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Developmental plasticity of the rubrospinal tract in the North American opossum, Didelphis virginiana

Xu, Xiao Ming, Ph.D.
The Ohio State University, 1990
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DEVELOPMENTAL PLASTICITY OF THE RUBROSPINAL TRACT
IN THE NORTH AMERICAN OPOSSUM, DIDELPHIS VIRGINIANA

DISSERTATION

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

By

Xiao Ming Xu, M.S.

* * * * *

The Ohio State University

1990

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TO MY COUNTRY, CHINA
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ABBREVIATIONS

aq, cerebral aqueduct
C.C. central canal
Coe, nucleus locus coeruleus
D.F., dorsal funiculus
Fac, facial nucleus
g, genu of facial nerve
Hg, hypoglossal nucleus
1FLM, interstitial nucleus of the medial longitudinal fasciculus
111, oculomotor nerve
1P, interpeduncular nucleus
L.F., lateral funiculus
OcM, oculomotor nucleus
Ol, inferior olive
ped, cerebral peduncle
RN, red nucleus
SO, superior olivary nucleus
trMo, motor nucleus of the trigeminal nerve
Vstl, inferior vestibular nucleus

xiv.
Chapter I.
EVIDENCE FOR DEVELOPMENTAL PLASTICITY OF THE RUBROSPINAL TRACT IN THE NORTH AMERICAN OPOSSUM

ABSTRACT

We have shown previously that rubrospinal plasticity, i.e. the ability of rubral axons to grow around a lesion of their pathway, could be observed in the developing opossum, Didelphis virginiana (Martin and Xu.'88). In the present report we expand on that observation and present evidence which suggests that the critical period for plasticity of the rubrospinal tract ends earlier at cervical than at thoracic levels. In addition, we present preliminary evidence which suggests that most rubrospinal neurons die as a result of axotomy during early stages of the critical period. The opossum was chosen for study because the development of its rubrospinal tract occurs after birth.

In one set of experiments the area containing the rubrospinal tract was lesioned at cervical or thoracic levels and after 30 days or more, retrograde transport techniques were used to determine if rubral axons had grown caudal to the lesion. When the lesions were made at rostral cervical levels
between estimated postnatal day (EPD) 26 and maturity (adult), neurons could not be labeled in the contralateral red nucleus by injections of retrograde markers ipsilateral to the lesion and caudal to it. We were not able to obtain adequate survival after cervical lesions made prior to estimated postnatal day 26. When the lesions were made at mid to caudal thoracic levels between estimated postnatal day 19 and 26, neurons could be labeled in the contralateral red nucleus. When comparable lesions were made at estimated postnatal day 40, there was usually a decrease in the number of labeled neurons and when they were made at estimated postnatal day 54, none were labeled. In selected cases, operated at estimated postnatal day 19, cell counts provided evidence for loss of neurons in the red nucleus contralateral to the lesion.

In orthograde transport experiments performed on animals with thoracic lesions of the rubrospinal tract made between estimated postnatal day 18-33, rubral axons could be labeled caudal to the lesion and they seemed to take the most direct route around it. Although they sometimes assumed abnormal positions caudal to the lesion, rubral axons appeared to reach areas of the grey matter appropriate to them. When lesions were made at estimated postnatal day 54, or in older animals, labeled axons could be traced to the lesion site but not caudal to it.

In still other experiments, rubrospinal neurons were
labeled by caudal thoracic or rostral lumbar injections of Fast Blue at estimated postnatal day 26 and 3-4 days later the injected animals were subjected to a lesion of the rubrospinal tract 3 segments rostral to the injection and ipsilateral to it. They were sacrificed 30 to 34 days after the lesion so that the red nucleus contralateral to it could be examined for labeled neurons. The results showed that only a few rubrospinal neurons survived axotomy, suggesting that the growth of new axons around the lesion is the primary factor in developmental plasticity.
INTRODUCTION

It is generally accepted that the central nervous system is capable of greater plasticity during development than in the adult animal (Weber and Stelzner, '77; Stelzner et al., '79; Goldberger and Murray, '85). This plasticity is well illustrated by the growth of pyramidal tract axons around or through lesions of their pathway at birth in the hamster, rat and cat (Kalil and Reh, '79; Bregman and Goldberger, '82, '83; Bernstein and Stelzner, '83; Schreyer and Jones, '83; Tolbert and Ti Der, '87). Such plasticity probably underlies the sparing of tactile placing seen after spinal cord lesions which include the pyramidal tract in neonatal cats (Bregman and Goldberger, '82). In contrast, rubral axons do not grow caudal to lesions of their pathway in neonatal rats (Prendergast and Stelzner, '76) or cats (Bregman and Goldberger, '82, '83). The rubrospinal tract may be relatively mature at birth, however, at least in comparison to the pyramidal tract (Bregman and Goldberger, '82, '83; Schreyer and Jones, '83), and it may simply have lost its potential for plasticity. If so, it should be possible to demonstrate plasticity of the rubrospinal tract at earlier stages of development. This hypothesis would be difficult to test in the rat or cat because rubrospinal development occurs prenatally (Prendergast and Stelzner, '76; Bregman and Goldberger, '82, '83), but it should be testable in the North
American opossum (*Didelphis virginiana*) because the development of its rubrospinal tract occurs after birth (Cabana and Martin, '84, '86). Birth takes place 12-13 days after conception in the opossum (McCready, '38; Cutts et al., '78).

