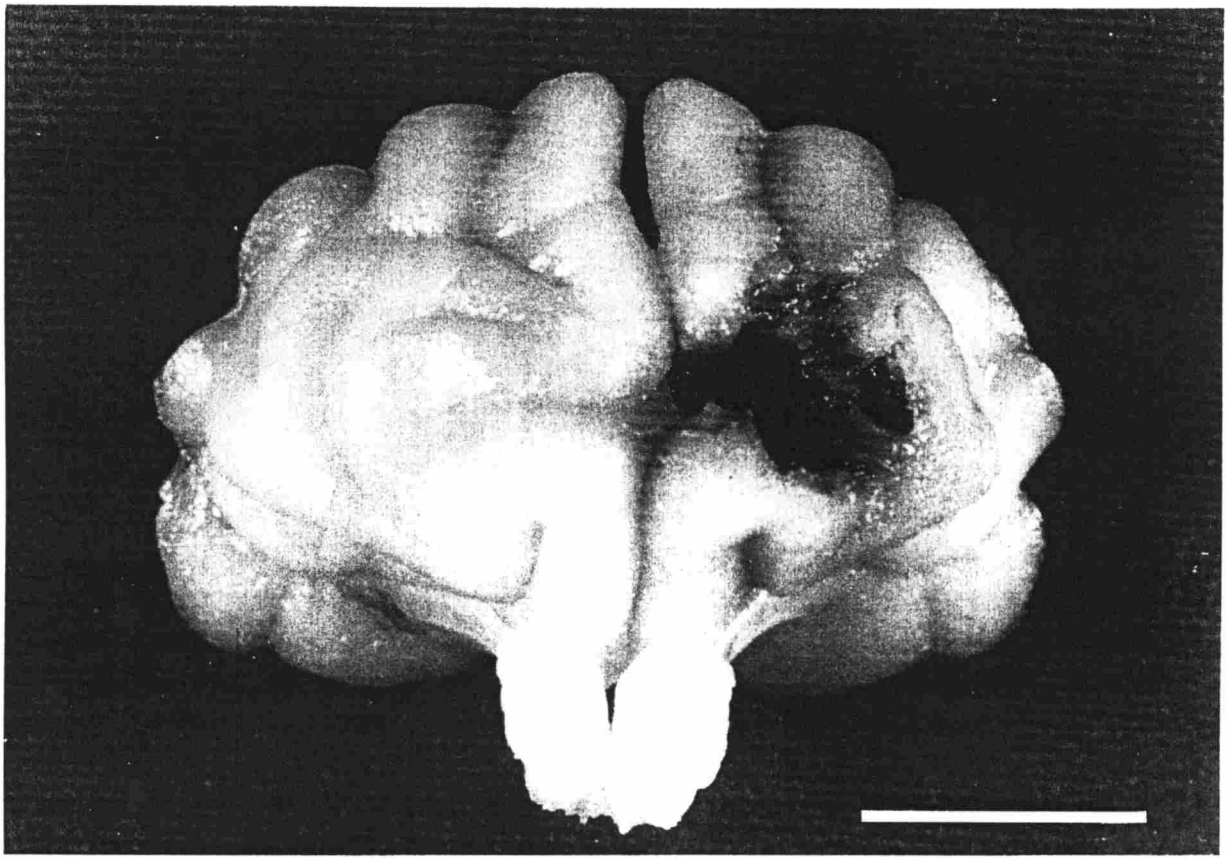

Fig. 1. Cerebral cortex of a hemispherectomized kitten. This animal was sarcificed 36 days after birth. Ablation includes areas 4, 3 and part of area 6, 5, 1 and 2 in this particular example. Bar: 1 cm.

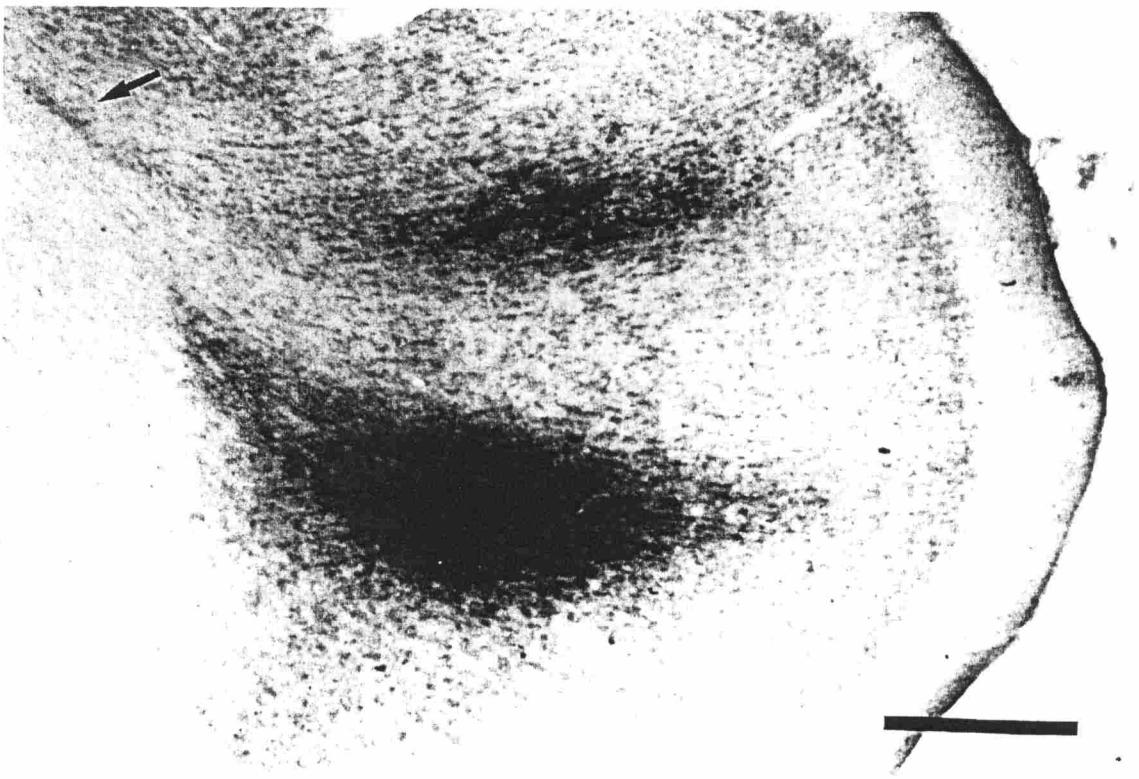

Fig. 2. Sagittal section of sensorimotor cortex injected with PHA-L. The injection sites shown here are centered in layer V of precruciate cortex. Two different injection sites can be seen. Labeled fibers leaving the cortex are also seen (arrow). Counterstained with Methylene blue. Bar=500um

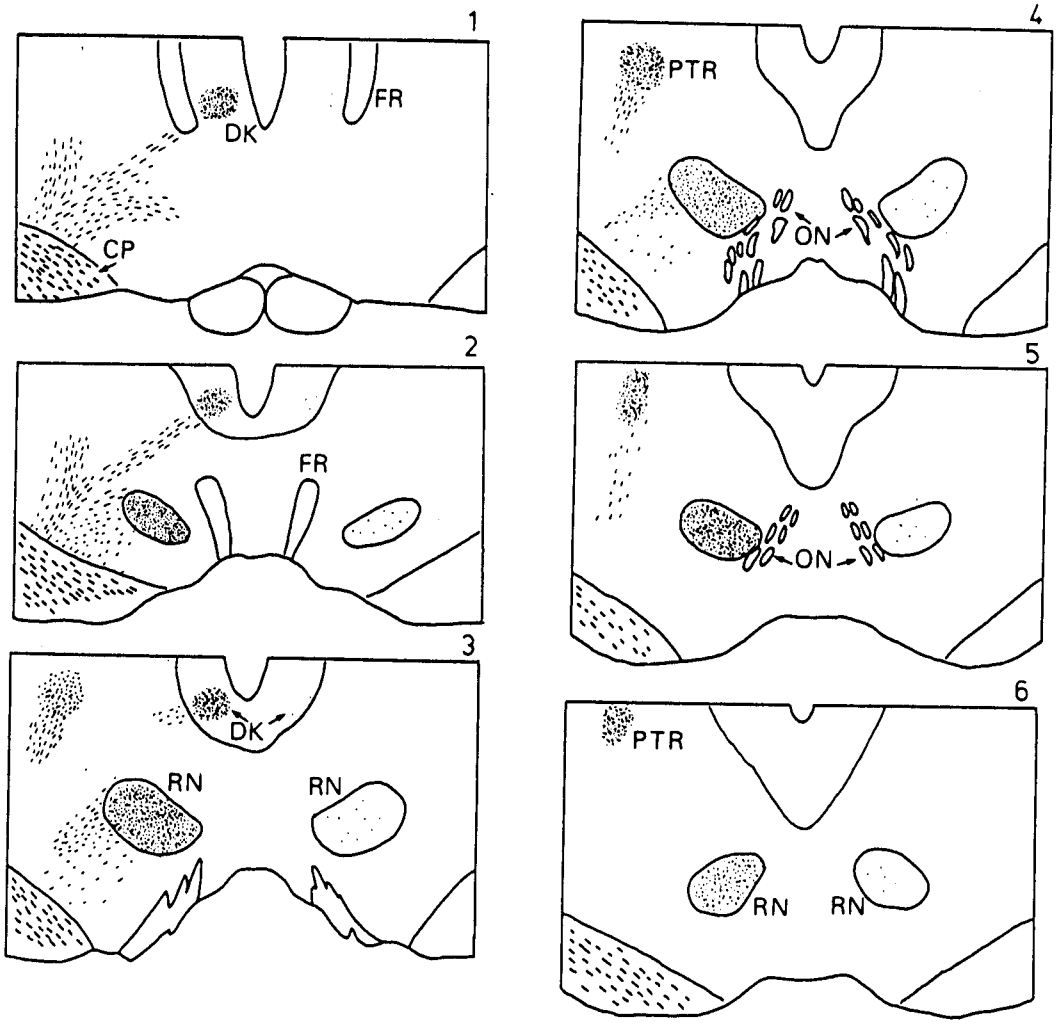

Fig. 3. Diagrammatic presentation of labeled corticofugal fibers in 6 sections through the mesencephalon of a kitten. The kitten received an injection of *Phaseolus vulgaris* leucoagglutinin into the right pericruciate cortex 1-2 weeks prior to perfusion. Dots represent labeled straight fibers with extensive branchings and swellings; dashes shemaize fibers with less branching. Sections are arranged from rostral to caudal. RN, red nucleus; CP, cerebral peduncle; PTR, pretectal region; ON, oculomotor nerve; DK, Darkschewitsch nucleus; FR, fasciculus retroflexus.

branch extensively and formed varicosities which appear in an en-passant and terminal way along the trajectory of the axons (Fig. 4A). The number of labeled fibers in the ipsilateral RN varied from animal to animal, which may probably depend on the site, depth, size and number of injections. In the RN contralateral to the injection some labeled fibers were also observed, although the fibers were much less frequently observed than in ipsilateral RN. Fig. 4B shows an example of labeled fibers oberved in the contralateral RN. Similar to the ipsilaterally observed fibers, the fibers in the contralateral RN also showed varicositiesas well as terminal end branches with bulbous swellings. They appeared to branch less than those observed ipsilateral RN in all of the normal kittens used in the present study.

The labeled fibers seemed to be somatotopically arraged as in adults (Mabuchi and Kusama, 1965). This will be described in next chapter.

Red nucleus -after lesion of sensorimotor cortex

In the animals which underwent cortical lesion, labeled fibers were also observed in the ipsilateral RN (Fig. 4C). In contrast to the RN of the normal kitten, many labeled fibers were seen in the RN contralateral to injection (Fig. 4D). Some of the fibers seemed to stem from the ipsilateral RN but others appeared to project directly from the cerebral peduncle to the contralateral RN. The fibers branched as extensively as those observed in the ipsilateral RN and formed varicosities along the trajectory of the axons. Fibers with large diameter (1 um<) which were not observed in control animals were often encountered.

In addition to the bilateral projection to the RN, other corticofugal fibers also distributed bilaterally. Extensive branching of fibers with abundant varicosities were present in the contralateral pretectal region and nucleus Darkschewitsch.

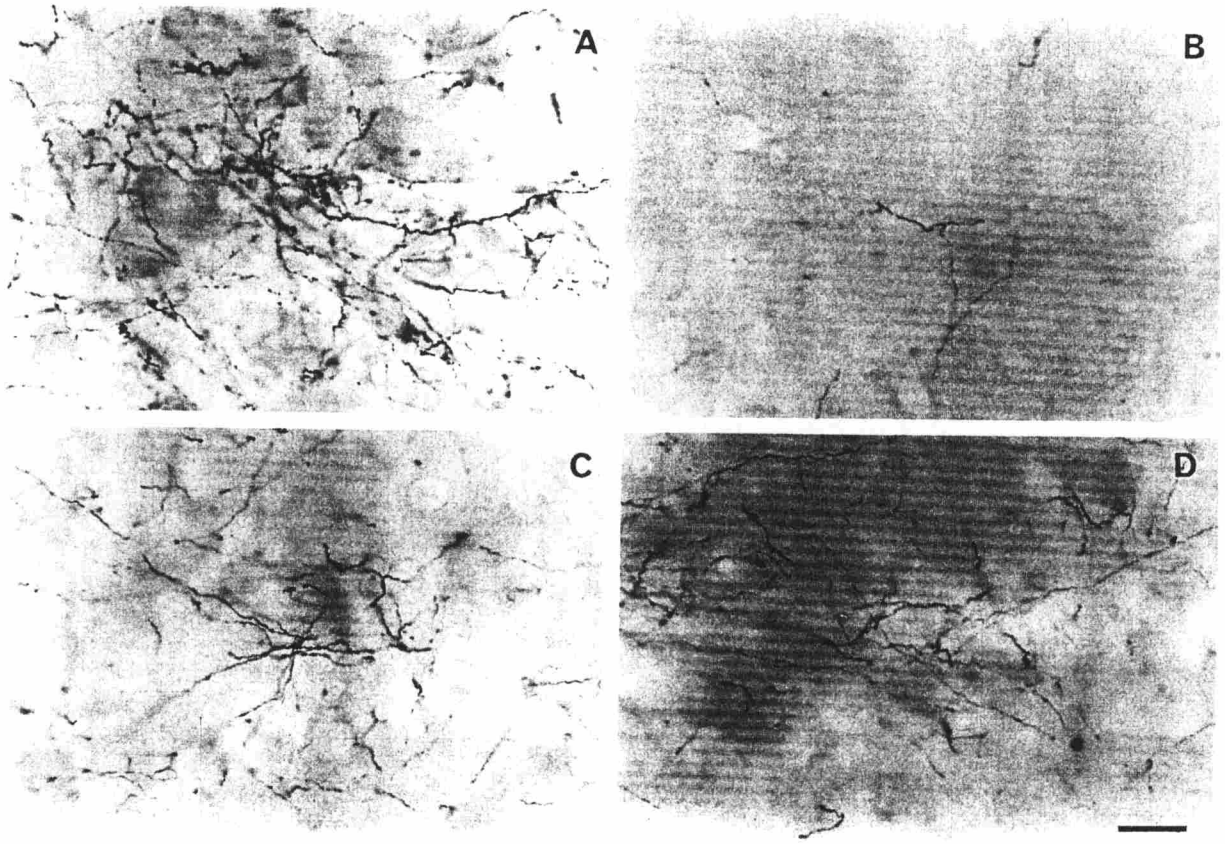

Fig. 4. Corticorubral fibers labeled with PHA-L. A: labeled fibers observed in the RN ipsilateral to the cortical injection in an intact animal. Many fibers with and without varicosities can be seen. Perfused one month after birth. B: RN on the contralateral side of the same animal. Few labeled fibers are seen. C: labeled fibers on the RN ipsilateral to the injectionsite. In this animal the sensorimotor cortex was ablated ipsilaterally 3 weeks after birth and it was sacrificed 26 days later. PHA-L was injected into the intact sensorimotor corex 14 days prior to prefusion. D: labeled fibers in the RN contralateral to that shown in C. Note the abundance of labeled fibers. Although the fibers were more extensively labeled in the ipsilateral RN in animals shown in A than that in D, fewer labeled fibers are seen on the contralateral side of the same animal (B). Bar=25 um.

Regardless of the variability of cortical lesionsamong operated animals, a large number of labeled fibers observed in the contralateral RN of every lasioned animal.

Increase of crossed corticorubral fibers

Although labeled fibers were found in the RN contralateral to the injection in every kitten, those fibers were much more numerous in lesioned animals than in intact ones. Fig. 5 shows drawings of labeled fibers in the RN of 3 normal kittens. These drawings are representative of the corticorubral projections in these animals. The labeled fibers in the contralateral RN (right row) are much fewer than those observed ipsilaterally (left row). The ipsilateral labeling was so intense in the case shown in A that it was not possible to draw all the fibers. Therefore, the actual difference in labeling in even more pronounced. By contrast, in the animals which underwent cortical ablation the number of labeled fibers observed in the RN contralateral to the injection sites was almost compare to that observed ipsilaterally (Fig. 6).

Axon terminal swellings

Cortical axonal endings which exhibit growth cone-like morphology were observed in the present study. Fig. 7 shows examples of growth cone-like axon terminals observed in the RN. These axon terminals were shaped as ovoid or triangular bulbs with irregular contours, 1-2.5 um in diameter by 2-10 um i length. These axon terminals are very similar to the tips of regenerating growing axons in their morphology (Kawaguchi et al., 1986; Scalla and Matsumoto, 1985), although there is no direct evidence to suggest that they represent actual sites of axonal growth. Growth cone-like axonal endings were observed on both sides of the RN of lesioned animals and in the RN ipsilateral to the injection site in normal animals.

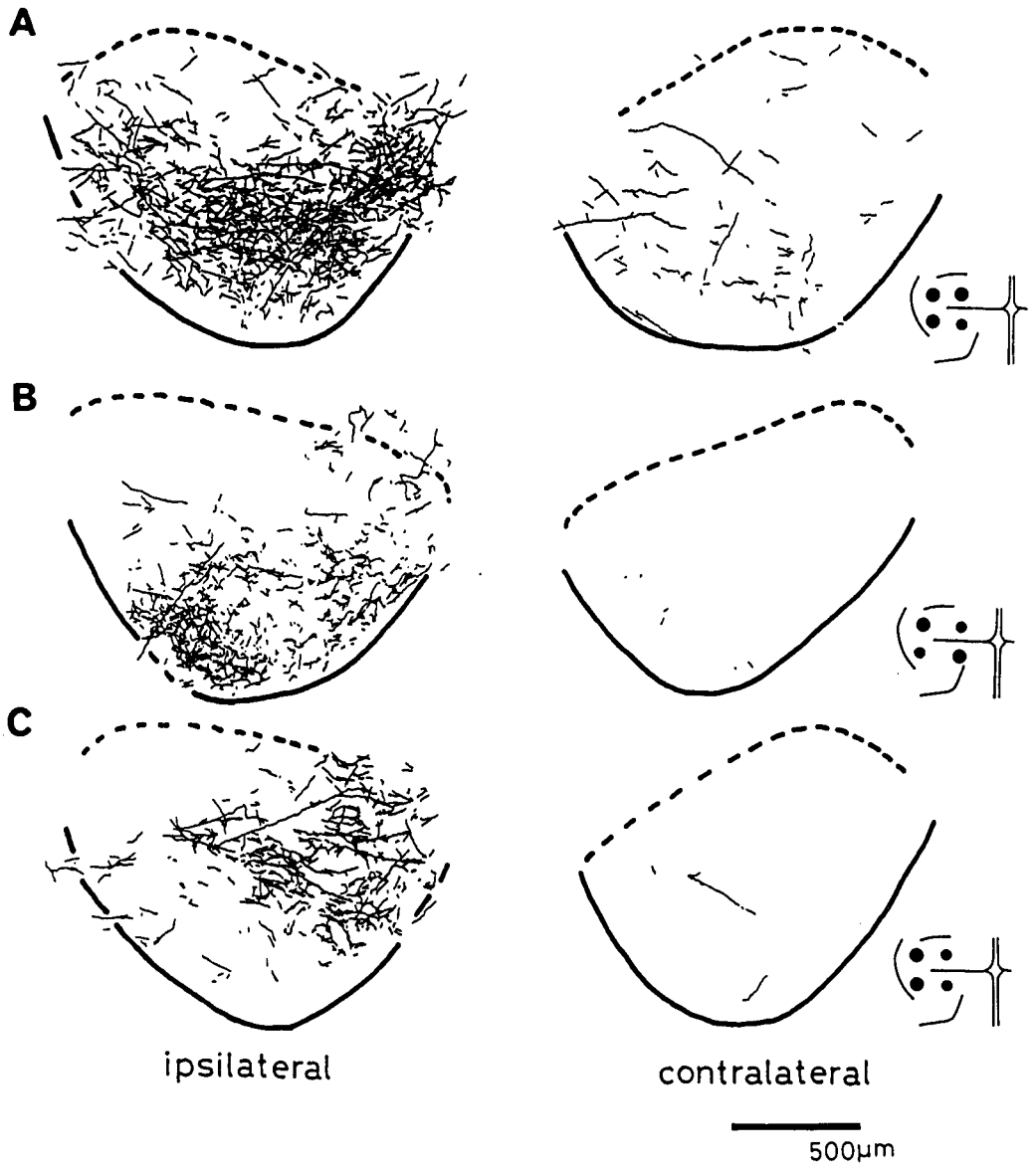

Fig. 5. Labeled corticofugal fibers in the RN of the intact kittens following injections of PHA-L into the sensorimotor cortex. A-C: camera lucida drawings of labeled fibers in th eRN. Frontal sections. Left row: ipsilateral to cortical injection. Right row: contralateral to cortical injection. The data shown in A were taken from the same animal as shown in Fig. 4 A, B. B and C are from different animals, 7 and 4 weeks of age respectively. Filled circles in the inset in each panel represent sites of injection in the sensorimotor cortex. Larger circles represent sites where many labeled cells were seen.