We have already reported that rubrospinal plasticity, i.e. the ability of rubral axons to grow around and caudal to a lesion of their pathway, occurs during early stages of development (Martin and Xu, '88). In the present communication we expand on that observation and present evidence which suggests that the critical period for plasticity of the rubrospinal tract ends earlier at cervical than at thoracic levels. In addition, we provide preliminary evidence which suggests that most rubrospinal neurons die as a result of axotomy during early stages of the critical period suggesting that the growth of new axons around the lesion may be a major factor in plasticity.
MATERIALS AND METHODS

Retrograde transport experiments

The area containing the rubrospinal tract was cut at rostral cervical or mid to caudal thoracic levels of the spinal cord in both developing and adult opossums (table I). The developing animals were obtained from females captured in the wild or bred in captivity at The Ohio State University. The snout-rump length (SRL) of each animal was measured by stretching it on a ruler and used to estimate age, when unknown, from the growth curve of Cutts et al. ('78). The birth of opossums takes place 12-13 days after conception (McCready,'38; Cutts et al.,'78), which was considered to be postnatal day one. Most of the developing animals were operated while they were still in the mother's pouch. The mother was anesthetized by a 1.2ml intramuscular injection of Ketamine followed by inhalation of Metofane and then placed on her back. During anesthesia the pouch sphincter relaxes, exposing the litter. The pouch-young, still attached to the nipples, were then anesthetized individually by hypothermia or Metofane inhalation, so that the desired level of the spinal cord could be exposed and the rubrospinal tract cut using a number 11 surgical blade and microscissors. After the incision was closed, the operated animals were returned with their mother to the vivarium. The adult animals were anesthetized by intraperitoneal injections of sodium
### TABLE I. Summary of Animals and Methods Employed

<table>
<thead>
<tr>
<th>METHOD</th>
<th>AVERAGE AGE AND LENGTH AT TIME OF LESION</th>
<th>AVERAGE SURVIVAL TIME IN DAYS AFTER LESION</th>
<th>NO. OF ANIMALS</th>
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<tr>
<td>EPD 19</td>
<td>SRL 40mm (36-45°) mid-low thor.</td>
<td>32 (30-40°)</td>
<td>5</td>
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<tr>
<td>EPD 26</td>
<td>SRL 50mm (49-52) mid-low thor.</td>
<td>34 (30-40)</td>
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<td>SRL 85mm (65-90) mid-low thor.</td>
<td>35 (33-40)</td>
<td>15</td>
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<tr>
<td>EPD 72</td>
<td>SRL 125mm (130-125) mid-low thor.</td>
<td>34 (30-35)</td>
<td>7</td>
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<tr>
<td>EPD 79</td>
<td>SRL 160mm (155-165) mid-low thor.</td>
<td>40 (40)</td>
<td>7</td>
</tr>
<tr>
<td>EPD 19</td>
<td>SRL 40mm (30-43) mid-low thor.</td>
<td>38 (30-50)</td>
<td>15</td>
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<td>F.B. retrograde labeling experiments</td>
<td>EPD 25  SRL 51mm (50-53) cervical</td>
<td>80 (60-90)</td>
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<td>EPD 119</td>
<td>SRL 260mm (200-280) cervical</td>
<td>30 (28-35)</td>
<td>5</td>
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<td>ADULT</td>
<td>cervical</td>
<td>30 (30)</td>
<td>2</td>
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<td>5</td>
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<tr>
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<td>SRL 40mm (38-42) mid-low thor.</td>
<td>57 (54-60)</td>
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<tr>
<td>EPD 33</td>
<td>SRL 60mm (60) mid-low thor.</td>
<td>57 (57)</td>
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<tr>
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<td>SRL 160mm (160) mid-low thor.</td>
<td>38 (38)</td>
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</tr>
<tr>
<td>ADULT</td>
<td>mid-low thor.</td>
<td>40 (40)</td>
<td>2</td>
</tr>
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a: EPD = estimated postnatal day  
b: SRL = snout-rump length  
c: range of snout-rump lengths  
d: range of survival in days
pentobarbital (40mg/kg) prior to surgical exposure of the appropriate level of the spinal cord. The lesion was made with a number 11 surgical blade and after closure of the surgical exposure, the animals were returned to the vivarium for recovery.

All animals were maintained for at least one month before being subjected to a second surgery and injections of wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) or Fast Blue (FB), two segments caudal to the lesion and ipsilateral to it. The objective was to label rubral neurons whose axons had grown caudal to the lesion. The developing animals were removed from the pouch for the second surgery and anesthetized either as described previously or by intraperitoneal injections of sodium pentobarbital (40-60mg/kg). After exposure of the spinal cord and stabilization of the vertebral column, the injections (0.15-0.20ul of 10% WGA-HRP or 2% FB) were made stereotaxically using a glass micropipette attached to a 1.0ul Hamilton syringe. The developing animals were maintained for 1-2 days postoperatively. The adult animals were anesthetized as described for the first surgery and after exposure of the spinal cord, they were placed in a spinal frame. The injections (0.2 - 0.25ul of 5% WGA-HRP or 2% FB) were then made as described for the pouch-young. After the injections were completed and the exposure closed, the adult animals were returned to the vivarium for a 2-3 day survival.
All of the above animals were sacrificed by an overdose of anesthetic and perfused intracardially with physiological saline, followed either by cold 1% paraformaldehyde-1.25% glutaraldehyde-1% sucrose in phosphate buffer and 10% sucrose in the same buffer (WGA-HRP experiments) or a citrate buffer-formaldehyde solution (FB experiments). The spinal cords and brains were removed and placed in either 30% sucrose phosphate buffer (WGA-HRP experiments) or the 30% sucrose citrate buffer (FB experiments) for refrigeration at 4°C. Frozen sections of the lesion site, the injection site and the brain were cut at 40um in all cases. The sections from the WGA-HRP cases were processed for HRP using tetramethyl benzidine as the chromagen (Mesulam, '78). Every third to fifth section was stained with Neutral Red. Examination and photography were carried out with a Leitz Dialux photomicroscope using bright and darkfield condensors. The sections from the FB cases were mounted immediately and coverslipped with Entellan (Merck) for viewing and photography with a Leitz (Orthoplan) fluorescence photomicroscope using the A cube of the Ploem illumination system. The positions of rubral neurons labeled by HRP or FB were recorded on drawings of the sections using an X-Y plotter attached to the microscope stage by potentiometers. In the FB cases, the coverslips were removed after plotting and photography so that the sections could be stained for Nissl substance.