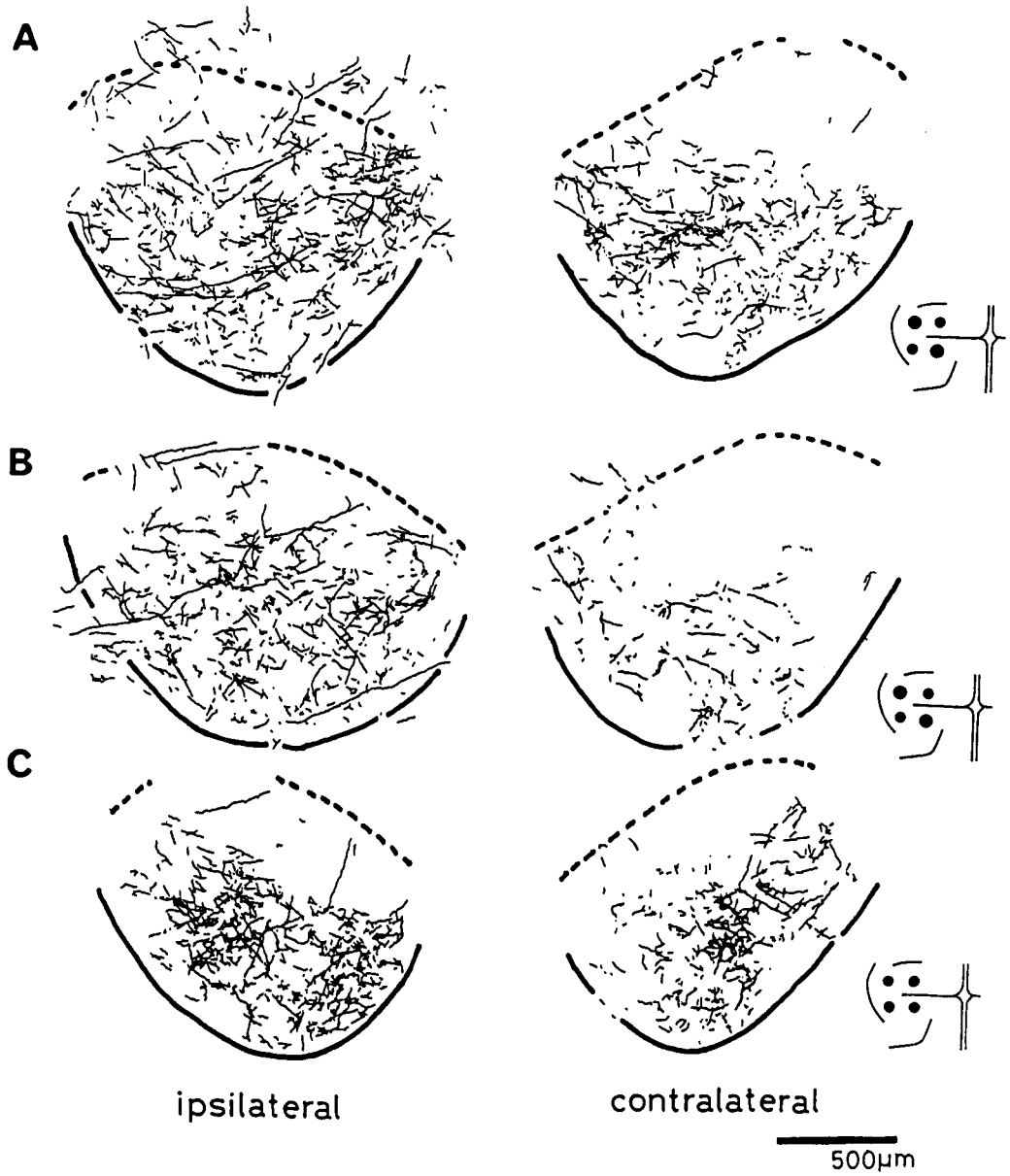

Fig. 6. Labeled corticofugal fibers in th RN of kitten which underwent cortical ablations. Camera lucida drawings of frontal sections. Sensorimotor cortex was ablated at 3, 3 and 4 weeks after birth in the case shown in A, B and C, respectively. Lef row, RN ipsilateral to injection. Right row, RN contralateral to injection. Note that the contralateral labeling is almost comparable to the ipsilateal labeling in each case. Insets refer to Fig. 5.

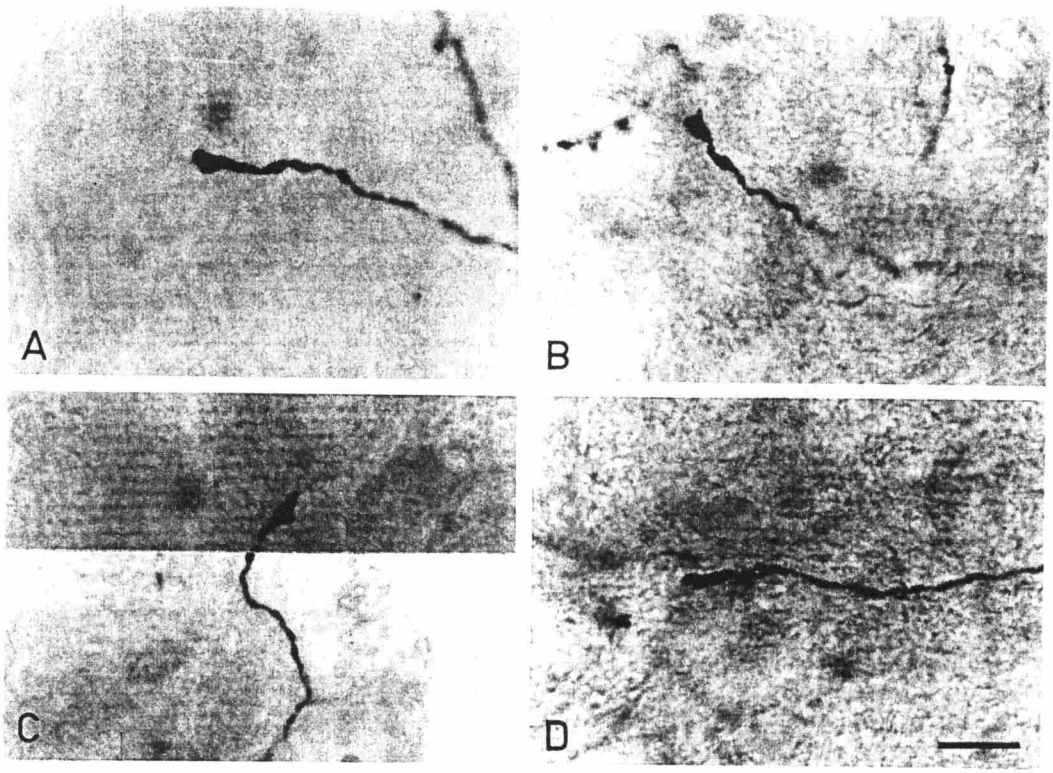

Fig. 7. Growth cone-like axonal endings observed in the RN of a kitten. Growth cone-like structures usually followed very thin fibers. They were ovoid or spherical in shape with irregular contours. Tips of the growth cone bifurcated in some occasions (D). Bar=10 um.

DISCUSSION

The results of the present study indicate that crossed corticorubral fibers are present at early developmental stages in the kitten and that unilateral cortical lesions result in an increase in the density of the crossed projection.

Presence of crossed corticorubral fibers in normal animals

Bilateral corticorubral fibers have been demonstrated in the adult cat using silver staining techniques (). According to Rinvik there are bilateral projections from th esupplementary motor area and proreus gyrus. It is unlikely that we happened to inject PHA-L into these areas in lesioned aimals, because we avoided these area in all of our injections (see Materials and Methods) and sites of injection in normal animals do not seem to differ from those in lesioned animals (compare insets in Fig. 5 with those in Fig. 6). Furthermore, in an experiment in which PHA-L was intentionally injected into the medial part of the precruciate cortex, there were hardly any labeled fibers in the contralateral RN (not shown). This is in agreement with the results of physiological experiments by Fujito et al. (1983) which failed to record any synaptic potentials in RN cells in response to stimulation of the contralateral supplemental motor area. Stimulation of proreous gyrus was ineffective even in producing ipsilateral EPSPs (Fujito et al., 1983).

Sprouting vs maintenance of preexisting crossed projection

Leonard and Goldberger argued that massive crossed corticorubral fibers found in animals with cortical lesions may be due to maintenance of preexisting crossed fibers (Leonard and Goldberger, 1987). This argument was based upon their finding that there were abundant labeled fibers in the contralateral RN in the 1-day-old normal kitten. We did not perform any experiments using such young animals. The age of the control animals ussed in the present study varied from 2 to 7 weeks when they were perfused. Therefore, we cannot independently confirm whether an increase number of

crossed ccorticorubral fibers, as reported by Leonard and Goldberger (1987), could be detected in younger animals or not. So far no correlation between the age of animal and the extent of labeling in the contralateral RN in the normal kitten has been observed in our laboratory.

If the massive crossed corticorubral fibers wre solely due to the prevention of a normally occurring process of retraction, then the number the number of crossed corticorubral fibers observed in intact animals should have been comparable to those observed in animals which received unilateral cortical ablations at corresponding ages. It was, however, not the case; in every normal animal, crossed corticorubral fibers were significantly fewer in number than those observed in lesioned animals which were operated at corresponding ages (compare Fig. 5A with Fig. 6C). Furthermore, Kosar et al. (1985) failed to find crossed corticorubral fibers in 7-30-day-old kittens utilizing anterograde transport of HRP, whereas they found abundant labeled terminals in kittens which underwent hemispherectomy 3.5- weeks after birth. In addition, it is to be noted that monosynaptic excitatory postnynaptic potentials (EPSPs) could be elicited in RN cells in respose to stimulation of the contralateral cerebral peduncle (Tsukahara et al., 1983) even in kittens in which the sensorimotor cortex was ablated 2 months after birth. The experiment using rats also suggest that aberrant crossed corticorubral fibers may not be the result of a persistent neonatal connection (Nah et al., 1981).

The present study results basically comfirm the findings described by Kosar et al. (1985). The failure of Kosar et al. (1985) to find crossed fibers could be due to the difficulty in discriminating very small amounts of anterograde transported HRP from non-specific precipitates of reaction product, particularly when the TMB method is utilized (Mesulam, 1982). It should be pointed out that the large amount of WGA-HRP (8-10 μl) which Leonard and Goldberger (1987) injected into the cortex could cause false-positive results due to the failure of WGA-HRP to remain within the

sensorimotor cortex. Concerning this point, PHA-L is far surperior to WGA-HRP; the injection site is very limited in size and the axons and their terminals are clearly labeled and can be reliably interpreted as arising form the neurons labeled at the injection site (Gerfen and Sawchenko, 1984

Possible mechanisms for the increase of crossed fibers

Although the present findings basically confirm the results of previous studies, the presence of even a few crossed corticorubral fibers in normal kittens may provide a different view for the molecular mechanisms responsible for aberrant projections. Kosar et al. (1985) needed to assume a "sprouting factor" which diffuses from the contralateral to the ipsilateral RN along a distance of about 3-4 mm. The presence of crossed fibers in the neonatal intact kitten, however, suggests that such diffusible molecules are not necessarily involved. Lesion may induce expression of a kind of adhesion molecule such as N-CAM on the surface of preexisting crossed fibers (Sanes and Covaut, 1985). These fibers may play a role in guding the sprouted fibers to their target.

In addition the presence of crossed fibers in normal animals suggests another possible mechanism for the increase of crossed projections after cortical lesions. Although the number of labeled fibers seemed to be increased in lesioned kitten, it is possible that terminal proliferation or increased branching of the corticorubral fibers already present in the contralateral RN may be occurring. This possibility is very intriguing, since it could explain the occurrence of the aberrant projections, observed following brain damage, in immature animals in which there are relatively diffuse projections. Retraction of exuberant projections during development would abolish the ability to induce aberrant projections such as the crossed corticorubral projection.

Chapter2

POSTNATAL DEVELOPMENT OF CROSSED AND UNCROSSED CORTICORUBRAL PROJECTIONS IN KITTEN: A PHA-L STUDY

absract

Morphological changes in individual corticorubral fibers and the patten of crossed and uncrossed corticorubral projections were studied during the postnatal development of cats in order to understand cellular mechanisms for restriction of corticorubral projections with development. The anterograde tracer phaseolus vulgaris leucoagglutinin (PHA-L)was injected into restricted areas of the pericruciate cortex in kittens and PHA-L-labeled axons in the red nucleus were examined at postnatal days (PND) 7-73.

In accord with our previous study (Murakami and Higashi, Brain Res. 1988;447:98-108), a crossed corticorubral projection was observed in addition to the uncrossed one in every experimental animal. During the early period of development (PND7-8), swellings of irregular shape were observed along the entire course of the axons and they were often interconnected with extremely fine axonal segments. These axons bifurcated only infrequently and often ended as growth cones. These features were common to both uncrossed and crossed corticorubral axons. At later stages of development (PND 28 or Later), the total number of swellings decreased and axonal swellings with smooth contours became dominant.

A quantitative examination of axonal branches indicated that axons on the ipsilateral side branch occurred more frequently at later stages of development. However, there was no substantial change in branching frequency for the crossed corticorubral fibers during development. In parallel with morphological changes in individual axons, the crossed projection that was initially relatively abundant was reduced during development. Since a PHA-L injection can be confined to a small region of cortex, topographic projections can easily be detected. At PND7-8 there was no well-defined topographic order in the ipsilateral corticorubral projection. Adult-like topography was first discernible at PND 13.

These observations suggest that the unilateral uncrossed corticorubral projection in the adult cat is achieved at least in part by the formation of axonal arbors in the uncrossed projection. This was accompanied by the failure of crossed fibers to form complex arbors. It is possible that a similar mechanism also operates in the formation of topographic maps.

INTRODUCTION

In mammals corticofugal as well as corticocortical projections have been shown to undergo considerable reorganization during development. A commonly observed phenomenon is the loss of exuberant projection and the resultant formation of specific synaptic connections. Anterograde tracer studies with radiolabeled amino acids or WGA-HRP demonstrate that initially diffuse corticocortical and corticofugal projections become segregated during prenatal and postnatal development (Wise and Jones, '76; Hollander et al., '79; Goldman-Rakic, '81; Tolbert and Panneton, '83; Mihailoff et al., '84; Tolbert et al., '84; Theriault and Tatton, '89). Segregation of inputs has been explained either by cell death or elimination of axon collaterals (see cowan et al., '84 for review) Retrograde tracer studies indicate that cortical neurons that initially have aberrantly projecting axons lose these "incorrect" axon collaterals as development proceeds (O'Leary et al., '81;Ivy and Killackey, '82; Innocenti and Clarke, '84; Stanfield and O'Leary, '85; O'Leary and Stanfield, '86). Although these studies provide valuable information on the development events for corticofugal and corticocortical projections, little is known about the developmental changes in axonal morphology within their target regions. The major purpose of the present study is to elucidate the morphological changes in corticofugal axons that underlie the restriction of projections.

We have used a *Phaseolus vulgaris* leucoagglutinin (PHA-L) method (Gerfen and Sawchenko, '84) to study developmental changes in corticorubral axons in the kitten. Since axonal labeling with PHA-L provides Golgi-like staining, the development of projection patterns and the morphology of individual axons can be studied concurrently. The corticorubral projection was chosen in the present study, because there is a wealth of knowledge, both electrophysiological and anatomical, on this projection in adult cats (Niimi et al., '63; Rinvik and Walberg, '63; Rinvik, '65; Mabuchi and Kusama, '66; Tsukahara and Kosaka, ' 68; padel et al., '73; Sadun, '75; Tsukahara et al.,

'75; Murakami et al., '82; Fujito et al., '83; Jeneskog and Padel, '83; Nakamura et al., '83; Robinson et al., '87). Furthermore, there is an extensive literature on the formation of aberrant corticorubral projections following early lesions of the cerebral cortex (Nah and Leong, '76a, b; Tsukahara and Fujito, '81; Villablanca et al., '82, '88; Tsukahara et al., '83; Kosar et al., '85; Naus et al., '85, '87; Leonard and Goldberger, '87; Murakami and Higashi, '88) Since exuberant projections in neonatal animals have been correlated with formation of aberrant projections after brain damage (Leonard and Goldberger, '87; Murakami and Higashi, '88), studies on the development of the corticorubral projection may also contribute to the understanding of cellular mechanisms of synaptic plasticity after early brain damage.

The results show that the crossed corticorubral projection, which is initially significant, becomes negligible with time and that sharpening of the topographic maps occurs during the first postnatal month. The morphology of individual corticorubral axons in the ipsilateral red nucleus is simple, and unbranched early in development, but become elaborated as development proceeds. These changes are not observed in the crossed corticorubral fibers.

MATERIALS AND METHODS

Nineteen kittens aged 7 to 73 days were used. Eleven of the animals came from Hyogo Prefectural Administration center for Animals and their age was estimated from the day of teeth eruption (Murakami and Higashi, '88). Others were from breeding colonies mostly in Abrahi Laboratories of Shionogi and Co. LTD. For These animals the date of birth was considered postnatal day (PND) O. Animals were divided into four groups and PHA-L injections were made into either the lateral sigmoid gyrus (group I), the medial part of the posterior sigmoid gyrus (group II), or the medical part of anterior sigmoid gyrus (group III) (see Fig. 1). In each of these three groups two injections were made within a distance of 2 mm and the forth group received four injections into the pericruciate cortex (Fig. 1E) as described previously (Murakami and Higashi, '88). Briefly, PHA-L, 2.5% in 0.05 M Tris-buffered saline, pH 7.6, was injected iontopholetically (5 uA, 7.5 seconds on and 7.5 seconds off) through glass micropipetts with a tip diameter of 20-40 um into anesthetized (see below) kitten. Five to 14 days after the injections the kittens were deeply anesthetized with Nembutal (30 mg/kg) and ketamine hydrochloride, perfused transcardially with phosphate buffered saline followed by 4% paraformaldehyde or a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde and then processed as previously described (Murakami and Higashi, 1988). Serial tissue section including the red nucleus (RN) were cut either coronally or horizontally at 50 um. The sensorimotor cortex was cut serially in the parasagittal plane at 100 um to examine the injection sites. PHA-L was visualized immunohistochemically using an ABC method as described previously (Murakami and Higashi, 1988). Part of the section were postfixed with OsO4, counterstained en bloc with Uranyl acetate, and flat embedded in Epon.