In 5 cases (table I), prelabeling experiments were
carried out to determine whether rubrospinal neurons survive axotomy at thoracic levels. The animals were anesthetized in the pouch as described above and subjected to injections of FB into caudal thoracic or rostral lumbar levels of the spinal cord. Three to four days later they and the mother were anesthetized again so that the rubrospinal tract of the pouch-young could be cut 3 segments rostral to the injection. The operated animals remained in the pouch for 30 to 34 days before being sacrificed and perfused as described above so that their spinal cord and brain could be removed and sectioned for fluorescence microscopy.

**Cell counts**

In 5 of the cases subjected to a thoracic lesion of the rubrospinal tract at EPD19 and FB injections 30-50 days later, neurons were counted in the red nucleus contralateral and ipsilateral to the lesion after they were stained with thionin.

The cell counts were made with the aid of an interactive computer-assisted image analysis system (Magiscan, Nikon/Joyce Loebl) from 1 out of every 3 sections cut at 40um. The signal of a black and white television camera (Dage-Mti Series 68, Newvicon, attached directly to a Leitz orthoplan, 6.3X) was fed through a real time video processor (Nippon Avionics, Model Image Sigma) and finally to the computer (Magiscan 2A, Joyce-Loebl) for digitization and analysis. The real time video processor was employed to: 1) transform the intensity
function of the video signal into a linear density function, 2) remove density aberrations introduced by the mounting medium, the illumination source, the microscopic optics and the camera by dynamically subtracting a reference image of those sources from the image of the sections and 3) amplify and offset the video signal into a range appropriate for digitization by the computer. The video amplification was adjusted so that the computer would assign a value of 0% for areas judged to contain no cells and a value of 100% for areas with the heaviest staining.

After digitization, the computer was instructed to separate dark areas (cells) from light areas (non-cells). This separation was verified for each case and adjusted accordingly by comparing the microscope image with that of the computer. Groups of dark pixels were identified as cells; groups with areas smaller than 25 pixels (1 pixel = 7.8 um²) were not counted while pixel groups larger than 100 were assumed to represent overlapping cells. The latter were counted according to the formula: grouping size/100 + 1 (using integer mathematics). Obvious artifacts were rejected from counting. Additionally the computer was instructed to restrict the areas of analysis by having the user identify them with a light pen. The output from the computer included estimates of the total number of cells in all of the sections counted and the number in sections from rostral and caudal halves of the nucleus only. The total area of the red nucleus from 1 out of
sections was counted in pixels and then transformed into mm$^2$.

Orthograde transport experiments

For these experiments pouch-young and adult opossums (table I) were subjected to lesions of the rubrospinal tract at mid to caudal thoracic levels as already described, but after 38 to 60 days, they were anesthetized as described previously for stereotaxic injections (0.12-0.15ul) of 10% WGA-HRP into the red nucleus contralateral to the lesion. After about 48 hours, the animals were anesthetized again and perfused so that the spinal cord and brain could be removed, sectioned on a freezing microtome at 40um, and processed for HRP as described above. Orthogradely labeled axons were photographed and their positions plotted using a Leitz Dialux photomicroscope equipped with light and darkfield condensors.
RESULTS

The results of experiments using retrograde transport techniques

When lesions of the rubrospinal tract were made at rostral cervical levels between EPD26 and maturity (adult), and injections of WGA-HRP or FB were made caudal to the lesion at least 30 days later, no neurons were labeled in the contralateral red nucleus. The case plotted in figure 1 shows the results obtained from an animal subjected to a C-4 lesion of the rubrospinal tract at EPD119 and 30 days later to injection of FB caudal to the lesion. Neurons were not labeled in the contralateral red nucleus (Fig. 1, A-D, arrows) although they were labeled in the superior colliculus (Fig. 1D), the interstitial nucleus of the medial longitudinal fasciculus (Fig. 1A-D), the locus coeruelus (Fig. 1E), the reticular formation, the raphe of the pons and medulla (Fig. 1E-H), and the vestibular nuclei (Fig. 1F). All of the latter regions project through the spared white matter (Martin et al, '79, '81). When the coverslips were removed and the sections stained for Nissl substance, fewer neurons appeared to be present in the red nucleus contralateral to the lesion (Fig. 2) than on the same side (Fig. 3). We did not obtain adequate survival when animals were subjected to cervical cord lesions prior to EPD26.