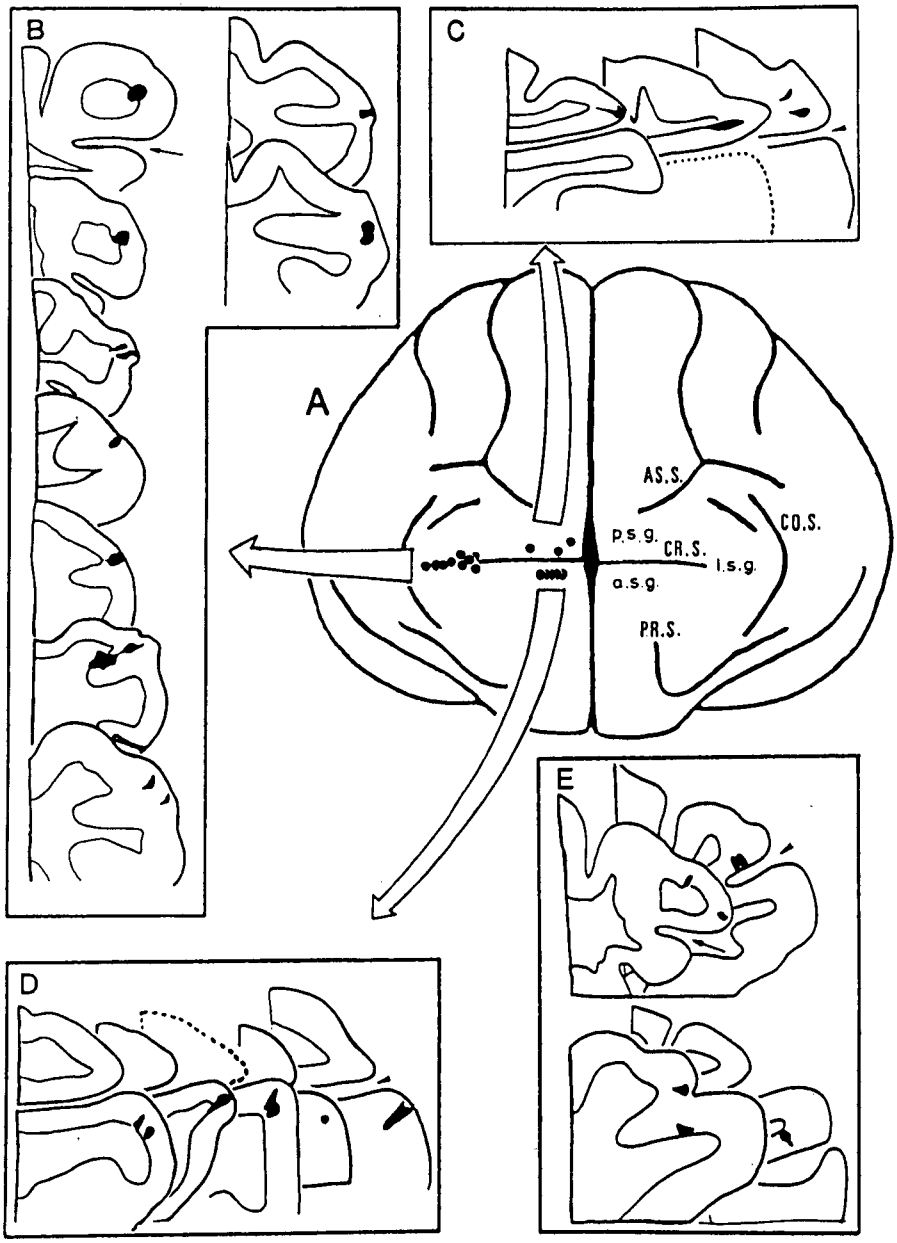

Fig. 1. Summary of PHA-L injection sites of all the experimental animals. A: Dorsal view of the cat cerebral cortex to show injection sites in 18 experimental animals with injections confined to one of the three cortical areas: lateral sigmoid gyrus (group I), medial part of posterior sigmoid gyrus (group II), and medial part of anterior sigmoid gyrus (group III). AS.S., ansate sulcus, CO.S., coronal sulcus, CR.S., cruciate sulcus, PR.S., presylvian sulcus, p.s.g., posterior sigmoid gyrus, a.s.g., anterior sigmoid gyrus, l.s.g., lateral sigoid gyrus. B-E: Drawings of sagittal sections showing center and size injections. The area occupied by darkly stained cells represented by dark spots. Each drawing in B-D represents the injection site of one experimental animals. In E, drawings of two parasagittal sections are shown for each animal. B, group I; C, group II; D, group III; E, group IV animal in which four injections were made around the pericruciate sulcus. Arrows in B and E point to coronal sulcus and arrowheads in C-E point to the cruciate sulcus. Missing portion of sections that were either lost or damaged during dehydration are illustrated by dotted lines.

Labeled axons were observed under a light microscope. Labeled fibers in part of the sections were drawn, with a camera lucida attached to the microscope, at x40-100 to study topography of projections and x400 for quantification of branching (see below). The size of axonal swellings was measured at x400 using an eyepiece micrometer attached to the microscope.

Table 1 summarizes animals used for the present study. Development of axonal morphology was for the most part based on observation from group I animals, since we had a concern that axonal morphology might vary among cells in different cortical areas. Four animals at postnatal days 7, 8, 29 and 28 were used for quantification of axonal branching. From the animals at PND 7 and 8, every second or every third section was sampled over a rostrocaudal distance of 1.5-1.7 mm. Every labeled axon fragments found within a 50 um tissue section of the RN was drawn at x400. From the kitten at 28 and 29 PND every fourth section was sampled over rostrocaudal distance of 2.4 mm and axon fragments were drawn. A total of 535 and 699 axon fragments were drawn from ipsilateral red nucleus of the kittens at PND 7-8 and PND 28, respectively. Similarly, 193 and 164 axon fragments were drawn from the contralateral red nucleus of the kittens that were PND 7-8 and PND 29, respectively.

TABLE I Summary of the experimental protocol for each kitten incruded in the present study

Animal no.	Injection site	Survival (days)	Age at sacrifice (PND)
108	l.s.g.	5	7
115	l.s.g.	7	8
104	l.s.g.	7	10
109	l.s.g.	11	13
103[2]	l.s.g.	10	17[3]
98	l.s.g.	10	21[3]
97[2]	l.s.g.	10	24[3]
78	l.s.g.	14	28[3]
73	l.s.g.	14	73[3]
99[2]	m.p.s.g.	13	24[3]
40	m.p.s.g.	14	52
83	m.p.s.g.	14	41[3]
125	m.a.s.g.	7	12
123	m.a.s.g.	7	16[3]
41	m.a.s.g.	14	24[3]
81	m.a.s.g.	11	39[3]
39	m.a.s.g.	14	48
46	(quadruple)	12	29[3]
15	(quadruple)	14	52

[1] l.s.g., lateral sigmoid gyrus; m.p.s.g., medial part of possterior sigmoid gyrus; m.a.s.g., medial part of anterior sigmoid gyrus.

[2] Red nucleus of these animals were cut horizontally.

[3] Age estimated by teeth eruption.

RESULTS

Injection sites

The drawings of Fig. 1 summarize the injection sites from all the experimental animals. Filled circles in Fig. 1A illustrate the surface view of the injection sites reconstructed serial sections of group I-III animals. Two injection sites are represented by a single dot here. Fig. 1B-D shows drawings of sagittal sections of the cortices; the center of injections is indicated by a dark spot. Injection sites of two group IV animals shown in Fig. 1E without their surface view. Although multiple injections were made in every case, it was not always possible to discriminate multiple sites. As can be seen in this figure, the injection sites were found in the intended cortical loci. Size of injection sites varied from 750 um x 500 um to 1,500 um x 1,000 um in group I, from 500 um x 380 um to 1,000 um x 500 um in group II, and from 630 um x 500 um to 1,500um x 750 um in group III animals. No correlation was found between an animal's age and size of the injection site.

As previously described (Murakami and Higashi, 1988), Golgi-like staining of neurons in the cortex was observed at the injection site in every animal. Staining of glial cells was rarely observed.

Ipsilateral projection

Morphology of corticofugal axons in the ipsilateral red nucleus.

At PND 7-8, growth-cone-like profiles were often encountered (Fig. 2A). The PHA-L-labeled axons in the RN were generally very thin and straight during the early postnatal days. The axons had swellings throughout the entire course of their trajectory. Axonal segments between swellings were often so thin that were beyond the resolution of the light microscope. Most of the axonal swellings were

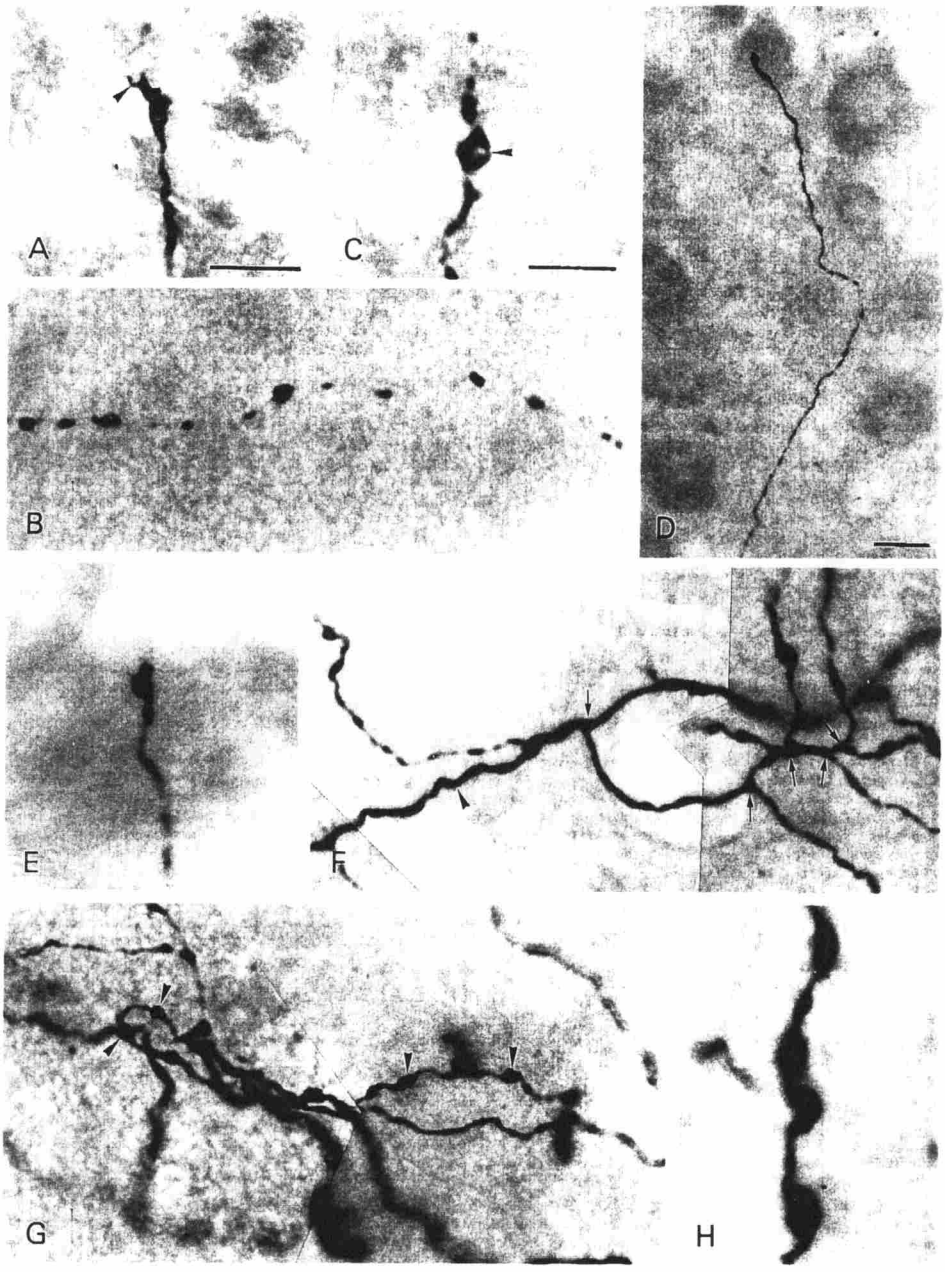

Fig. 2. Light micrographs of PHA-L labeled fibers in the red nucleus. A-D, labeled axons at PND 8 (A-C) and PND 7 (D). A: A growth-cone like axonal ending with microspike-like protrusion (arrowhead). Axons at this stage mostly had irregular-shaped swellings that were interconnected with extremely fine axonal segments (B). Some of the axonal swellings appeared to have a lighter region in their center (arrowhead)(C). D: A low power light micrograph illustrates that axons at this stage usually course straight without branching extensively. E-G, labeled axons at PND 28. A growth-cone like axonal ending (E) was still observed. Axonal swellings (arrowheads in G) at this stage were mostly smooth in contour and were connected by thick axonal segments. Thick axons never observed at PND 7-8 were now apparent (arrowhead in F). Furthermore, axons bifurcated very often (arrows in F) and coursed tortuously. At a later developmental stage (PND 73), axonal swellings with larger diameter were observed (H). Bar = 10 um in A. Bar in A applies to B,E,F and G. Bar = 5 um in C and applied to H. Bar = 20 um in D.

small-sized and exhibited rugged or crenulated contours (Fig. 2B, C). It was uncommon to encounter axonal bifurcations (e.g., Fig. 2G, H). The number of axons labeled at this stage was generally few.

At later ages, axons changed from straight to tortuous in form and rugged to smooth in contour (Fig. 2F, G). They branched more frequently and axonal swellings of a larger diameter appeared (Fig. 2H). The labeled fibers observed in the ipsilateral RN and the cerebral peduncle appeared to be fewer in younger animals.

Increase in branching frequency.

Fig. 3A shows drawings of axon fragments observed in 50 um thick section of the RN ipsilateral to the injection at PND 7-8 (#108 and #115). Out of the 535 axon fragments, the ten axons that exhibited the most frequent branching are shown here. Similarly, the eight most frequently branched axon fragments from an animal at PND 28 (#78)are shown in Fig. 3B. These drawings suggest that corticorubral axons that are initially simple in form come to have extensive arborizations as development proceeds. Fig. 3C summarizes the data on the frequency of axonal branching within the ipsilateral RN. Open columns represent data from kittens at PND 7 and 8, and shaded columns at PND 28. This graph indicates that the proportion of the axon fragments that have branches within 50 um thick sections is much larger at PND 28 than at PND 7-8 (see discussion). Less than 2% of the axons exhibited more than two branches at this stage. In contract, branching was much more frequent (8.6%) at PND 28. A statistical analysis (Kolmogorov-Smirnov test) indicated that the frequency distribution of axonal fragments at PND 7-8 is significantly different from that at PND 28 ($P < 0.001$).

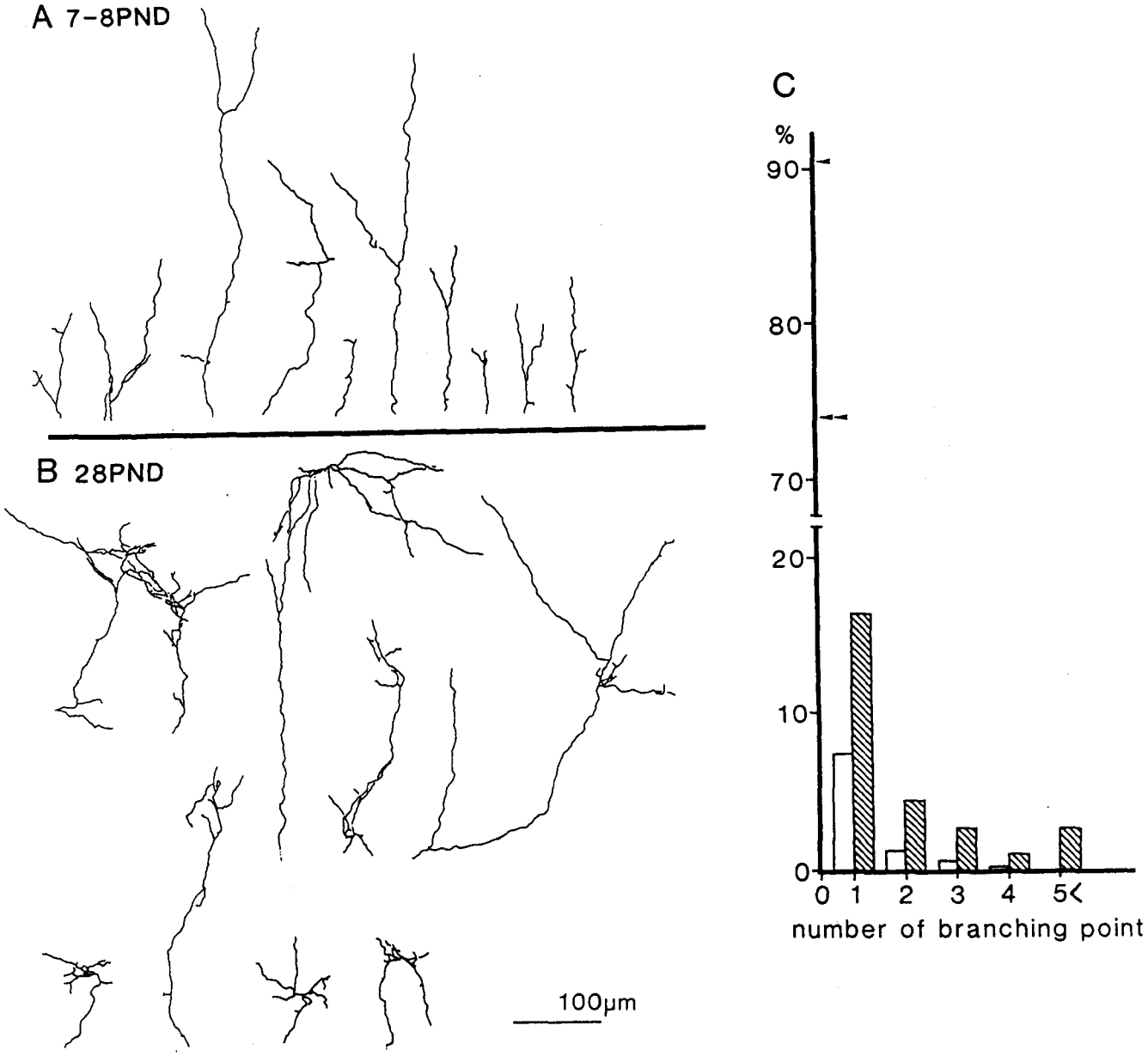

Fig. 3. Comparison of axonal morphology in the ipsilateral red nucleus between PND 7-8 and 28. Twenty-six frontal sections cut at 50 um were selected from two animals at PND 7 and 8, and every labeled axon in the red nucleus in these sections was drawn with the aid of a camera lucida (see Materials and Methods for detail). The number of branching points was measured from 539 axonal fragments drawn in this manner. Similarly, a total of 699 axons was drawn from seven sections of the red nucleus at PND 28 and the number of branches was measured. The most frequently branched axons among the 539 axonal fragments at PND 7-8 are shown in A and the most frequently branched axons among the 699 axonal fragments at PND 28 are shown in B. C: Histogram showing the relationship between the number of axonal fragments and frequency of branching. Ordinate: the percentage of axonal fragments. Abscissa: number of branching points of the labeled axonal fragments within 50 um thick sections. Open columns represent data at PND 7-8 and hatched columns at PND 28. The percentage of axons without branches is indicated by an arrowhead and double arrowheads for the kitten at PND 7-8 and 28, respectively.

Topography of corticorubral projection.