When rubrospinal lesions were made at mid to caudal
thoracic levels between EPD54 and maturity and injections of FB or WGA-HRP were made caudal to the lesion at least 30 days later, no evidence for retrograde labeling was found in the contralateral red nucleus. As in the cervical cases, labeling was found in all of the nuclei known to have axons within the spared white matter (Martin et al., '79, '81). The results from a case subjected to a lesion at EPD54 which involved all of the lateral funiculus and some of the adjacent grey matter is illustrated in figure 6. When comparable lesions were made between EPD19 and 43, rubral neurons were labeled contralateral to the lesion (Fig. 6), suggesting that their axons had grown caudal to it. As in control animals (Cabana and Martin, '84), neurons were labeled throughout the red nucleus although they were most numerous caudally. It should be noted that the opossum's red nucleus is not well organized somatotopically (Martin et al, '81). Neurons were also labeled in the red nucleus ipsilateral to the lesion, probably because the marker spread to both sides of the cord. Photomicrographs of the labeling, or lack of it, in the contralateral red nucleus are supplied in figures 7-10. Although the number of labeled neurons generally decreased with increasing age at the time of lesion, it varied even between cases subjected to lesions at the same age. Such variation may be due to differences in the extent of the lesion and/or the size of the injection.

Although it appeared that fewer neurons were present in
the contralateral red nucleus (Fig. 4) than in the ipsilateral one (Fig. 5) after thoracic lesions, this was not as obvious as after cervical lesions. Our qualitative impression was supported, however, by the results of cell counts made on animals subjected to thoracic lesions at EPD19 (Fig. 11). The mean of the total number of neurons was 26% (range = 15-31.6%) less on the side contralateral to the lesion than on the ipsilateral side. The results were statistically significant (P<0.01). It can also be seen from Fig. 11 that the number of neurons was less in both caudal and rostral halves of the nucleus although cell loss was somewhat greater caudally than rostrally. As might be expected from the cell loss, the area of the red nucleus contralateral to the lesion was also less than that on the ipsilateral side (mean decrease = 17.9%; P<0.01).

When FB was injected into the caudal thoracic or rostral lumbar (T11-L1) cord at EPD26 and the rubrospinal tract was cut 3 to 4 days later, only a few neurons were labeled in the contralateral red nucleus 30-34 days later. In the case illustrated in Figs 12-15, the injection spread to both sides of the cord labeling the red nucleus ipsilateral as well as contralateral to the lesion. The paucity of labeled neurons on the contralateral side was in marked contrast to the large number on the ipsilateral side.

The results of experiments using orthograde transport techniques
When rubrospinal lesions were made at thoracic levels on EPD54 or in the adult animal, and the contralateral red nucleus was injected with WGA-HRP approximately one month later, labeled axons could be traced to the lesion site, but not beyond it (Figs. 16-19). When the lesions were made between EPD19 and EPD33, however, rubrospinal axons could be labeled caudal to the lesion. A plot of the labeling produced in three cases is illustrated in Figure 20. Rostral to the lesion, rubral axons were labeled contralaterally in the dorsal part of the lateral funiculus, where they are found normally, and terminal labeling was present in areas of the grey matter expected from previous studies (Cabana and Martin, '86). At the lesion site and caudal to it, however, rubral axons were labeled in different areas depending on the extent of the lesion. When the lesion extended across the midline, rubral axons were labeled at the edge of the remaining cord (Figs. 20, left side, and 21). Caudal to the lesion most of the labeled axons formed a tract in the dorsal funiculus, rather than in the dorsal part of the lateral funiculus where they are found normally. In spite of their abnormal position, rubral axons appeared to reach areas of the grey matter appropriate to them. When the lesion was less extensive, labeled axons were still present at the edge of the cord (Figs. 20 and 22). In such cases, labeled axons were found in the lateral funiculus caudal to the lesion and they appeared to terminate in appropriate areas. In the younger animals
labeled axons could be traced to at least lumbar levels of the cord, but in older ones, labeling could only be followed several segments caudally.
DISCUSSION

The techniques employed for our study are sensitive, but problems of interpretation exist. For example, WGA-HRP can be transported transneuronally (Gerfen et al., '82; Itaya and van Hoesen, '82; Ruda and Coulter, '82; Peschanski and Ralston, '85; Itya, '87) and it might be argued that the labeling in the red nucleus in our experiments resulted from transport from labeled areas of the spinal cord or brainstem rostral to the lesion. The density of the rubral labeling suggested that was not the case, however, and comparable results were obtained using Fast Blue. In any case, the presence of rubral axons caudal to the lesion was validated in the orthograde transport experiments. Transneuronal transport may also occur in the orthograde direction and labeled neurons were present in the spinal cord after injections of WGA-HRP into the red nucleus. On the basis of their location and labeling density, however, we interpreted them to be neurons which were retrogradely labeled by spread of the injection into the mesencephalic reticular formation.

The results of the retrograde transport experiments suggested that rubrospinal axons can grow around lesions of their pathway at some stages of development but not others, and that the critical period for plasticity ends earlier at cervical than at thoracic levels. The experiments using orthograde transport methods showed that rubral axons can
indeed grow caudal to lesions of their normal pathway in apparently large numbers, and that they can follow abnormal routes to reach their normal targets.

We know from the results of previous experiments (Cabana and Martin, '86) that rubral axons grow into the thoracic cord prior to the time of our earliest lesions and that they continue to do so well after that. Since the results of the prelabeling experiments suggest that relatively few rubrospinal neurons survive axotomy, at least during early phases of the critical period, rubrospinal plasticity may have resulted primarily from the growth of new axons around the lesion. A few rubrospinal neurons survived axotomy, however, so regeneration of cut axons cannot be excluded. It is possible, of course, that the axons of the surviving neurons never grew caudal to the lesion. Kalil and Reh ('79, '82) have suggested that true regeneration occurs when pyramidal tract axons grow around lesions of their pathway in neonatal hamsters (see, however, Merline and Kalil, '90), but Tolbert and ti Der ('87) have concluded that such plasticity results from new growth in the cat.

When the rubrospinal tract is transected in neonatal rats (Prendergast and Stelzner, '76) or cats (Bregman and Goldberger, '82, '83), many axotomized neurons appear to undergo retrograde degeneration (the so-called Gudden effect, see review by Cowan, '70). A similar phenomenon occurs in mature rubrospinal neurons (Barron et al., '76; Goshgarian et
al., Prendergast and Stelzner, '76), but to a much lesser extent. It has been suggested, therefore, that rubrospinal plasticity does not occur in newborn rats and cats because rubrospinal neurons die as a result of axotomy (Bregman and Goldberger, '83). It appears, however, that many rubrospinal neurons fail to survive axotomy even when plasticity can be documented (present study). The results of the cell counts suggest that 26% (mean) of the neurons within the red nucleus die as a result of a lesion of their axon at mid-caudal thoracic levels, but that is probably an underestimation since many of the intact neurons project exclusively to areas of the spinal cord and brainstem rostral to the lesion (Martin et al., '74; Martin et al., '81). The results of the prelabeling experiments also suggest that most neurons die after axotomy, at least during early phases of the critical period. It appears, therefore, that the critical issue in developmental plasticity of the rubrospinal tract may not be neuronal survival, although that would be necessary if true regeneration were to occur, but whether new axons are added to the tract after the lesion is made.

We have emphasized the potential for axonal growth in developmental plasticity, but we recognize that other factors, including the environment encountered by regenerating or late growing axons, may play an important role (Reier et al., '83; Aguayo, '85; Gorio et al., '85; Nieto-Sampedro and Cotman, '85; Olson, '85). In the neonatal rat, transplants of fetal
spinal cord rescue injured rubrospinal neurons, apparently by preventing their degeneration (Bregman and Reier, '86). It is possible that glia in such transplants are immature and do not form a reactive scar. The development of an astrocytic response might explain loss of plasticity (Ramon y Cajal, '28; Brown and McCouch, '47; Clemente, '55; Guth, '56; Puchala and Windle, '77; Reier, et al., '83), including that described here. It is also possible that immature glia in the transplants produce factors which encourage axonal growth.

Bregman and Goldberger ('82, '83) have shown that rubral axons cannot grow around lesions of their spinal pathway at birth in the kitten, although cortical axons do so readily. The results of our study suggest that the rubrospinal tract is capable of plasticity, only at earlier stages of development than represented by birth in the kitten. Since rubral axons grow into the spinal cord before cortical ones (Cabana and Martin, '84, '85), it seems likely that at any stage of development, the rubrospinal tract is more mature than the corticospinal tract and thus less likely to show developmental plasticity. It is our working hypothesis that all descending spinal pathways are capable of plasticity at some stage of development, but that the critical period for each may be different.
Fig. 1  Plot of neurons (dots) labeled in selected sections of the brainstem by the C-6 injection of Fast Blue (FB) illustrated at the lower right. The injection was placed 30 days after the lesion (C-4, les) which was made at EPD119. Note that FB labeled neurons are not present in the contralateral red nucleus (arrows, sections A-D). The scale bar equals 1.5mm.
Figs. 2-5  Photomicrographs of Nissl stained sections through the red nucleus (RN) contralateral (Figs. 2 and 4) and ipsilateral (Figs. 3 and 5) to lesions of the rubrospinal tract made 30 days earlier. The sections in Figs. 2 and 3 are from an animal lesioned at cervical levels on EPD119, those in Figs. 4 and 5 are from an animal lesioned at thoracic levels on EPD19. The bar in Fig. 2 equals 90um, that in Fig. 4 equals 80um.
Fig. 6 Plots of the labeling produced in the red nucleus (RN, sections A, B) at different stages of development by injections of WGA-HRP (inj) made approximately 30 days after lesions (Les) which included the rubrospinal tract. The midbrain sections are stacked from rostral (A) to caudal (B); the red nucleus contralateral to the lesion and injection is indicated by arrows.
Fig. 6
Figs. 7-10  Darkfield photomicrographs of rubral labeling produced by WGA-HRP injections approximately 30 days after rubrospinal lesions made at EPD21 (Fig. 7), EPD28 (Fig. 8), EPD43 (Fig. 9) and EPD54 (Fig. 10). The labeling in Fig. 7 is shown at higher power in the insert and some of the labeled cells are indicated by arrows in Figs. 7-9. The bar equal 160um in each figure.
Fig. 11 Graphs of the total number of rubral neurons from 1 out of 3 sections and the number of neurons in the rostral and caudal halves of the red nucleus contralateral and ipsilateral to thoracic lesions of the rubrospinal tract made at EPD 19. The animals were sacrificed 30-50 days later and the tissues were prepared for cell counts.
Fig. 11
Figs. 12-15 Photomicrographs of labeled neurons in the red nucleus contralateral (Contralat.) and ipsilateral (Ipsilat.) to a caudal thoracic injection of Fast Blue (FB) made at EPD 26. Three days later, a lesion of the rubrospinal tract was made 4 segments rostral to the injection. The animal was sacrificed 30 days later. Although the injection was large enough to label neurons in the ipsilateral red nucleus (Figs. 13 and 15), only a few rubral neurons were labeled on the contralateral side (Figs. 12 and 14). In cases with comparable injections but no lesions, the number of labeled neurons in the contralateral red nucleus far exceeded that in the ipsilateral one. The areas included within the rectangles in Figs. 12 and 13 are shown in Figs. 14 and 15 respectively. The bar in Fig. 12 equals 100um and it can be used for figure 13. The bar in Fig. 14 also equals 100um and it can be used for Fig. 15.
Figs. 16-19  Darkfield photomicrographs of rubrospinal labeling in the cervical enlargement (Fig. 16), just rostral to the deepest part of the lesion (Fig. 17), at the deepest part of the lesion (Fig. 18) and caudal to it (Fig. 19) in an animal subjected to a lesion of the rubrospinal tract in the caudal thoracic cord at estimated postnatal day 54 and an injection of WGA-HRP into the contralateral red nucleus 60 days later. The labeled rubrospinal tract is indicated by arrows in Figs. 16 and 17. The bar in Fig. 16 equals 100μm and can be used for all figures.
Fig. 20 Plot of the rubrospinal labeling (arrows) present well above the lesion (T1, Cl and C5), 5mm above the lesion, at the deepest part of the lesion (thoracic or Low Thor), 5mm below the lesion, and at lumbar (Lumb) levels in 3 cases subjected to lesions at EPD 18, 33 and 20 and injections of WGA-HRP into the contralateral red nucleus approximately 30 days later.
Fig. 20
Figs 21 and 22  Darkfield photomicrographs of rubrospinal labeling (arrows) at the lesion site in the cases plotted on the left and right in Fig. 20. The bars equal 100um in both figures.
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Chapter II.
THE RESPONSE OF RUBROSPINAL NEURONS TO AXOTOMY AT DIFFERENT STAGES OF DEVELOPMENT IN THE NORTH AMERICAN OPOSSUM