It is well established that there are somatotopic arrangements for the corticorubral projection in adult cats (Rinvik and Walberg, 1963; Mabuchi and Kusama, 1966; Padel et al., 1973; Jeneskog and Padel, 1983); the neurons in the lateral sigmoid gyrus project to the medial part of the RN, which contains cells projecting the upper spinal segments, while those on the posterior bank of the medial sigmiod gyrus project to the lateral part of the RN, which contains cells project to the lateral part of the RN, which contains neurons projecting down to the lower spinal cord (Pompeiano and Brodal, 1957).

A similar topographic organization of the ipsilateral corticorubral projection is already found at about 1 month after birth (Fig. 4). PHA-L injections into the lateral sigmoid gyrus (group I) resulted in the labeling of axons in the medial portion of the caudal RN. Projections to the anterior part of the RN tended to be lass restricted (see Fig.4Aa2 and a3). Projections were found to the nucleus of Darkshevich and the anterior and posterior pretectal nuclei, too. The morphology of axons in these projection areas was characterized by frequent branching, tortuousness, and swelling of axons. Most of the swellings occurred as dilations along the axons.

When PHA-L was injected into the medial part of posterior sigmoid gyrus (group II), axonal labeling was localized to the ventrolateral part of the RN as well as the nucleus of Darkshevich and the anterior pretectal nucleus (Fig. 4B). Projection to the anterior RN was weak (Fig. 4b1-2).

In contrast with the dense projections from these cortical areas, injections into the medial part of the anterior sigmoid gyrus (area6, group III) produced very weak labeling in the RN, nucleus of Darkshevich, and pretectal nuclei. However,

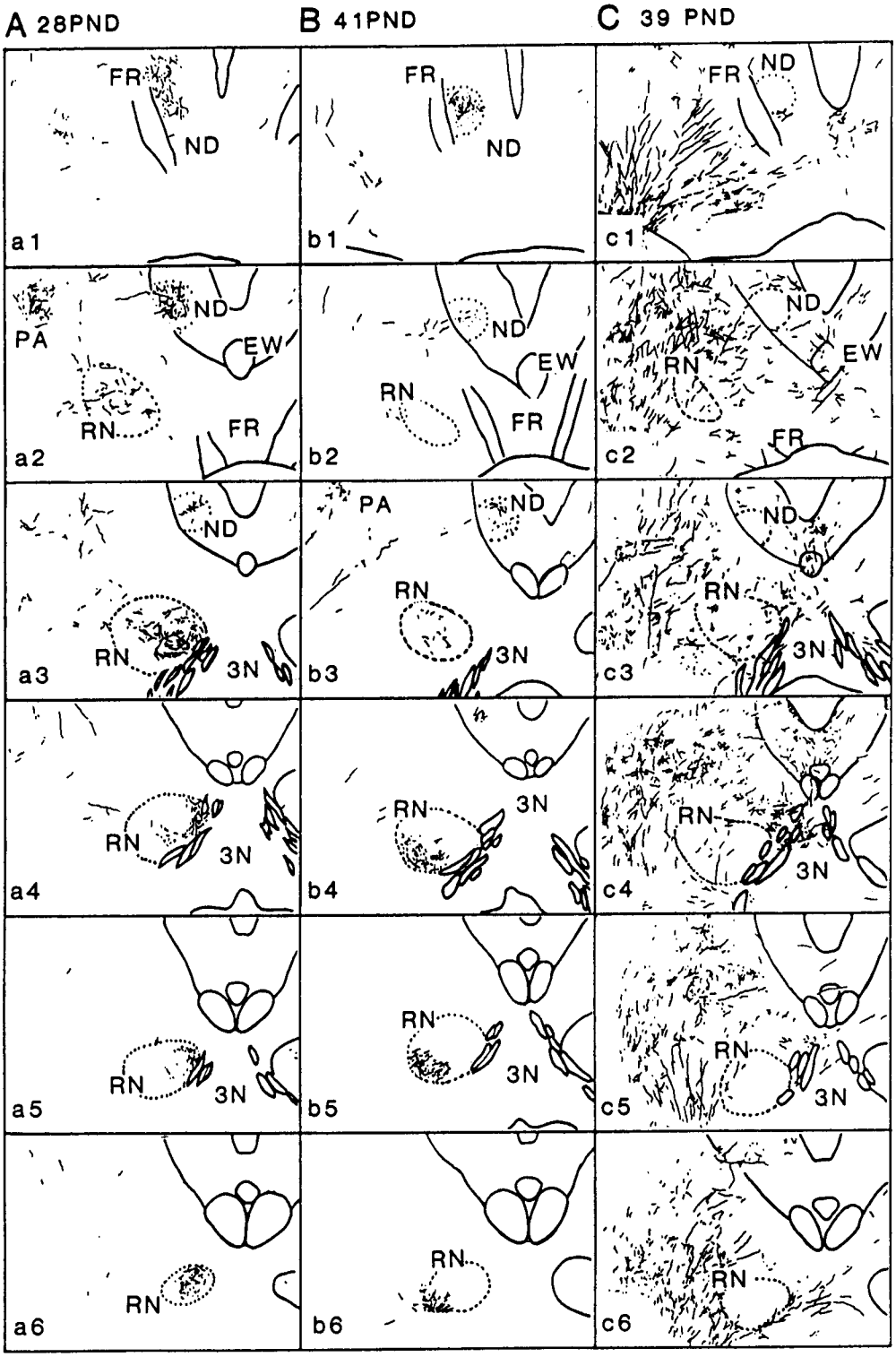

Fig. 4. Topographic projections to the red nucleus and nearby midbrain areas from three different parts of the pericruciate cortex. Equally spaced drawings of labeled fibers in frontal sections of the kittens in which PHA-L was injected into either the lateral sigmoid gyrus (B) (see Fig. 1C), or the medial part of the anterior sigmoid gyrus (C, see Fig. 1D). Rostral is to the top and caudal is to the bottom. A, B and C were from animals at PND 28, 41 and 39, respectively. ND, nucleus of Darkshevich; PA, pretectal area; EW, Edinger-Westphal nucleus; RN, red nucleus; 3N, oculomotor nerve; FR, fasciculus retroflexus.

many labeled axons were observed in the perirubral reticular formation, pretectal region, periaqueductal gray, and nucleus of Edinger-Westphal (Fig. 4C) following these injection. In rare instances, labeled fibers were observed in the RN. However, they were usually straight in shape and did not branch extensively, suggesting that they are passing fibers.

Axonal swellings.

In order to demonstrate that the localization of labeled fibers within the RN does indeed represent localization of boutons, we examined the distribution of the axonal swellings that are likely to be synaptic boutons (see discussion). Fig. 5 compares the distribution of PHA-L-labeled axons and axonal swellings in a 73-day-old kitten. Fig. 5A depicts a drawing of labeled fibers in the RN. Axonal swellings (> 1 um diameter) observed in the same sections are indicated in Fig. 5B. Drawings from three serial frontal sections are superimposed. Comparison of Fig. 5A ans B indicates that the localizations of fibers and axonal swellings are fairly similar within the RN.

Topographic refinement.

Fig. 6 shows labeled fibers in the RN of three group I kittens at three different PNDs. The fibers were drawn at three or five different levels along the rostrocaudal axis. The topographic distribution of the projection that is well established at PND 73 (Fig. 6C) seems to be less defined at PND 13 (Fig. 6B). At PND 8, there was no clear topographic organization (Fig. 6A). The immaturity in the topography observed in the early postnatal days is supported by observations in two other kittens in which injections were made into area 6 (#125 and #123). In these animals labeled fibers were frequently observed within the

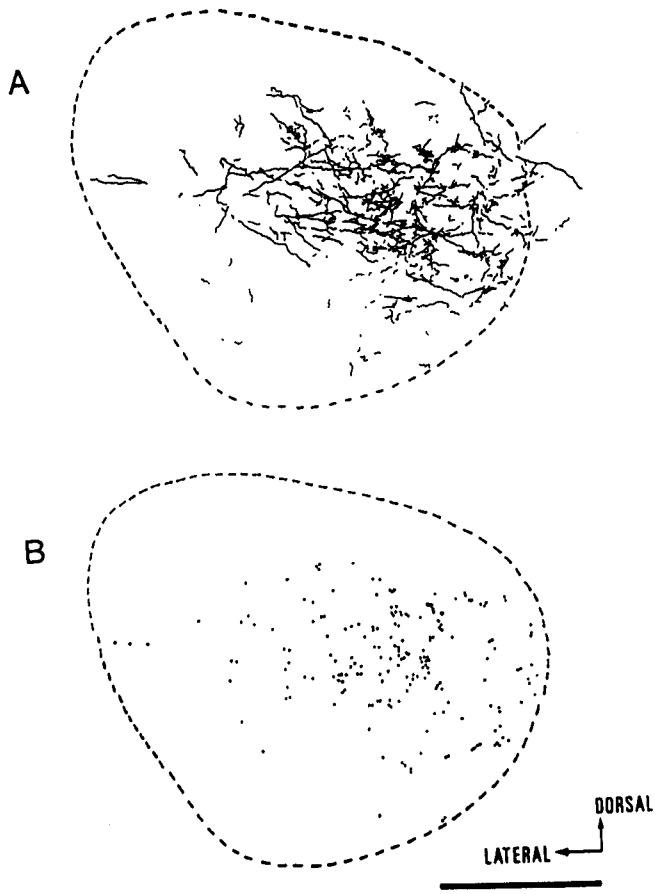

Fig. 5. Comparison in localization of labeled fibers and axonal swellings in the red nucleus. A: Drawing of labeled fibers in the red nucleus of a kitten (PND 73) in which PHA-L was injected into the lateral sigmoid gyrus. Drawings from three serial frontal sections are superimposed. Axonal swellings (diameter: >1 um) found along the labeled fibers in A are shown in B as closed circles. Note localization of labeled fibers is essentially the same as that of axonal swellings in the red nucleus. Bar = 500 um.

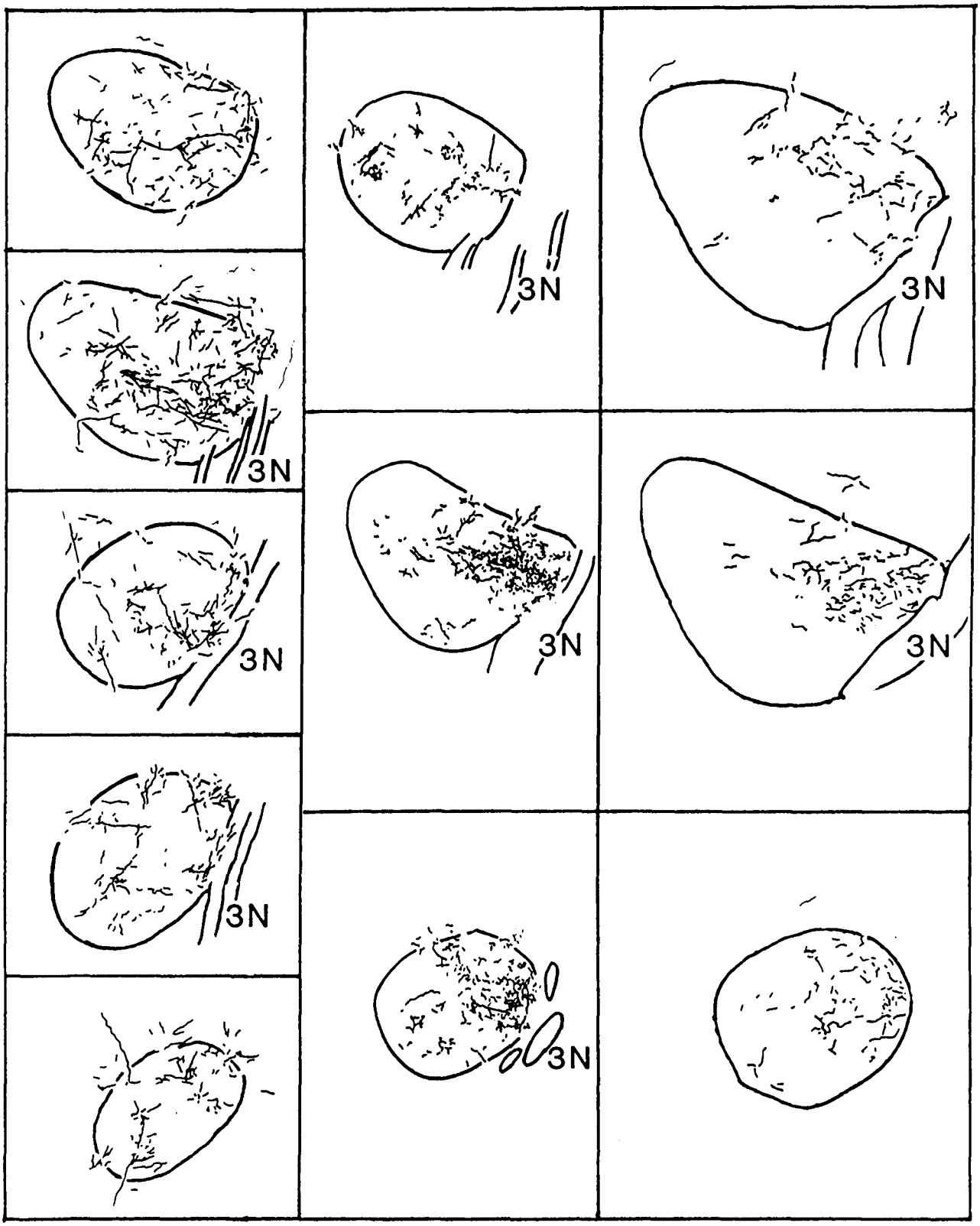

Fig. 6. Topographic refinement of corticorubral projection during postnatal development. Drawings of labeled fibers in and near the red nucleus at PND 8 (A), 13 (B), and 73 (C). Drawings are equally spaced at 250 um, 500 um, 750 um intervals in A, B, and C, respectively. Drawings in A were made from superimposed tracings of three successive sections. Rostral is to the top and caudal is to the bottom. Lateral is to the left and dorsal is to the top.

RN, whereas in older animals there is only a very weak projection from area 6 to this region (see Fig. 4).

Crossed corticorubral projection.

Morphology of axons.

As described previously, a sparse projection was found to the contralateral RN in every animal (Murakami and Higashi, 1988). There was no morphological difference between the crossed and uncrossed ipsilateral axons in the early postnatal days (Fig. 7A). A morphological difference between the corticorubral axons on the two sides becomes evident as development proceeds. Although axons on the contralateral side do not show an increase in the frequency of branching, large diameter axons with a smooth contour that were not observed at earlier stages were found (Fig. 7C) in addition to those with immature morphology (Fig. 7B).

Fig. 8 illustrates the axon fragments that showed the most frequent branching patterns from two kittens at PND 7-8 (A) (#108 and #115) and from one at PND 29 (B) (#46). No distinct difference was noted in the frequency of branching between PND 7-8 and 29. In Fig. 8C the percentage of axon fragments was plotted against the frequency of branching. Open columns represent axon fragment from the animals at PND 7-8 and shaded columns represent those at PND 29. Although the proportion of axons without any branches is smaller and those with only one branch is largest at PND 29, no axon fragment with a branching frequency >4 was found at this stage (compare the graph with Fig. 3C). This may indicate that although there is an increase in frequency of branching with development, it is less than that on the ipsilateral

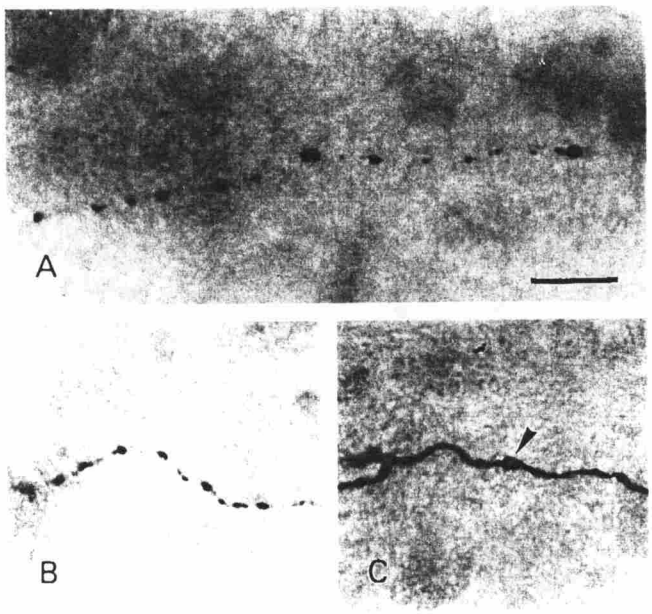

Fig. 7. Light micrographs of PHA-L labeled crossed corticorubral fibers. A: Axon at PND 8. Note that the axon has irregular contour due to numerous swellings and does not show any bifurcation. B and C: Axons at PND 28. Although irregularly shaped axons can still be observed (B), thicker axons with smooth contours appear at this stage (C). An axonal swelling with smooth contours can be seen (arrrowhead). Bar = 10 um.

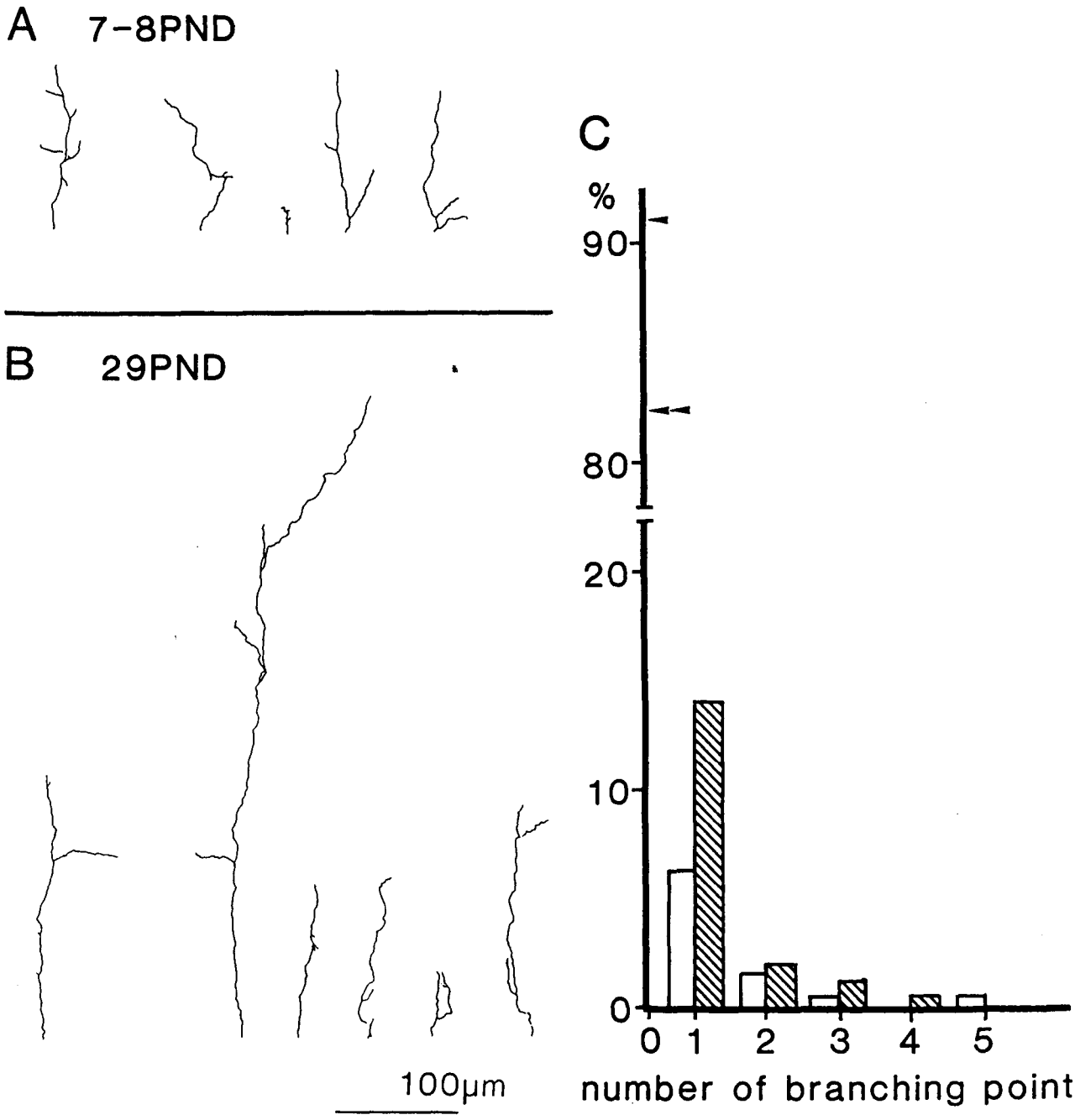

Fig. 8. Comparison of axonal morphology in the contralateral red nucleus between PND 7-8 and 29. Thirty-five frontal sections cut at 50 um were selected from two animals at PND 7 and 8, and every labeled axon situated within the red nucleus in these sections was drawn with the aid of a camera lucida. A: Drawings of five axons having the most frequently branched axonal fragments among 193 drawings from the same animals as in Fig. 3. B: Drawinds of six axons having the most frequently branched axonal fragments among 164 drawings from a PND 29 kitten. C: Explanation of this histogram is in Fig. 3 C legend. Open columns represent data at PND 7-8 and hatched columns at PND 29. Axons without bifurcation are shown by an arrowhead (PND 7-8) or a double arrowhead (PND29).

side. (No statistical difference in frequency distribution of branching frequency was found between the two groups of animals.)

Relationship abundance of crossed corticorubral projection at an early postnatal period.

Although crossed corticorubral axons were observed irrespective of animal age, the contralateral /ipsilateral ratio in the number of labeled axons tended to be larger in young animals. This is indicated in Fig. 9, where the corticorubral axons at PND 13 (A) and PND 52 (B) are compared. The columns on the left indicate sections from the ipsilateral side and columns on the right show sections from the contralateral side. More labeled fibers can be seen at PND 52 than at PND 13 ipsilaterally, while fewer fibers exist on the contralateral side at PND 52.

Since the crossed corticorubral projection was weak, it was impossible to determine whether or not it is topographically organized.

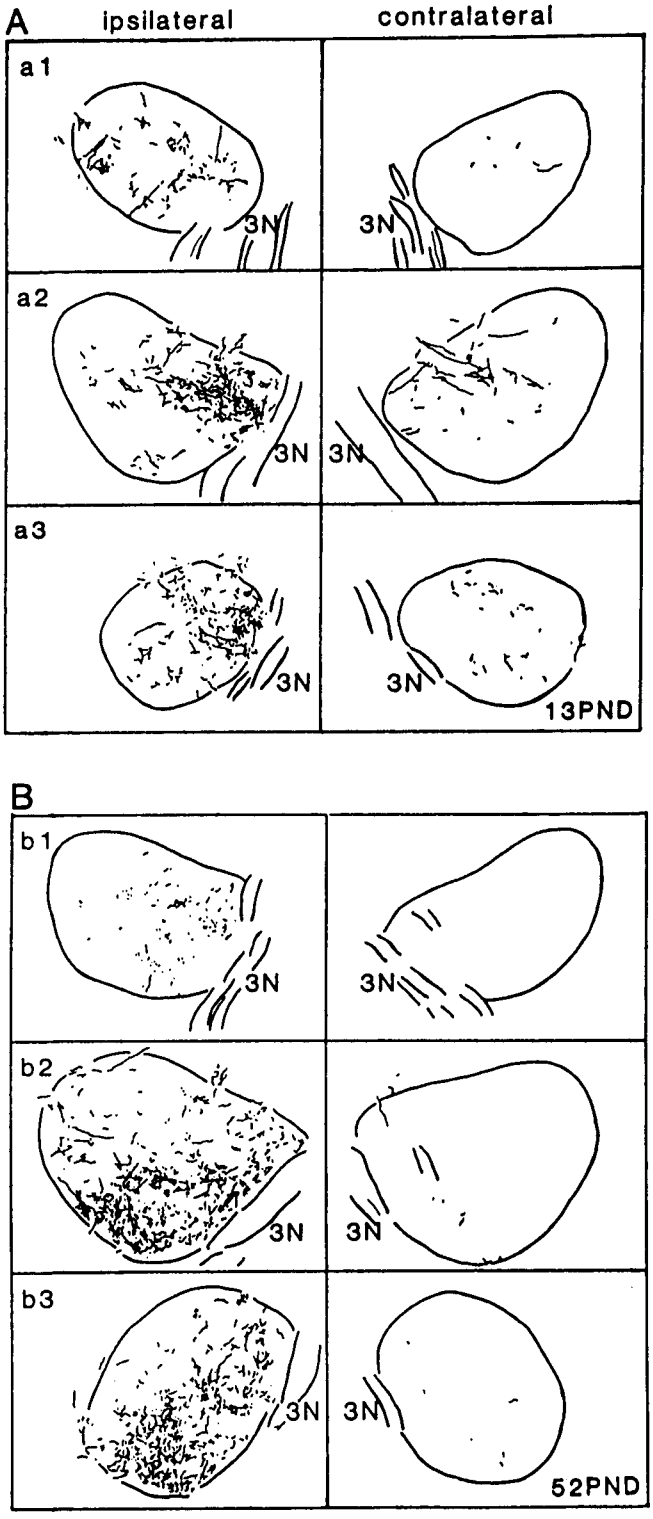

Fig. 9. Developmental change in the ratio of contralateral and ipsilateral corticorubral projection. Equally spaced drawings of labeled fibers in frontal sections of the red nucleus at PND 13 (A) and PND 53 (B). The red nucleus was cut serially at 50 um and every tenth section picked was drawn. This animal (#15) received four cortical injections. Rostral is to the top and caudal is to the bottom. Left row, ipsilateral red nucleus; right, contralateral red nucleus; 3N, oculomotor nerve.

DISCUSSION

The present study demonstrates that corticorubral fibers arrive at the RN at least as early as PND 7. The morphology of fibers at this stage of development differed from that in older animals in several respects; they were straight, branched very in frequently, and were irregular in contour. At PND 7-8, in addition to the fibers observed in the ipsilateral RN, a relatively large number of fibers was seen in the contralateral RN, where virtually no projection exists in adults. Topographic organization that was clear established by PND 73 was not well defined at PND 8.Z

Methodological considerations

It seems well accepted now that the PHA-L method is an excellent technique for tracing axonal projections (Gerfen et al., 1984; Woutelood and Groenewegen, 1985; Murakami and Higashi, 1988). A problem, however, arises when applying this method to a developing animal in which axonal elongation is still occurring. During the survival period following PHA-L injection, growth of axons may occur concurrent with the transport of PHA-L. It is possible that the rate of transport of PHA-L may be unable to catch up with that of axonal elongation due to the relatively slow transport rate of PHA-L. This appears to be unlikely, since even in the animal with the shortest survival period, growth-cone-like axonal endings were frequently observed in the RN.

A shorter survival period for animals at the early postnatal days (see Table 1) would be in sufficient for transport of PHA-L to the distal end of corticorubral axons. Thus the observed poor arbolization of axons at PND 7-8 could be ascribed to insufficient PHA-L transport due to the short survival period. However, this is unlikely, because even in the animal with 5 days' survival time PHA-L was transported down to the cervical spinal cord (not shown). Furthermore, we could follow extremely fine axons from one end of the tissue section to the other or to their endings, mostly growth cones, in such an animal.

Injections were very small compared to the size of the sensori-motor area with the PHA-L method and only a subpopulation of cortical neurons were labeled in each experiment. Since axonal morphology might vary among neurons were carried out using animals with injections into the lateral sigmoid gyrus. Further, we made an effort to inject the tracer into identical loci of the cortex in animals of the same group to minimize the possible variation of axonal morphology derived from different neuronal subpopulation. The fact that no difference was noted in axonal morphology between group I and group II animals of similar ages (not shown) suggests that axons of the neurons indifferent cortical areas are similar in morphology.

Projections to the red nucleus and other midbrain areas

Projections to the RN and other midbrain areas revealed in the present study are in good agreement with the results obtained in adult monkey (Hartmann-von Monakov et al., 1979) and cat (Nakamura et al., 1983) with autoradiography . Robinson et al. (1987) made multiple injections of WGA-HRP into the pericruciate cortex of adult cats and observed relatively weak labeling of the caudal magnocellular RN. Similar results were reported by Sadun who used degeneration method (Sadun, 1975). However, in our study the projection from the medial part of the posterior sigmoid gyrus was even denser in the magnocellular region than in the parvocellular region (Fig. 4B). Since 1) corticorubral fibers course rostrocaudally and 2) HRP or degeneration methods do not allow the discrimination of passing fibers from axon terminals, it is likely that in the earlier studies some passing fibers were mistaken for termination zones. The possibility that the projection pattern along rostrocaudal axis undergoes a developmental redistribution seems unlikely, because an experiment using an adult cat did not support the earlier results either (unpublished observation).

Topographic organization of corticorubral projection

The topography of corticorubral projection has been extensively studied in adult cats both anatomically (Rinvik and Walberg, 1963; Mabuchi and Kusama, 1966) as well as electrophysiologically Padel et al., 1973; Jeneskog and Padel, 1983). We confirmed the reported topography, expect in animals studied in the earliest postnatal days, using PHA-L, which is selectively taken up only from cell body and dendrites of the neurons in well-defined injection sites. In this respect our results are probably more reliable than those of others, since with these other methods lesions (Rinvik and Walberg, 1963; Mabuchi and Kusama, 1966) or stimulation (Padel et al., 1973; Jeneskog and Padel, 1983) of passing fibers cannot be avoided.

In agreement with some studies (Rinvik and Walberg, 1963; Mabuchi and Kusama, 1966; Padel et al., 1973; Jeneskog and Padel, 1983) but in disagreement with others (Giuffrida et al., 1983; Futami et al., 1986), we found only weak corticorubral projections from area 6. The neurons in this area sent diffuse projections to the midbrain structures surrounding the RN and the nucleus of Darkshevich, but axonal morphology that is typical projection area was not observed (Fig. 4C). Probably in physiological studies in which corticofugal cells were antidromically activated (Giuffrida et al., 1983; Futami et al., 1986), stimulation with the electrode placed in the RN activated the axons of area 6 neurons projecting to perirubral areas. Pompeiano and Brodal (1957) found using retrograde degeneration that rubrospinal fibers to the cervical cord originate from the dorsal and dorsomedial part, and those to the lumbosacral cord from the ventrolateral parts of the caudal three-fourths of the RN in 6-12-day-old kittens. Taken together with our results, it seems likely that the somatotopical arrangements of the cortico-rubro-spinal system is already established soon after birth, although electrophysiological experiments are necessary to confirm the establishment of functional connections.

Development of ipsilateral corticorubral axons

Corticospinal projections in the cat develop prenatally, but corticotectal, corticopontine and corticobulbar projections seem to develop perinatally (Wise et al., 1977). It is very likely that the corticorubral projection also develops perinatally. Therefore, the axons observed in the RN at PND 7-8 are probably newly arrived. Failure to label many axons at this stage can, at least in part, be ascribed to a small number of axons having arrived at the RN.

Branching of axons.

In the present study it was not possible to reconstruct entire axons due to the presence of numerous labeled axons in each section. Only the morphology of axonal fragments within 50-um-thick sections was revealed. Therefore, the data shown in Fig. 3 and 8 represent the morphology of axons cut into pieces. This situation would reduce the actual complexity of the axonal arbor and would produce many non-branching axons. In spite of this situation, differences in frequency of branching of ipsilateral axons between a late postnatal stage and an early stage were evident. Axons at PND 28 showed, in addition to a general tendency to branch more frequently, axonal arbors never observed at PND 7-8 (Fig. 3B). This suggests that the arborization of individual axons at PND 28 is actually more complex than that at PND 7-8. Considering that axons were viewed in frontal planes and that stem axons course rostrocaudally (data not shown), the results shown in Fig. 3 might be at least partly attributable to the formation of side branches that run mediolaterally. In accord with our results Sretavan and Shatz (1986) demonstrated that axons, possibly derived from the eye, showing simple unbranched morphology in the lateral geniculate nucleus at early embryonic days come to have complex arbors as development proceeds. Recent studies on corticopontine (O'Leary and Terashima, 1988) and retinotectal projections (Nakamura and O'Leary, 1989) with DiI and DiO staining also support the present view.

Axonal swellings.

Axonal swellings, which are observed throughout the entire course of axons at early stages of postnatal development, decrease in number as development proceeds, and at later stages their number increases again. However, the axonal swellings observed at PND7-8 were generally small in size, rough in shape and were connected by extremely thin intervaricosity axonal segments. By contrast, those observed later than PND 28 typically showed morphology with smooth contours and larger diameter sometimes exceeding 2 um. Since increase in axon diameter occurs in parallel with a reduction in the number of axonal swellings, this apparent reduction might to a certain extent be attributed to the growth of the intervaricosity segment.

Axonal swellings have been correlated with synaptic boutons in electron microscopic studies (Lagerback et al., 1981; Conradi et al., 1983; Wouterlood and Groenewegen, 1985). The morphological similarities of the axonal swellings observed here at later stages of development to those observed in other studies suggest that they are probably synaptic boutons. A large number of swellings possessing a different morphology, i.e., small and rugged were observed at the earliest postnatal stages. It would be quite interesting to know whether such structures also correspond to synaptic terminals or whether they have completely different ultrastructural correlates. We are currently performing electrophysiological studies to determine whether functional connections are already established at this stage.

Topographic projection.

No clear topographic projection was observed in the animals at PND 7 and 8. Since the brain is smaller in younger animals, the size of the injection relative to that of the brain could be larger in younger animals than in older ones even if absolute injection size remained constant. Therefore, less defined topography in very young animals might be due to differences in brain size. We think this unlikely, because 1) the PHA-L injection site covered only

a very small portion of the whole pericruciate cortex and its size did not correlate with animal age and 2) a slight difference in position of the injection site within the presently studied three cortical areas provided no noticeable difference in the area of projection. Furthermore, 3) there appears to be an abrupt change in topographical projection from PND 7-8 to PND 13, although there was no sudden increase in brain size from PND 7-8 to PNd 13.

Topographic refinement during development ha been demonstrated in many other systems (see Udin and Fawcett, 1988 for review). Two possible mechanisms that could underlie topographic refinement have been considered: one is cell death and other is elaboration and/or collateral elimination (Cowan et al., 1984). We have no evidence either to reject or support cell death of corticorubral cells. However, we favor the later mechanism, because 1) development of topographic projection occurred in parallel with an increase in frequency of branching, and 2) elimination of transient corticocortical or corticofugal projections during postnatal development has in many instances been ascribed to collateral elimination (e.g., O'Leary et al., 1981; Innocenti and Clarke, 1984; Tolbert and Panneton, 91984; see Innocenti, 1988 for review).

Crossed projection to the contralateral red nucleus

The present study confirmed our previous findings that there are weak crossed corticorubral projections in 1-month-old kittens (Murakami and Higashi, 1988), but considerable crossed projections exist soon after birth (see also Leonard and Goldberger, 1987). Kosar et al. (1985) did not find appreciable bilateral corticorubral connections in 7-day-old to 1-month-old kittens. In those experiments, the small injections of HRP (30%, 0.1 ul x 3) applied were probably insufficient to label the small number of crossed fibers. The crossed corticorubral fibers are probably eventually eliminated, since no crossed projection has been reported in adult cats (Rinvik and Walberg, 1963; Fujito et al., 1983; Villablanca et al., 1988).

Morphological differences between crossed and uncrossed corticorubral projections become obvious as development proceeds; crossed fibers fail to develop complex axonal arbors, whereas uncrossed ones develop axonal arbors. This could contribute establishment of laterality in corticorubral projection. Sretavan and Shatz (1986) suggested that similar cellular mechanisms for segregation of eye-specific layers within the lateral geniculate nucleus of the cat, i.e., the elaboration of terminal arbors and the elimination of side branches of retinogeniculate axons. However, the exact origin of terminal arbors was unknown in their study, because the axons were labeled by HRP injection into the optic tract near lateral geniculate nucleus.

The elimination of crossed axons (which could be caused by either cell death or collateral elimination) may take place, possibly because poorly developed axon terminals are disadvantageous for the uptake of putative trophic factor(s)or synaptic transmission (Hebb, 1949; Stent, 1973; Cowan et al., 1984). Alternatively, lack of axonal arbors may be an early sign of axonal elimination, which could be caused by other mechanisms. In this context it would be quite interesting to know whether the crossed corticorubral fibers from synapses or not. Intracellular recordings from the kitten RN cells suggest that they form functional synapses (Song et al., in press).

Early hemispherectomy causes a significant and functional crossed corticorubral projection (Nah and Leong, 1976a, b; Tsukahara and Fujito, 1981; Villablanca et al., 1982, 1988; Tsukahara et al., 1983; Naus et al., 1985, Kosar et al., 1985; Leonard and Goldberger, 1987; Murakami and Higashi, 1988). We previously showed that the lesion-induced crossed projections cannot be explained by the retention of early crossed projections that in normal animals eliminated (Murakami and Higashi, 1988). Our preliminary study indicates that the crossed corticorubral fibers in hemispherectomized animals have complex axonal arbors similar to those projecting to the ipsilateral RN (Murakami et al., in press), suggesting that

cortical lesions induce the proliferation of axon terminals. Evidence supportive of this idea has been obtained in a recent study on the interpositorubral projection of the kitten (Song and Murakami, in press).

Competitive interactions between convergent inputs has been reported as a possible mechanism for the growth of projections after denervation (Guillery, 1988). There is a wealth of evidence suggesting that activity-dependent mechanisms such as those proposed by Hebb (Hebb, 1949) underlie the interactions (Meyer, 1982; Reh and Constantine-Paton, 1985; Stryker and Harris, 1986; Sretavan et al., 1988; Shatz and Stryker, 1988). Interestingly, several lines of evidence suggest the formation of new synapses in cats in which classical conditioning was established by pairing stimulation of the cerebral peduncle and skin of the forelimb (Murakami et al., 1987; Oda et al., 1990). Thus, one attractive hypothesis is that conjunctive activity within corticorubral synapses and in those conveying peripheral inputs strengthens these synapses but eliminates those without conjunctive activity, through Hebb-type mechanism (Hebb, 1949; Stent, 1973).

To summarize, postnatal development of the corticorubral projection seems to be associated with an elaboration of axonal arbors. However, although there are substantial number of incorrectly projecting axons early in development, their number is dramatically reduced, as development proceeds, probably due, largely, to a failure to develop complex arborizations.

Chapter3.

Lesion-induced establishment of the crossed corticorubral projections in kittens is associated with axonal proliferation within the red nucleus and topographic refinement

Abstract

The aberrant crossed corticorubral projection of the cats, which is very weak compared to the uncrossed one at about one month postnatal, becomes pronounced following unilateral lesions of the sensorimotor cortex. In order to determine whether or not terminal proliferation of preexisting axons underlie this enlargement, the morphological changes of the crossed axons were examined, using the anterograde tracer *Phaseolus vulgaris*-leucoagglutinin (PHA-L). The crossed corticorubral axons in normal kittens were mostly simple in morphology with infrequent branching and did not often exhibit growth-cone like axonal endings at one month postnatal. Two to five days after unilateral lesions of the sensorimotor cortex placed at this age, the axons were as simple as those in normal animals but ended in growth cones more frequently. Seven to ten days post-lesion, the axons often bore side branches which ended in growth cones. Two to three weeks post-lesion axons with sprays of finger-like fine sprouts occurred throughout the projection zone. There was no clear topography for the crossed projection in normal animals, but at one to two weeks post-lesion the axons started to show a certain amount of localization in the regions of the red nucleus which corresponded to the densely innervated region on the ipsilateral side. The topography of the crossed projections roughly mirrors that of the ipsilateral projection at about one month post-lesion. Thus, the lesions of the sensorimotor cortex induce substantial growth and proliferation of the crossed corticorubral axons. The post-lesion changes in axonal morphology and topographic refinement are reminiscent of developmental events. It is likely that the lesions permit the crossed axons, which normally fail to develop, to develop as do the uncrossed ones.