ABSTRACT

Rubral axons can grow around a lesion of their spinal pathway in the developing opossum, *Didelphis virginiana*, and a critical period exists for that plasticity (Martin and Xu, Dev. Brain Res., 39:303-308). Such plasticity may be a result of true regeneration of cut axons and, if so, rubrospinal neurons would be expected to survive axotomy. Rubrospinal plasticity may also result from the late growth of axons into the spinal cord that were not injured by the lesion. In an initial attempt to choose between these alternatives, we have studied the degree to which rubrospinal neurons survive axotomy during the critical stage for plasticity and, for comparison, during later stages of development.

Five groups of pouch young opossums received bilateral or unilateral injections of Fast Blue into the T12-L2 segments of the spinal cord at different ages to label rubrospinal neurons and 3-4 days later, the rubrospinal tract was cut unilaterally 3 segments rostral to the injection(s). In cases with
unilateral injections, the lesion was made ipsilateral to the injection. The animals were retained for approximately 1 month after the lesion before being anesthetized and perfused so that sections through the red nuclei could be examined for labeled neurons.

As suggested by preliminary observations (part I of the dissertation), relatively few neurons were labeled in the red nucleus contralateral to the lesion when it was made during the critical period for plasticity. We interpret these results to mean that most neurons failed to survive axotomy. Although some of the surviving neurons may have supported regenerating axons, it appears that late growth of new axons is a major contributor to rubrospinal plasticity. The number of labeled neurons increased with age, however, and we have suggested that collaterals established rostral to the lesion may be instrumental in neuronal survival.
INTRODUCTION

It is well known that neonatal mammals show greater anatomical and functional recovery after spinal lesions than do adults (see Bregman and Goldberger,'82,'83; Goldberger and Murray,'85 for reviews). For example, when a lesion of the corticospinal tract is made in neonatal rats, cats and hamsters, cortical axons grow around (Bernstein and Stelzner,'81; Bregman and Goldberger,'82,'83; Kalil and Reh,'79a,'79b,'82) and possibly through (Schreyer and Jones,'83) it to innervate appropriate areas more caudally and such plasticity probably underlies sparing of function (Bregman and Goldberger, '82). The growth of cortical axons around a lesion of their pathway could result from regeneration of cut axons, as suggested by Kalil and coworkers (Kalil and Reh,79a,'82; Kalil,'84; see however, Merline and Kalil, 1990), or new growth (Bryz-Gornia and Stelzner,'86; Tolbert and Der,'86,'87). It is possible, of course, that both occur.

We have shown that rubrospinal axons can also grow around a lesion of their pathway in developing opossums (Martin and Xu,'88; Xu and Martin,'89) and have provided preliminary evidence for death of most rubral neurons after axotomy during the critical period for plasticity (Xu and Martin,'89). The study described here was an attempt to expand upon those observations and to extend them to later stages of
development. If most rubral neurons die after axotomy during the critical period, it suggests that rubrospinal plasticity results primarily from growth of new axons around the lesion, not from regeneration of cut axons. We first injected Fast Blue (FB), a long-lasting fluorescent marker, into caudal thoracic or rostral lumbar levels of the cord and 4 days later, lesioned the rubrospinal tract 3-4 segments rostral to the injection. Thirty days later, the animals were sacrificed so that the red nucleus on both sides could be searched for labeled neurons. Our results show that most axotomized neurons do indeed degenerate during the critical period, supporting our previous suggestion (Xu and Martin, '89) that rubrospinal plasticity results primarily from growth of new axons.
MATERIAL AND METHODS