INTRODUCTION

Although it has been well established that partial denervation causes axonal sprouting in the central nervous system of adult mammals, it appears far more prominent when lesions are made in neonates (see Cotman et al., 1981; Lund, 1978; Tsukahara, 1981 for reviews). This could indicate that maturing axons lose their capacity of forming new connections. However, findings of exuberant projections in developing brains (see Purves and Lichtman, 1980; Shatz and Sretavan, 1986; Villablanca et al., 1982 for reviews) led to the suggestion that aberrant projections induced by early brain lesions resulted from arrested retraction of exuberant projections rather than growth of axons(Hubel et al., 1977; Land and Lund, 1979; McLoon, 1982). In support of this idea anatomical studies in rodent retino-collicular projections with retrograde tracers, for example, suggested that early removal of one eye results in rescue of many ipsilaterally (aberrantly) projecting cells from naturally occurring death (Cotman et al., 1984; Insausti et al., 1984; Jeffery and Perry, 1982).

The studies of the corticorubral and interpositorubral systems of the rat and cat, however, demonstrated that aberrant projections are markedly enlarged by early unilateral lesions of the cerebral cortex (McLoon, 1982; Murakami et al., 1991) or the interpositus nucleus (Song and Murakami, 1990). These results suggest that axonal growth, rather than the arrested retraction of axons, is responsible for lesion-induced plasticity in developing animals. However, these studies failed to show axons which are in the course of sprouting or proliferation, the most convincing evidence for occurrence of axonal sprouting. Further, it has remained unclarified whether or not such axonal growth repeats events occuring during development of normal projections.

The present study was attempted to obtain direct morphological evidence for axonal sprouting by examining the changes in axonal

morphology after partial denervation. The corticorubral projection was chosen for the analysis. This projection is uncrossed in adult cats (Fujito et al., 1983Rinvik and Walberg,1963), but a crossed one is also present in kittens (Higashi et al., 1990; Murakami and Higashi, 1988). Unilateral lesions of the sensorimotor cortex in kittens, but not in adults, results in establishment of the crossed projection (Murakami and Higashi, 1988; Villablanca et al., 1982) (but see Villablanca et al., 1988). We labeled the crossed corticorubral axons with *Phaseolus-vulgaris* leucoagglutinin (PHA-L) and observed the changes in morphology of the axons following unilateral lesions of the sensorimotor cortex, at varying post-lesion period. The results show that the crossed axons change progressively in morphology to eventually form complex axonal arbors, indicating that terminal proliferation actually takes place. Interestingly, the crossed projections which are normally disorganized gradually form topographic order which mirrors the topography of ipsilateral projections, after the lesions.

MATERIALS AND METHODS

A total of 30 kittens were used. Eighteen of them were obtained from Hyogo Prefectural Administration Center for Animals and their ages were estimated from the day of the teeth eruption. Others were from breeding colonies mostly in Abrahi Laboratories of Shionogi Company LTD. and the day of their birth was termed as postnatal day (PND) 0.

Eighteen of the kittens underwent ablation of the left sensorimotor cortex at PND 24 to 39 under anesthesia with Nembutal (25mg/kg. i.p.) supplemented with Ketamin hydrochloride when required, and were perfused transcardially with a fixative (see below) 2 to 47 days later as previously described (Murakami and Higashi; 1988) (see Table I). Others were intact controls 21 - 52 PND of age (Table II). In most of the normal and lesioned kittens PHA-L injection was made

into the lateral sigmoid gyrus (forelimb area (Kusama et al., 1966; Padel et al., 1973; Sachs et al., 1963) (group I) or the medial part of the posterior sigmoid gyrus (hindlimb area (Kusama et al., 1966; Padel et al., 1973; Sachs et al., 1963)) (group II). In the animals of

Table I. Summary of experimental protocol for each lesioned animal included in the present study. l.s.g.: lateral sigmoid gyrus. m.p.s.g.: medial part of posterior sigmoid gyrus. * age estimated by teeth eruption.

Animal number	PHA-L injection	Plane of section	Age of lesion	Survival days
# 263	l.s.g.	horizontal	30 PND*	2 days
# 48	multiple	frontal	36 PND	3 days
# 261	l.s.g.	horizontal	26 PND*	5 days
# 47	multiple	frontal	29 PND	7 days
# 137	l.s.g.	frontal	29 PND*	7 days
# 266	l.s.g.	horizontal	28 PND*	7 days
# 91	m.s.g.	frontal	33 PND	10 days
# 32	multiple	frontal	32 PND	14 days
# 267	l.s.g.	horizontal	30 PND*	14 days
# 37	l.s.g.	frontal	24 PND*	21 days
# 36	multiple	frontal	24 PND*	24 days
# 69	m.p.s.g.	frontal	31 PND	27 days
# 138	l.s.g.	horizontal	28 PND*	36 days
# 82	l.s.g.	frontal	32 PND*	42 days
# 258	l.s.g.	frontal	39 PND	42 days
# 4	l.s.g.	horizontal	24 PND	45 days
# 87	m.p.s.g.	frontal	31 PND*	46 days
# 140	l.s.g.	horizontal	34 PND	47 days

Table II. Summary of the experimental protocol for each normal kitten included in the present study. See legends to Table I for abbreviation and the meaning of *.

Animal number	PHA-L injection	Plane of section	Age at sacrifice
# 135	l.s.g.	horizontal	14 PND
# 136	m.p.s.g.	frontal	14 PND
# 103	l.s.g.	horizontal	17 PND*
# 98	l.s.g.	frontal	21 PND*
# 97	l.s.g.	horizontal	24 PND*
# 99	m.p.s.g.	horizontal	24 PND*
# 78	l.s.g.	frontal	28 PND*
# 46	multiple	frontal	29 PND*
# 83	m.p.s.g.	frontal	41 PND*
# 6	l.s.g.	horizontal	48 PND*
# 40	m.p.s.g.	frontal	52 PND
# 15	multiple	frontal	52 PND

groups I and II, two injections were usually made within a distance of 2 mm. Care was taken for making injections into identical loci of the cortex particularly in group I in which injections were attempted near the lateral end of the cruciate sulcus. In the remaining animals (group III), three to four injections were made, two into the lateral sigmoid gyrus and others into the lateral part of the pre- and postcruciate sulcus. In some animals, antidromic field potentials elicited by stimulation of the corticospinal tract and the red nucleus (RN) were utilized for making accurate injections.

Procedures of PHA-L injections and visualization have been previously described (Higashi et al., 1990; Murakami and Higashi,1988). Briefly, PHA-L (Vector labs.) was injected iontophoretically into the right sensorimotor cortex of anesthetized animals with a glass micropipette. Five microampere-positive currents (7.5 sec on and 7.5 sec off) were injected for 15 minutes in each injection. After 7 to 17 days' (mostly 14 days) survival, the animals were perfused with a mixture of 2.5 % glutaraldehyde and 2 % paraformaldehyde or 4 % paraformaldehyde under deep anesthesia with Nembutal. Brain stem containing the RNs was cut either frontally or near horizontally at 50 μm with a Microslicer (Dosaka EM, Kyoto) or a freezing microtome. The cerebral cortex including the injection site was cut parasagittally at 100 μm with a freezing microtome. All the sections were incubated in 1/2000 goat anti-PHA serum (Vector labs.) overnight, reacted with biotinylated 2nd antibody and processed with the avidin-biotin-peroxidase complex method. Most of the sections containing the RN were counterstained with Methylene Blue. Others were post-fixed with O_sO_4, and flat-embedded in Epon. All of the cortical sections were counterstained with Methylene Blue.

Labeled axons were observed under a light microscope and the axons in part of the sections were photographed. The axons were drawn, with a drawing tube attached to the light microscope, at x40 to x100 magnification to study the topography, at x200 for reconstruction of axonal trajectories, at x400

for analyzing axonal branching (see below) and at x1000 for reconstruction of axonal arbors. Nomarski optics (Olympus) were used as required. A gelatin filter (Kodak Wratten filter No.45A) was used to facilitate drawings and photography.

Two animals, one normal (#103, see Table II) and one lesioned (#138, see Table I), were chosen for comparison of local axonal branching. These animals were chosen, because injection sites were very close to each other between these two animals and individual fibers were clearly labeled. In the lesioned animal, a moderate number of axons were labeled so that it was possible to distinguish each axon. From the normal animal, every third section was sampled over a ventrodorsal distance of 750 to 800 μm. From the lesioned animal every fourth section was sampled over a ventrodorsal distance of 1200 to 1250 μm (the difference in the distance is due to different sizes of the brains between the normal and the lesioned animal). Every axonal fragment in the RN contralateral to the injection was drawn (the terms "ipsilateral" and "contralateral" refer to the injection site throughout the text). A total of 205 and 247 axonal fragments were drawn from the normal and the lesioned animal, respectively, and the number of branching point in each axonal fragment was counted.

RESULTS

Lesion and injection sites

The lesions of the sensorimotor cortex were centered on area 4 and included areas 6, 3a, 3b, 1, 2 and 5(Hassler and Muhs- Clement, 1964). No damage was observed in either the dorsal thalamus or the right sensorimotor cortex.

All injection sites were found within the pericruciate cortex. Cortical neurons including layer V pyramidal cells were heavily stained as shown

previously (Higashi et al., 1990; Murakami and Higashi, 1988) No difference was found in the size of injection between normal and lesioned animals.

Course of axons projecting to the contralateral red nucleus

Both in normal and lesioned animals there appeared to be three different pathways for the axons to reach the contralateral nucleus. 1) Many descending axons gave off collaterals at a right angle to the corticospinal tract at the level of the rostral part of the RN (Fig. 1A). Observations of frontally-sectioned samples suggest that some of such axons project to the contralateral RN, while most of them project to the ipsilateral RN; it was sometimes possible to follow axons from the tract, coursing ventrally to the ipsilateral RN towards the contralateral nucleus (Fig.1B). 2) Numerous fibers were also found to deflect from the tract more rostrally and course towards the ipsilateral RN. Some fibers further deflected towards the midline and appeared to have projected to the contralateral nucleus as judged by observations of horizontally-sectioned brains. Reconstruction of an axon from serial sections demonstrate that at least some of such axons do project to the contralateral RN (Fig. 1C). 3) The third pathway of the axons appeared to be one via the ipsilateral RN (see Fig. 1D).

All axons crossing the midline were very thin in diameter (< 1 μm) in normal animals. By contrast, some of these axons were thick (1.5 μm <) in lesioned animals with survivals of ≤ 1 week (Fig. 1E). More axons appeared to cross the midline in lesioned animals compared to normal ones.

Morphology of crossed corticorubral axons

i) normal animals

In agreement with our previous study (Higashi et al., 1990), the axons in the contralateral RN of normal animals were mostly straight and did not show extensive branchings along a long course both in frontal and horizontal

sections (Fig. 2A). Smooth-surfaced axonal swellings, though present, were not encountered so often. The axonal endings exhibited slender and blunt (Fig. 2B-1), bulbous structures (Fig. 2B-2), or growth cone-like (Fig. 2B-3) structures. These features did not show noticeable changes with increasing ages of animals, although in younger animals growth cone-like axonal endings were more frequently encountered and the axons were often rugged in contours and thin compared to older ones.

ii) Lesioned animals

In the kittens which received neonatal sensorimotor-cortex ablation, strengthening of the crossed corticorubral projections was observed as reported previously (Murakami and Higashi, 1988).

The crossed axons progressively changed in morphology with increasing survival periods after the lesion. Two to five days after the lesions the morphology of axons was not substantially different from that of normal animals. But the axons ending as growth cones were more frequently encountered than in normal animals (Fig. 2C). The axons at this stage resembled those in the ipsilateral RN in newborn (≤ 1 week) kittens.

Seven to ten days after the lesions, the axons were still largely straight and ended as growth cones quite often. Axonal swellings with a few fine sprouts were occasionally observed (Fig. 2D). A notable feature at this stage was that the axons often bore short side branches which also ended in growth cones (Fig. 2E and Fig. 2F). Complex axonal arbors were not found, however. The number of the labeled axons relative to the uncrossed ones appeared to be appreciably higher than that in normal animals.

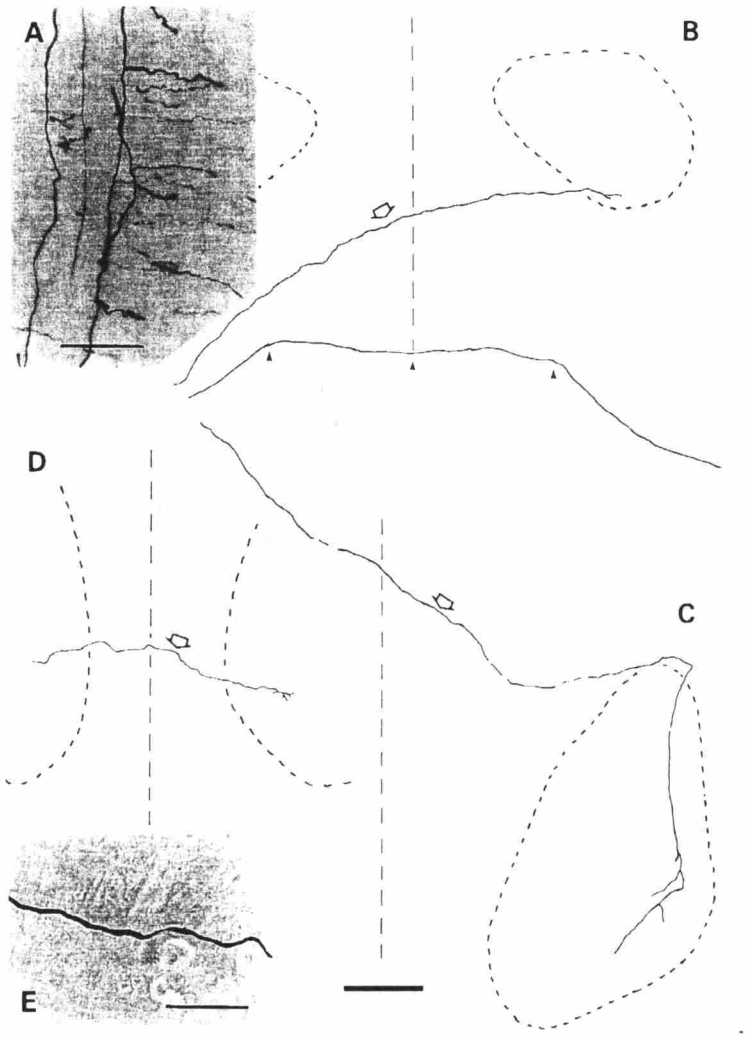

Fig.1. Course of axons to the red nucleus. (A) A photomicrograph of corticofugal axons at the cerebral peduncle yielding axon collaterals at a right angle. This was observed at the level of the rostral half of the red nucleus. A horizontal section. Lateral is to the left. Bar = 50 μm. (B) A drawing of a corticorubral axon (open arrow) coursing beneath the ipsilateral red nucleus towards the contralateral red nucleus. A partial reconstruction from 4 frontal sections. Upper is dorsal. (C) A drawing of an axon (open arrow) leaving the corticospinal tract rostrally to the red nucleus and end in the contralateral red nucleus. A partial reconstruction from 30 horizontal sections. The axon was discontinuously labeled but was assumed to be the same axon, since no other labeled axon was found nearby. (D) A drawing of an axon (arrow) coursing through the ipsilateral red nucleus. A partial reconstruction from 3 horizontal sections. In C and D upper is rostral. The continuous line pointed by arrow heads represent ventral border of the brain. Bar in C is 500 μm and applies to B through D. (E) A photomicrograph of an axon coursing between the ipsi- and contralateral red nuclei. Seven days post-lesion. Bar = 20 μm.

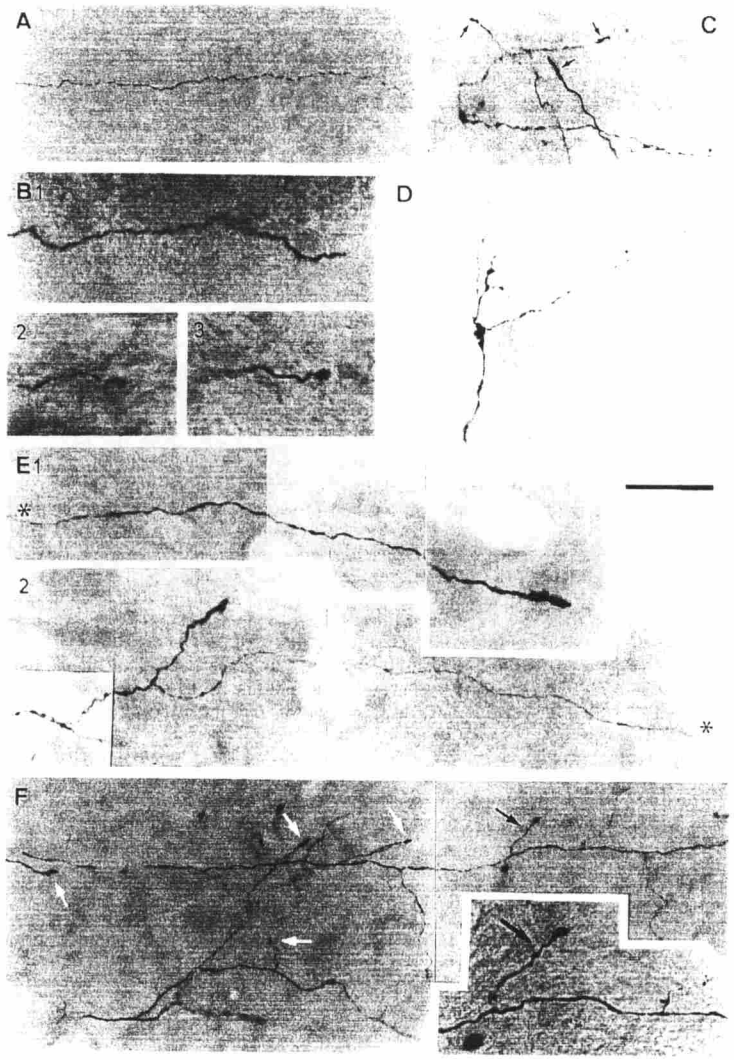

Fig.2. Photomicrographs of crossed corticorubral axons in normal and lesioned kittens. (A,B) normal kittens. (A) an axon coursing without any bifurcation. (B) axons with slender (B1), bulbous (B2) and growth-cone like (B3) endings. (C) crossed axons two days after lesion. Note axons frequently end as growth cones (arrows). (D - F) crossed axons seven days post-lesion. (D) an irregularly shaped axonal swelling with a few sprouts. (E) growth cones arising from a side branch. The left end of Figure 4E1 continues to the right end of E2 (asterisks). Note the primary side branch also ends as a growth cone. In F many growth cones arising from side branches (white arrows) can be seen. The growth cone pointed by the black arrow is shown in the inset at a higher magnification. Bar = 50 μm in A and F, and 20 μm in B, D ,E and the inset of F.

Two to three weeks after the lesion somewhat complex axonal arbors with many fine sprouts were encountered (Fig. 3A and B, see Fig. 4A also), though infrequently. Such sprouts usually emerged from a swelling with irregular shape and often ended as growth cones (Fig. 3A and Fig. 4A arrows). Axons with short side-branches were also found.

About one month post-lesion, axons with a spray of finger-like fine sprouts were observed throughout the RN (Fig. 3C and D, see Fig. 4B also) and axons ending as growth cones decreased in number. The axons were tortuous and very often showed branchings. Some of the axons were very thick (1 μm <) (Fig. 3E, inset).

Morphological complexity further proceeded with longer survival periods and smooth-surfaced axonal swellings, which may possibly correspond to synaptic boutons were then frequently encountered (Fig. 3E). Growth cone-like axonal endings were still observed (Fig. 3F), although less frequently. The morphology of the axons was indistinguishable from that of uncrossed ones at this stage (see ref. Higashi et al., 1990).

iii) Comparison of local axonal branchings

Drawings in Fig. 5 are axonal fragments showing the most frequent branchings among those used for the analysis (see Materials and Methods). It is obvious that axons in the normal animal (Fig. 5A) have much fewer branches and lack terminal-like arbors compared with those in the lesioned one (Fig. 5B).

Considering that each drawing represent axonal fragments contained in 50 μm-thick sections, the results indicate that the axonal branching proceeded locally withing the RN following ablation of the sensorimotor cortex.

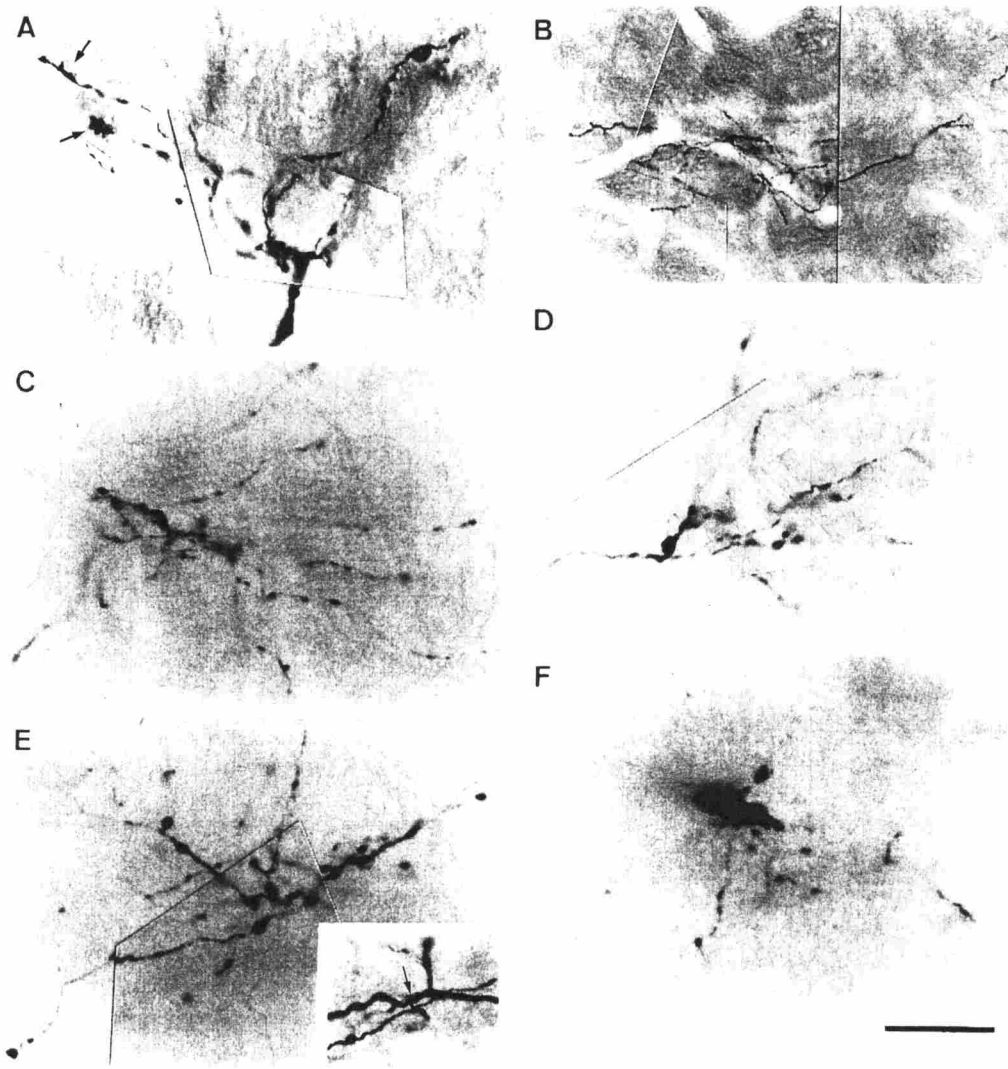

Fig. 3. Axonal arbors formed by crossed corticorubral axons of lesioned animals. (A) a thick axon forming many fine sprouts which end as growth cones (arrows). See Fig. 4A also. Fourteen days post-lesion. A Nomarski optics was used. (B) an axonal arbor in a lesioned animal 21 days post-lesion. (C) an axonal ending with numerous fine sprouts. Thirty-six days post-lesion. (D) finger-like fine sprouts from a swelling. Thirty-six days post-lesion. See Fig. 4B also. (E) an axonal arbor with many smooth-surfaced swellings and fine sprouts. Forty-two days post-lesion. Inset, a thick axon in the red nucleus. (F) a growth cone with many fine sprouts. Forty-two days post-lesion. Bar = 20 μm in C, D and E, 50 μm in B and 16 μm in F.

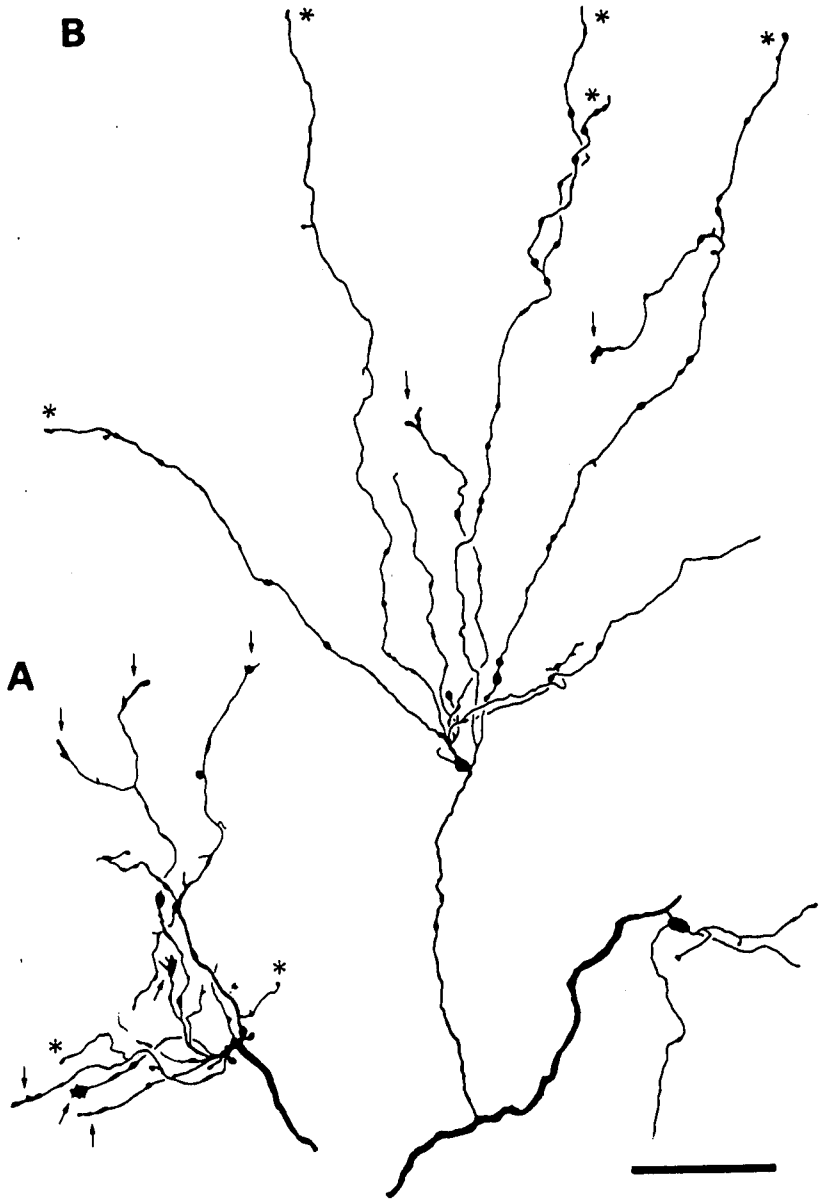

Fig. 4 Camera lucida drawings of axonal arbors in the contralateral RN of lesioned animals. (A) a partial reconstruction of the axon shown in Fig. 3A. Fourteen days post-lesion. Note that most of the endings of axonal sprouts are crenulated or irregularly shaped (arrows), and thus may possibly be growth cones. Some axonal endings have spherical or oval shape (asterisks). (B) a finger-like axonal sprouts arising from a side branch. This is a partial reconstruction of the axon shown Fig. 3D and was drawn from three horizontal sections. Thirty-six days post-lesion. Unlike the axon terminal shown in A, most of the sprouts end as smooth-surfaced swellings (asterisks). Only two of them end as growth ones (arrow).

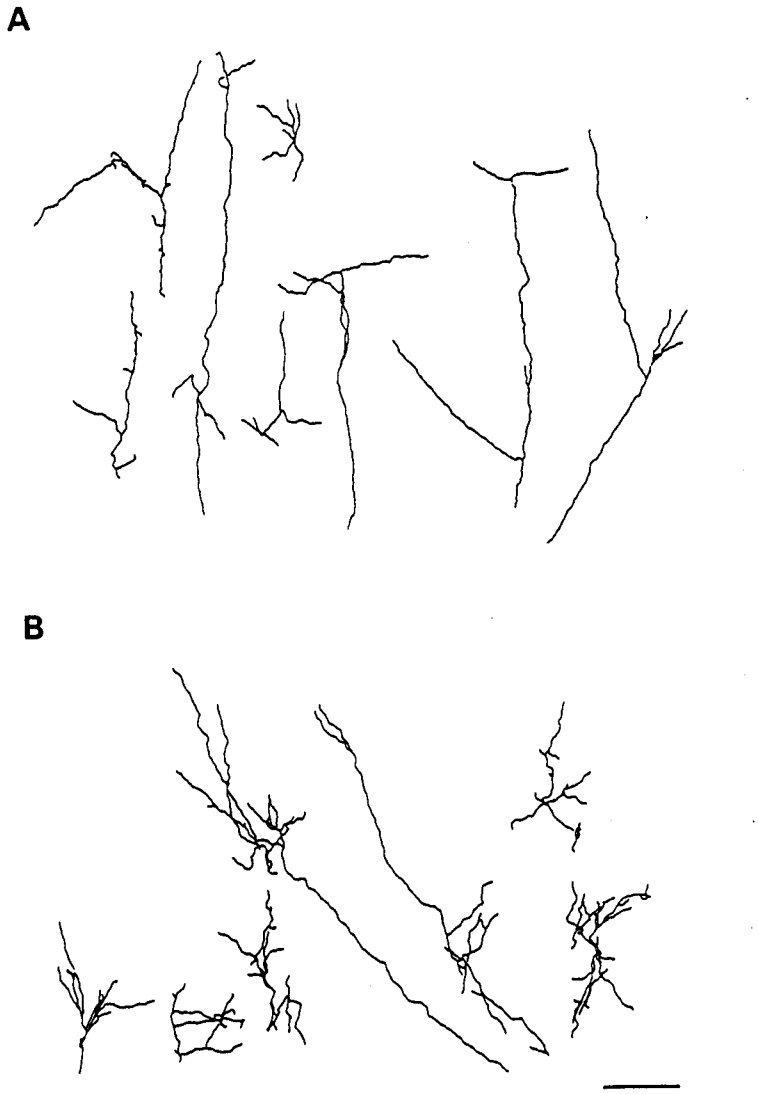

Fig. 5 Comparison of axonal branchings in the crossed corticorubral axons between the normal (A) and lesioned animals (B). (A) a total of 205 axonal fragments were drawn from 50 μm thick horizontal sections of a PND 21 normal animal with a camera lucida attached to a light microscope and the eight most frequently branching axons are shown. (B) a total of 247 axonal fragments were similarly drawn from a lesioned animal (36 days post-lesion) and the seven most frequently branching axons are shown. Bar = 100 μm.

Topography of the crossed corticorubral projection

i) Topography in normal animals

Shown in Fig. 6 are drawings of the labeled fibers in horizontal sections of the RNs of a group I normal animal. As exemplified here, no obvious localization of fibers can be noted in the contralateral RN (Fig. 6B), while a clear localization of axons is found in the ipsilateral RN of normal animals (Fig. 6A). Similar results were obtained in animals whose RNs were cut at frontal planes (not shown).

ii) Post-lesion refinement of topography

In lesioned animals with survival periods of two to five days there was no obvious topography.

After one to two weeks' survival, crossed axons appeared to show certain localization in the RN. Axons were often labeled in the region of the contralateral RN which corresponds to the heavily innervated regions in the ipsilateral RN, but a substantial number of axons were also labeled in other regions. In the example shown in Fig. 7 (#267), the axons were most densely labeled in the rostromedial RN on both sides, but the labeling of the caudal RN was notable on the contralateral RN. Growth cone-like axonal endings were observed irrespective of the location of the axons. Three weeks after the lesions crossed axons appeared to be more localized in topographically appropriate regions.

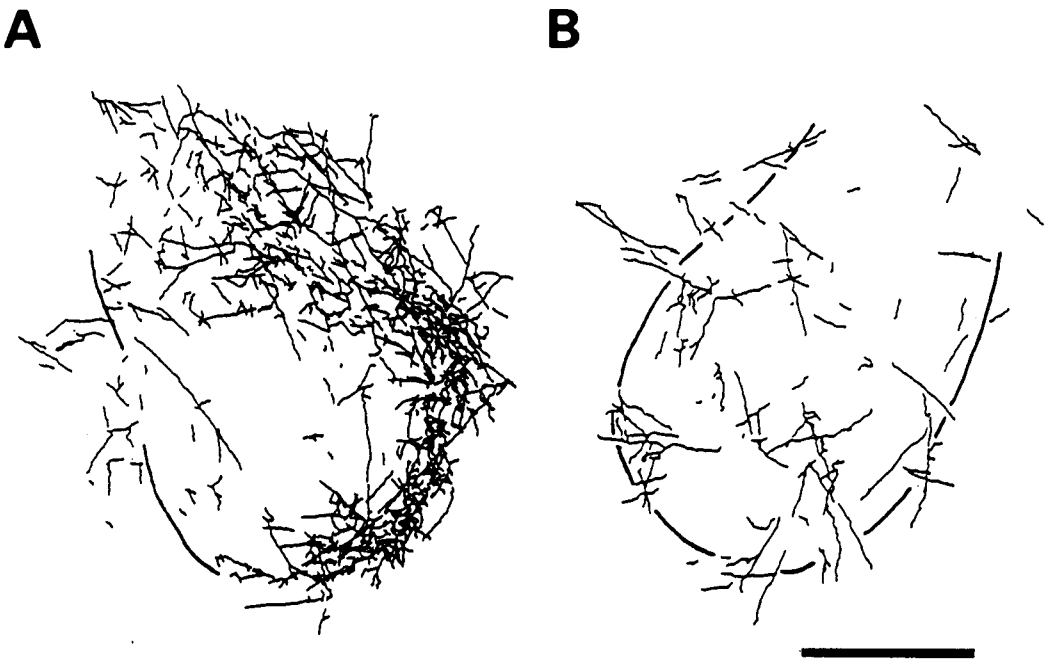

Fig. 6 Topography of uncrossed (A) and crossed (B) corticorubral projections in a normal animal (14 PND). Drawings of labeled axons in and near the ipsilateral (A) and contralateral (B) red nucleus. (A) a drawing of a representative horizontal section. (B) drawings of two neighboring sections were superimposed. The uncrossed axons show clear preference of the medial border of the red nucleus, while the crossed axons appear to be uniformly situated. In both A and B upper is rostral. In A the medial but in B the lateral is to the right. A thick continuous line delineates the border of the red nucleus. Bar = 500 μm.

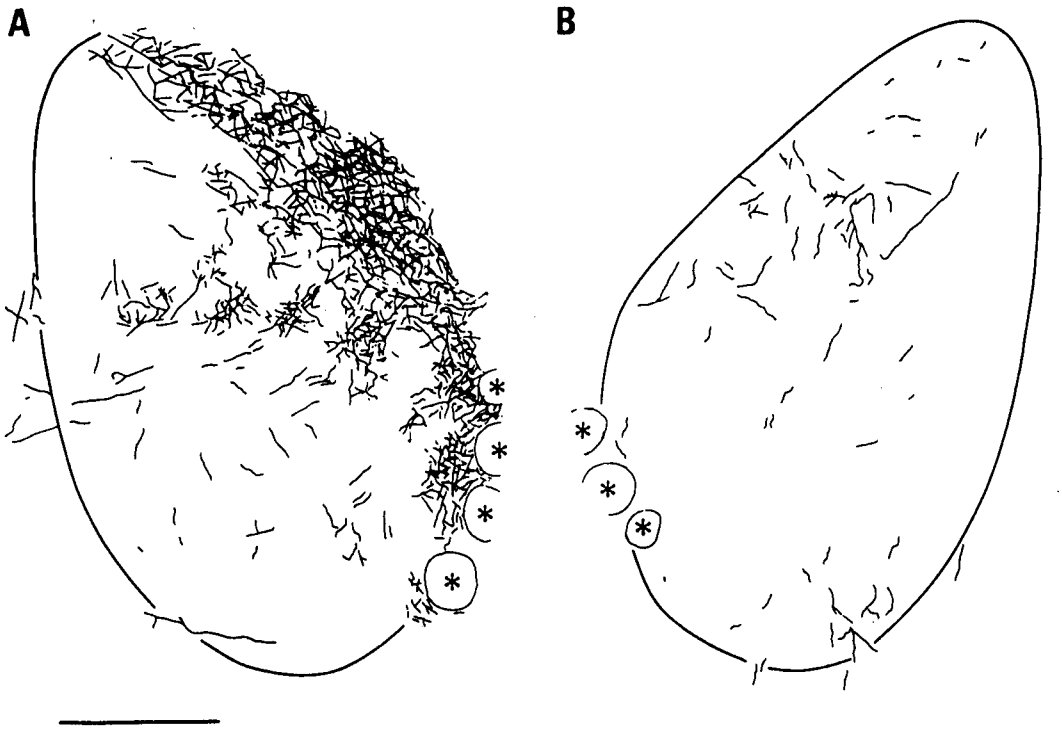

Fig. 7 A semischematic drawing of labeled axons in the ipsilateral (A) and the contralateral (B) red nucleus in a group I animal 14 days post-lesion. Axons are localized to the anteromedial portion of the red nucleus on the ipsilateral side. The axons are drawn schematically in densely innervated regions. Many labeled axons are found in the corresponding region in the contralateral red nucleus, but a notable number of axons are also seen caudally. A horizontal section. Upper is rostral. Left is lateral for the ipsilateral red nucleus and medial for the contralateral red nucleus. Continuous lines delineate the red nucleus and asterisks point to oculomotor nerves. Bar = 500 μm.

About one month after the lesions or later, the crossed projection roughly mirrors the ipsilateral projection. Figure 8A shows labeled fibers drawn at three different rostrocaudal levels of the RN of a group I lesioned animal which was observed 42 days after the lesion; the left column of Fig. 8A shows fibers on the ipsilateral RN and the right column shows fibers on the contralateral RN. Labeled fibers tended toward localization to the medial and rostral aspects of the RN, indicating that the projection to the ipsilateral RN occurs with topography similar to that in normal animals[11]. It is noted that this topography is roughly maintained for the projection to the contralateral side (right column of Fig. 8A). Figure 8B shows similar drawings from a group II lesioned animal 46 days post-lesion. The fibers appear to prefer the ventrolateral aspects of the caudal RN on both sides.