Pouch young opossums obtained from females captured in the wild or bred in captivity at the Ohio State University were chosen for study. The snout-rump length (SRL) of each animal from the wild was measured by stretching it on a ruler so that its age could be estimated (estimated postnatal day, EPD) using the growth curve of Cutts et al. ('78). The birth of opossums takes place 12-13 days after conception (McCrady,'38; Cutts et al.,'78), which was considered to be postnatal day one. Five groups of animals were operated at different ages while still in the mother's pouch (Table II.). The mother was anesthetized by a 1.2 ml intramuscular injection of ketamine (100 mg/ml) followed by inhalation of Metofane and then placed on her back. During anesthesia, the pouch sphincter relaxes, exposing the litter. The pouch-young, still attached to the nipples, were then anesthetized individually by hypothermia or Metofane inhalation so that the caudal thoracic or rostral lumbar cord (T12-L2) could be exposed and 0.15-0.25 ul of 3% FB injected into the cord bilaterally or unilaterally on the right (Fig. 23, FB injection). After the incision was closed, the animals were returned with their mother to the vivarium. Three to four days later, they were anesthetized again so that the rubrospinal tract could be cut 3-4 segments rostral to the injection (Fig. 23, lesion). For animals with unilateral injections, the
<table>
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Table II. Summary of Animals Employed
lesion was made ipsilateral to the injection. Most of the animals remained in the pouch for 30-34 days before being sacrificed, but a few of the ones lesioned at EPD 22 were sacrificed at 1, 2, and 3 week(s). All animals were sacrificed with an overdose of the anesthetic and perfused transcardially with hypertonic saline followed by a 0.2 M citrate buffer-20% formaldehyde solution. The brain and spinal cord were dissected out and immersed in 30% sucrose citrate buffer for approximately 24 hours at 4°C. The brains were scored by a shallow cut on the side of the lesion so that laterality of the tissue sections could be determined after mounting. Frozen sections of the injected segment of the spinal cord, the lesion site and the brainstem were cut in the coronal plane at 40 um. The sections were mounted immediately and coverslipped with Entellan (Merck) for viewing and photography with a Leitz (Orthoplan) fluorescence microscope using the A cube of the Ploem illumination system (excitation wavelength = 340-380 nm).

In those cases with bilateral injections, the positions of labeled neurons were recorded from every 3rd section through the red nucleus using an X-Y plotter attached to the microscope stage by position transducers. In some of the cases with unilateral injections, FB diffused across the midline and rubral labeling was almost equal on the two sides. In such cases we plotted the positions of labeled neurons in the red nucleus ipsilateral as well as contralateral to the lesion.
All labeled neurons were recorded regardless of their labeling intensity, including those not sectioned through the nucleus. Four groups of developing opossums with bilateral injections and excellent labeling (lesioned at EPD 22, n=7; EPD 30, n=7; EPD 40, n=7; and EPD 54, n=6), were selected for counts of labeled neurons in every 3rd section. Since 99-99.5% of the rubral neurons which innervate the lumbar cord project contralaterally (Xu and Martin, '90b), most rubral neurons ipsilateral to the lesion were not axotomized. For that reason, they were used as controls. A paired student t-test was used to determine if differences in the number of labeled neurons on the two sides were statistically significant. We did not obtain sufficient postoperative survivals for animals lesioned at EPD 12, so they were not included in the statistical analysis.

After the labeled neurons were plotted, photographed and counted, the coverslips were removed and the sections stained with cresyl violet. Nissl stained neurons were recorded using the X-Y plotter referred to previously and counted. The same sections were used for counts of FB labeled and Nissl stained cells. Only neurons with clear nuclei and nucleoli were counted in the Nissl stained sections. Cytological criteria were used to distinguish neurons from neuroglia, so it is not likely that we failed to count neurons because of shrinkage. The same statistical methods mentioned above were used to determine if the difference in number of rubral neurons on the
two sides was statistically significant.
RESULTS

Twenty-five animals (lesioned at EPD12, n=4, and EPD 22, n=21) were subjected to injections and lesions during the critical period for rubrospinal plasticity. Fig. 24A shows the injection from one case which was made into the upper lumbar cord at EPD 18. The lesion (Fig. 24B) was made 4 days later (EPD 22), 3 segments rostral to the injection. The opossum's rubrospinal tract is located in the dorsal part of the lateral funiculus (Cabana and Martin, '86a,'86b; Martin et al.,'81), so it was completely transected by the lesion. Since the spinal cord is very small at this age, the injection spread to both sides, resulting in bilateral labeling of the red nucleus. Fig. 25 shows the labeled neurons in the red nucleus contralateral (contra.) and ipsilateral (ipsi.) to the lesion one week after it was made. Although labeled neurons were numerous contralateral to the lesion, many of them appeared to be degenerating (Fig. 25A, arrow heads). In contrast, labeled neurons on the ipsilateral side were normal in appearance (Fig. 25B). The number of labeled neurons contralateral to the lesion decreased dramatically in cases sacrificed 2 to 3 weeks after the lesion.

One month after the lesion, only a few neurons were labeled in the contralateral red nucleus. Fig. 26 illustrates the labeling present in a case lesioned at EPD 22. The photomicrographs show labeled neurons contralateral (left
column, contra.) and ipsilateral (right column, ipsi.) to the lesion from every 4th section through the red nucleus and it can be seen that labeled neurons were fewest in number on the contralateral side. In counts made from every 3rd section, only 65 neurons were labeled in the contralateral red nucleus, whereas 240 were labeled on the ipsilateral side. In the opossum, projections to the lumbosacral cord arise throughout the length of the red nucleus and neurons giving rise to them are not restricted to lateral and ventral parts of the nucleus as in the rat and cat (Cabana and Martin,'86; Martin et al.,'74,'81,'86a,'86b).

Fig. 27 shows higher power views of sections adjacent to those illustrated in Fig. 26I and J and it can be seen that the neurons labeled contralateral to the lesion (Fig. 27A, C) were not only fewer in number than on the ipsilateral side (Fig. 27B, D), but some of them were less intensely fluorescent. Small, intensely fluorescent profiles were present, however (Fig. 27C, arrows), and it is our interpretation that they were macrophages which phagocytosed degenerating neurons labeled by FB. We conclude from this and other cases that most rubrospinal neurons failed to survive axotomy during the critical period for rubrospinal plasticity.