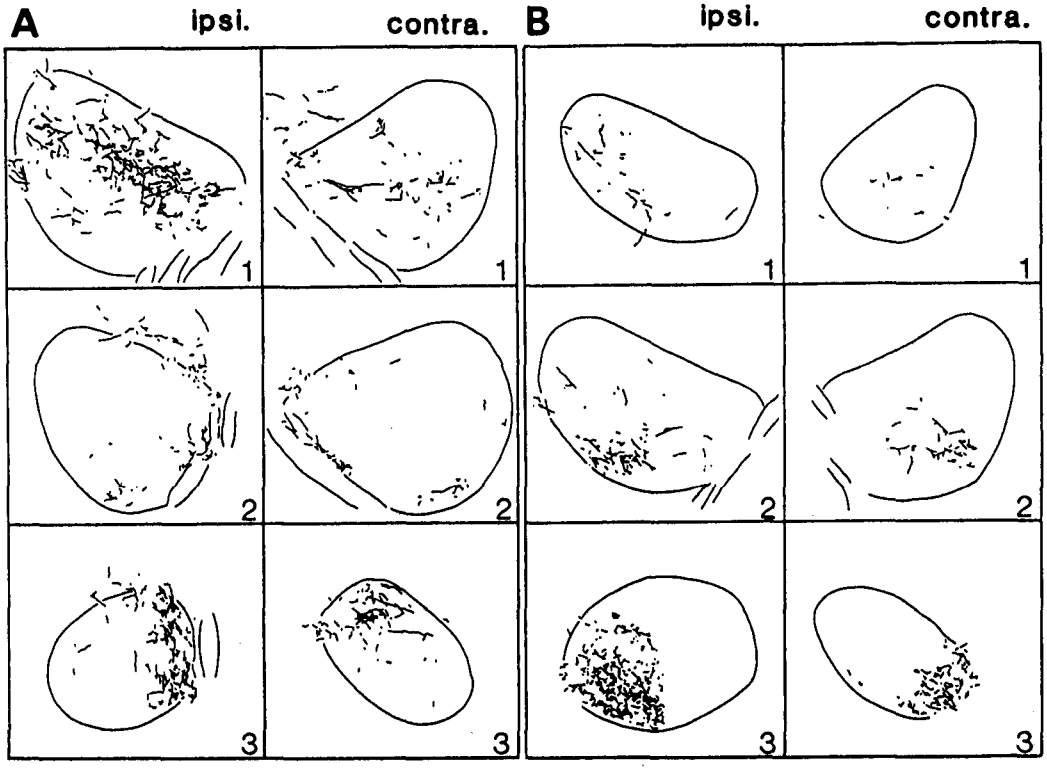

Fig. 8. Topography of uncrossed and crossed corticorubral projections. (A and B) equally-spaced drawings of labeled axons in frontal sections of the RN. Sections are shown from rostral (upper) to caudal (lower). A is from a group I (42 days post-lesion) and B from group II (46 days post-lesion) animal. Upper is dorsal in each panel. Lateral is to the left for the ipsilateral red nucleus and right for the contralateral red nucleus. The left row is ipsilateral and the right row is contralateral to the injection. Note that the topography of the crossed projections roughly mirrors that of the uncrossed ones.

DISCUSSION

The present study unequivocally demonstrated that the early unilateral lesions of the sensorimotor cortex causes the growth of crossed corticorubral axons in the kitten. Unilateral lesions of the cortex induce growth of the crossed axons, formation of side branches and sprays of finger-like fine axonal sprouts. These changes take place progressively during one month after the lesions leading to pronounced crossed corticorubral projections. Associated with these changes in axonal morphology, topographic refinement of the projection takes place; initially disorganized crossed projections begin to show topography which mirrors that of the ipsilateral projection. The results are summarized in Fig. 9.

Changes in axonal morphology

In lesioned animals with short post-lesion survivals, PHA-L was injected prior to the lesion. Nevertheless, PHA-L may probably be transported into growing tips of axons caused by the lesions, since numerous growth cone-like axonal endings were clearly labeled even in animals with short post-lesion survivals.

The growth cone-like axonal endings observed in the present study were in general compact and simple in morphology, but displayed the range of form observed in the regenerating frog optic nerve (Scalia and Matsumoto, 1985) developing mouse optic nerve (Bovolenta and Mason, 1987), hamster corpus callosum (Norris and Kalil, 1990), *Xenopus* spinal cord (Nordlander, 1987)and resembled those in mouse superior colliculus (Sachs et al., 1986). The observations that their number suddenly increased after the lesions and that gradually decreased as axonal arbors mature suggest that the presently described growth cone-like axonal endings are actually growing tips of axons.

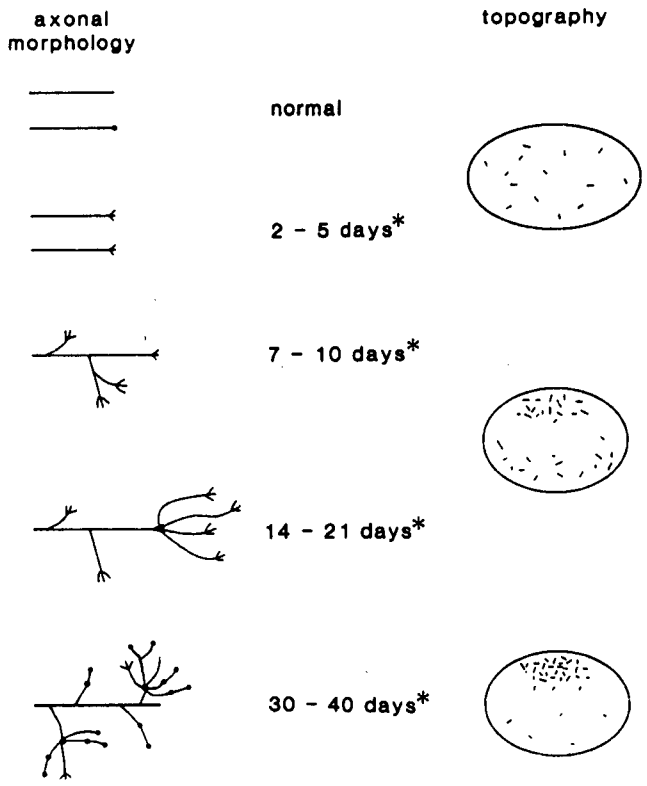

Fig. 9. Diagram summarizing the changes in axonal morphology and topography of the crossed corticorubral projection after unilateral lesions of the sensorimotor cortex. Asterisks:post-lesion days. The ellipses represent the red nuclei. See text for details.

They exhibit such a simple morphology possibly because they are located in the target region (see ref. Bovolenta and Mason, 1987).

The age of the normal animal used for the comparison of axonal branchings was somewhat younger than the age-at-lesion of the lesioned animal (see Materials and Methods). This should not affect the results, however, because development of axonal branching proceeds only slightly from PND 7 to 29 for the crossed projection (Higashi et al., 1990). The actual difference in the morphological complexity of axons between normal and lesioned animals should be larger than that shown in Fig. 5, since the number of branching points was estimated from the morphology of axon fragments drawn from 50 μm-thick sections. The effect of sectioning axons into fragments on branching number may be more significant for highly branched axons than those with infrequent branching.

It appears that the presently demonstrated post-lesion changes in axonal morphology resemble those occurring during the development of the ipsilateral corticorubral (Higashi et al., 1990), corticopontine (O'Leary and Terashima, 1988) and the murine and chick retinocollicular projections (Nakamura and O'Leary, 1989; Sachs et al., 1986). This supports the notion that the crossed axons normally cease development at a certain stage, but they resume development following the lesions.

Cellular mechanisms for the establishment of aberrant corticorubral projection

The aberrant crossed corticorubral projection found in animals with early unilateral lesions of the cerebral cortex was also initially suggested to be due to collateral sprouting (Kosar et al., 1985; Nah and Leong, 1986; Tsukahara et al., 1983). Subsequent findings of crossed corticorubral projections in newborn kittens led to a suggestion that lesions of the cortex arrested the retraction of crossed axons (Leonard and Goldberger, 1987). The present study, however, provided direct morphological evidence that

early unilateral lesions of the cortex cause the growth and proliferation of crossed corticorubral axons.

It remains to be elucidated whether the enlargement of the crossed projections is due to the proliferation of axons which were present in the RN at the time of lesions, or to axons newly entered into the RN, or both. The observation that the number of labeled axons crossing the midline appeared larger in lesioned animals compared to normal ones might mean occurrence of new-axon ingrowth into the contralateral RN. However, the same results could have been obtained without assuming actual changes in number of axons crossing the midline; since the formation of terminal arbor might require elevated axonal transport, more PHA-L could be transported after the lesions, resulting in an increase in number of labeled fibers. Presence of larger-diameter axons in lesioned animals is consistent with this view (Fig. 3E). Since the formation of axonal sprouts takes place so extensively, terminal proliferation of preexisting axons would be sufficient to explain the lesion-induced enlargement of the crossed corticorubral projections. Coincidence of axonal trajectories between normal and lesioned animals is in accord with this view.

It should be noted that terminal proliferation of preexisting axons can by itself explain the remarkable post-lesion plasticity in younger animals (Song ans Murakami, 1990); the aberrant projection might be necessary as a source of sprouting, and the developmental disappearance of exuberant projections may abolish the plasticity. In this context, it is quite interesting that in the adult nervous system an afferent appears to reinnervate a denervated zone, but only if its field overlaps that of a damaged afferent (Cotman et al., 1981).

There is a wealth of evidence suggesting that the segregation of initially overlapping inputs occurs in an activity-dependent fashion. Particularly, the possible participation of Hebb-type interaction has been repeatedly argued (Hebb, 1949) (see ref. Constantine-Paton et al., 1990 for a review). The

growth of the crossed axons could also be a result of perturbing such an interaction; the synapses of the crossed projections could be weakened, since they are unable to activate RN cells due to poor convergence of axons. Lesionings of the cortex might cause a decrease in incidence of uncorrelated activity of pre- and postsynaptic elements in the contralateral RN, leading to the prevention of synaptic elimination. The axons which are prevented from retraction would start to develop axonal arbors due to their intrinsic growth capability. Actually, the relative strengthening of functional synaptic connections has been recently demonstrated in the geniculocortical system in which pre- and postsynaptic elements were simultaneously silenced by eye-lid closure and muscimole application (Reiter and Stryker, 1988). The mechanism discussed here postulates that the crossed projections form functional synapses; preliminary electrophysiological and electron microscopic studies suggest that crossed axons actually form synapses (Murakami et al., 1991; Song et al., 1990).

Topographic refinement and maintenance of topography

No clear topography was found for the crossed projection of normal animals. Although the number of labeled axons in the contralateral RN might be insufficient to define the topography, in every experimental animal in which clear topography was found in the ipsilateral RN, an obvious localization of labeled axon in the contralateral nucleus could not be found. The topographic refinement which follow the lesions provides further support to the view that sprouting actually takes place.

The aberrant crossed corticorubral projections mirrored the topography of the uncrossed projections after appropriate survival periods. Such maintenance of topography for lesion-induced aberrant projections has been reported by other authors in the same (Villablanca et al., 1988) and other systems (Lund, 1978; Naus et al., 1984), but the mechanism has remained

unclarified. Non-selective local axonal sprouting from the crossed axons cannot explain the results, since no obvious topography was found for the crossed projection in normal kittens. An alternative possibility is that branch formation and proliferation of preexisting axons selectively take place specifically in topographically appropriate regions. The occurrence of growth cones and axonal endings with fine sprouts did not appear to be confined to topographically appropriate regions in animals observed shortly after the lesions. Therefore, it might be necessary to presume that, after lesions, crossed axons grow into topographically appropriate regions and then form terminal arbors. Further studies are, however, needed to verify this idea.

Although we did not observe any signs of axonal degeneration, it is not clear from the present results whether retraction of topographically inappropriately located axons takes place or not (see ref. Nakamura and O'Leary 1989; O'Leary et al., 1986).

The absence of topographic order in the crossed axons in normal animals makes it unlikely that the localization of crossed corticorubral axons are determined by positional markers (Sperry, 1963), although the lesions could unmask such markers on the axons. Another possible mechanism for the formation of topographic map would be an activity-dependent stabilization of the synapses. Evidence has been accumulating suggesting that activity-dependent mechanisms operate in the formation of topographic order of the retinotectal projections and eye-specific layers (e.g. Chapman et al., 1986; Cline et al., 1987; Scherer and Udin, 1989; see Constantine-Paton, 1990 and Fawcett, 1988 for reviews). It will be quite interesting to test whether the same mechanisms operate in the present system.

In conclusion, the changes observed in the crossed corticorubral projection following the lesions are similar to those occurring during postnatal development (Higashi et al., 1990); initially simple and unbranched axons

become tortuous and highly branched forming complex arbors, and the initially disordered projection begins to show topographic order. This suggests that lesion-induced formation of aberrant projections repeats events occurring during development. Thus, studying the lesion-induced synaptogenesis may be useful in understanding the development of corticorubral projection. Since various brain structures are well-developed and sizes of neurons are significantly larger at the time of lesions compared to at birth, both anatomical and physiological studies may be easier in the studies of lesion-induced synaptogenesis.

General Discussion

It is well known that neural plasticity in immature animals is remarkable (Lund, 1978; Tsukahara, 1981). It has not been, however, clear what kinds of cellular mechanisms induce the modification of neural connections. My study suggests that transient projections existing in immature stages have an important role for neonatal neural plasticity. These pre-existing projections that remain after the operation cannot, however, fully explain the remarkable neural plasticity at neonatal stages. Furthermore, my study suggests that besides the blockade of axonal elimination, induction of axonal proliferation occurs after lesion, and then aberrant projections, which are not observed in adults, may appear in neonatally lesioned animals.

Remarkable plasticity in neonatal animals

Neural plasticity seems to be more significant in neonatal animals than in adults (Lund and Lund, 1971; Lund, 1978; Tsukahara, 1981). If axonal elongation occurs in neonatal animals as well as in adults, does this remarkable neural plasticity in neonatal animals result from a higher property of axonal elongation during the neonatal stage as compared to the adult stage? It is still difficult for the present study to reject this possibility. It is, however, obvious that there is some relationship between the period when pre-existing projections exist in neonatal animals and the period when manipulation induces modulation of the neural connection. This relationship suggests that formation of aberrant projections depends on existence of transient projections that must be eliminated during development. In other words, in immature animals many redundant projections are formed first and then eliminated during development. After a lesion, however, these projections not only remain but also elongate even in inappropriate areas. In contrast, in adults, aberrant projections cannot be formed because the crossed projections that should cause produce the aberrant projections have

already been eliminated. Therefore, corticorubral projections cannot form bilaterally in adults. Although this idea might be speculative, it may well explain why only neonatal lesion form aberrant projections. All axons have a potential of extension in the brain at initial developmental stage, but the ability of redundant axons to extend is inhibited by some mechanism soon after development and these axons are gradually eliminated. When an animal suffers a lesion, however, this inhibition of redundant axons is somehow restricted and axonal elongation can occur as it does in developmental stages. From these results, we can see that although plasticity in neonatal animals is based on axonal proliferation as well as it is in adults, axonal elongation during the neonatal stage depends much more on the existence of pre-existing axons than it does in an adult stage. I also suggest that mechanisms for axonal elongation may not differ between neonatal stages and adult stages, except of existence where pre-existing projections exist.

My next point is that morphological properties of the crossed projections observed after lesions might be similar to those of the uncrossed ones in normal development; A topographical refinement of projections and an increase of branching frequency occur after a lesion. Besides this, the crossed projections in lesioned animals have similar electrophysiological properties to those observed in normal projections (Tsukahara et al., 1983). These results suggest that the formation of aberrant projections by manipulation at neonatal stages is due to similar cellular mechanisms as in those formed during normal development of neural connections. Some investigators suggest that neural activity have an important role for the refinement of neural connection (Constantine-Paton et al., 1990; Nelson et al., 1990; Shatz, 1990; Udin and Fawcett, 1988). It is, however, unknown whether neural activity plays a role in the formation of topographical order in the corticorubral projections. Therefore, it is a reasonable way to study the mechanisms causing topographical order in this system.

Mechanisms for elimination and sprouting

The present study suggests that the elimination of redundant projections serves as an important cellular mechanism for formation of precise neural connections. Although the elimination phenomena of redundant projections are well known (Cowan et al., 1984; Panneton, 1986; Ivy and Killackey, 1982; Innocenti, 1981; O'Leary et al., 1981; Mariani and Changeux, 1981), the mechanism for the elimination remains unknown. This study also shows that the crossed corticorubral axons had already entered the red nucleus when the lesions were done. They, however, failed develop their terminal branches. This suggests the possibility that although axons intend to form synapses during the developmental period, target areas might eliminate redundant synapses or prevent them from increasing the number of synapses from certain developmental stages. If there is a relationship between the development of axonal branching and an increase in the number of synapses, it might be preferable to think that undesirable synapses are prevented from increasing in the unmatched area. Accordingly, besides mechanisms for increasing the number of synapses during development, some mechanisms preventing the increase of undesirable synapses may exist at the same time. In the visual cortex, it is suggested that synapses are reorganized by visual experience during the neonatal stage (Hubel and Wiessel, 1963). Many models that increase synaptic strength when presynaptic neural activity synchronize with postsynaptic one have been already proposed (e.g., Hebb, 1949; Stent, 1973; Changeux and Dachin, 1973; Miller et al., 1989). An idea proposing that those mechanisms exist in the nervous system is widely accepted. For instance, long-term potentiation or depression of transmission efficacy is observed in the cerebrum and the cerebellum (Artola and Singer, 1987; Bliss and Lømo, 1973; Komatsu et al., 1981; Komatsu and Iwakiri, 1993; Ito, 1989; Mullkey and Malenka, 1992; Teyler and DiScenna, 1987).

Furthermore, Kandel's group has shown that the long-term modification of synaptic efficacy caused morphological changes of axon terminals (Baily and Chen, 1988; Glanzman et al., 1990). These phenomena may be, at least indirectly, related to an increase or a decrease of synapses. In other words, phenomena of potentiation or depression may trigger next mechanisms concerning an increase or a decrease in the number of synapses.

Although elimination itself is well known, there are many aspects of its mechanisms that are unknown. In the crossed projections, the axons that could be eliminated during the postnatal period in the case of normal animals can regrow after manipulation in the lesioned animals. This result suggests that mechanisms for elimination are inhibited and mechanisms for elongation are expressed after manipulation. Moreover, the mechanisms causing axonal proliferation on pre-existing axons seem to be the same as those expressed in the uncrossed axons during the normal developmental stage. The question is how the developing brain or a damaged brain changes system from the elimination process to the elongation process. To answer this question, it is necessary to reveal what cellular event occurs during the elimination phenomena, as well as the elongation phenomena. To do this, first it is necessary to emulate, *in vitro* environment, the elimination phenomena observed *in vivo*.

Regulation of expression of molecules concerning axonal elongation

Mechanisms for axonal proliferation in the developmental stage and for plasticity exhibit similar processes. If they are similar, what kinds of molecular mechanisms control those processes? One of the most influential ideas currently is that second messengers (e.g., Ca^{2+} or cAMP) may change its concentration, depending on neural activity and that this change controls gene expressions concerning synaptic formation (Kandel and Schwartz,

1982). For instance, gene elements regulated by internal concentration of cAMP have already been identified (Goodman, 1990). If there is a similar gene regulation concerning axonal elongation in development and in plasticity of mammals, then it may be safe to assume that two kinds of genes regulated by second messengers exist; one concerning the enhancement of axonal growth (Dodd and Jessell, 1988; Heffner et al., 1990) and the other concerning the blockade of axonal growth (Schwab, 1990; Pini, 1993). The increase or decrease of these factors may be controlled during the developmental stage. Similar things may occur during lesion-induced plasticity at neonatal stages. Future studies should focus on which of these possible genes is expressed in plasticity. It is also useful to understand the nature of the factors related to axonal proliferation (e.g., diffusible or substrate-bound) to reveal mechanisms for axonal elongation. Besides, it is also important to establish a good assay system that shows the long-term modification of the neural structure. The crossed projection formed after a neonatal lesion may become one of the most useful ways to approach this. Because if we find a candidate gene related to axonal elongation, we can assay its action in this system, using transgenic animals.

Conclusion

In my study, the appearance of aberrant projections after neonatal brain damage is related to the existence of pre-existing projections that seem to be eliminated during the developmental stage. I also found that the appearance of aberrant projections is due to axonal proliferation of pre-existing axons rather than simply due to the inhibition of elimination process by the lesion. Furthermore, this study suggests that cellular mechanisms inducing axonal elongation in the crossed projections are quite similar to those mechanisms formed in the formation of neural connection during normal development.

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