It appears that the older the animal, the more neurons survived axotomy. Fig. 28 shows the labeled neurons on the side contralateral (contra.) and ipsilateral (ipsi.) to the lesion from animals lesioned at EPD 22 (Fig. 28A, B), EPD 30
As noted above, only a few neurons were labeled in the contralateral red nucleus when the lesion was made during early stages of development (Fig. 28A, C). When the lesion was made at later stages, however, more neurons were labeled (Fig. 28E, G), indicating that they survived axotomy. In some cases, small, heavily fluorescent profiles were numerous in the contralateral red nucleus, indicating that microglia or other cells had phagocytosed degenerating neurons (arrows, Fig. 28E). In many cases, labeled phagocytes were seen ventral and lateral to the red nucleus. Although labeled neurons contralateral to the lesion increased in number with age, they were smaller and less intensely fluorescent (arrow heads, Fig. 28G) than on the ipsilateral side. This phenomenon was also seen in the adult red nucleus after rubrospinal tractotomy (Xu and Martin, '90).

The results from animals lesioned at EPD 26, 40 and 54 are plotted in Fig. 29 and it can be seen that the number of labeled neurons contralateral to the lesion was always less than on the ipsilateral side and that it increased with age. These differences are reflected in the counts of labeled neurons from the four groups provided in Fig. 30.

The upper histogram in Fig. 30 shows the mean numbers of labeled rubrospinal neurons contralateral (hatched area) and ipsilateral (non-hatched area) to the lesion when the lesions were made at EPD 22 (n=7), EPD 30 (n=7), EPD 40 (n=7) and EPD
54 (n=7). In these cases, the injections were made bilaterally in an attempt to maximize rubral labeling on both sides. The results show that the labeled neurons on the contralateral side were much fewer in number than on the ipsilateral side, although their number tended to increase with age. The student t test showed that the differences between the two sides were statistically significant in all groups (p<0.01).

Since it might be argued that the relative lack of labeled neurons contralateral to the lesion reflected transport failure rather than cell death, we counted all of the neurons whose nucleoli could be identified in the sections used to count labeled neurons. In order to accomplish that, the coverslips were removed and the sections stained with cresyl violet. The results are shown in the lower histogram of Fig. 30 where it can be seen that rubral neurons were fewer in number contralateral to the lesion at all ages. The student t test showed that the differences between the two sides in all groups were statistically significant (p<0.01). The numbers of Nissl stained neurons in the red nucleus contralateral to the lesion in comparison to those on the ipsilateral side were higher than for FB labeled neurons. This might be expected since many neurons in the red nucleus do not project to the caudal cord and, consequently, their axons were not transected.
DISCUSSION

Fast Blue proved to be a good marker for this study because it transported rapidly and remained in labeled neurons for a long time. Since a long term survival was required for our experiments, the possibility of transneuronal labeling must be considered. Such labeling could not have been a major factor, however, since the number and distribution of labeled neurons in the normal red nucleus after long survival times appeared comparable to those seen after shorter times. It has been reported that neurons in the ipsilateral red nucleus also respond to lesions of the rubrospinal tract in the rat (Barron, '88); if so, we may have underestimated the percentage of neurons which died or shrunk on the contralateral side. We counted all of the FB labeled neurons, including those not sectioned through the nuclei or nucleoli, because it was difficult to record the necessary detail without losing fluorescence. Labeled neurons from every 3rd section were recorded and counted to give the total number of labeled neurons from all the sections counted. No correction factors were employed to estimate the total number of labeled neurons.

It is possible that some rubral neurons project ipsilaterally to the lumbar cord. If so, it would be reasonable to infer that some of the rubral neurons labeled contralateral to the lesion projected ipsilaterally and were
labeled by spread of the marker at the injection site. This is not a problem, however, because only 0.05-0.1% of the rubral neurons which project to the lumbar cord do so ipsilaterally (Xu and Martin, '90b).

It is well known that neonatal animals show greater anatomical and functional plasticity after spinal lesions than do adults (see Bregman and Goldberger,'82,'83; Goldberger and Murry,'85 for review), even though lesion induced neuronal death is most severe in neonatal animals (Bleier,'69; Bregman and Goldberger,'82; Lieberman,'74; Prendergast and Stelzner,'76b). In the present study, quantitative evidence for a significant loss of axotomized rubrospinal neurons was observed, especially during the critical period for plasticity (Martin and Xu,'88; Xu and Martin,'89). For animals lesioned at EPD 22, more than 75% of the axotomized neurons had degenerated 30 days after the lesion. Although lesion induced neuronal death may have limited the potential for true regeneration during early stages of development, it did not do so at later stages (present study) or in the adult animal (Xu and Martin,'90a), when most rubral neurons survive axotomy. It has been reported that most rubral neurons also survive axotomy in the adult rat (Barron et al.,'89; McBride et al.,'88), although they may exhibit cytoplasmic, nuclear, and nucleolar atrophy as early as 7-10 days after the lesion (Barron et al.,'89).

During the critical period, axotomized rubrospinal
neurons showed signs of degeneration 1 week after axotomy. Although the number of labeled neurons contralateral to the lesion was almost equal to that on the ipsilateral side, fluorescent-intense granules were seen within their cytoplasm. It is our interpretation that such neurons were undergoing degeneration. The number of labeled neurons appeared to decrease dramatically 2 and 3 weeks after the lesion and one month later, only a few of them were found.

It is of interest to ask why most rubrospinal neurons failed to survive axotomy during early stages of development. One possibility is that they had been deprived of some trophic factor supplied by their targets (see review by Sofroniew et al.,'90). For example, nerve growth factor (NGF), a target-derived substance, is required to sustain neural crest-derived neurons (Barde,'89). NGF is also active in the central nervous system (Gage et al.,'88, Hagg et al.,'88,'89, Korsching,'86, Kromer,'87, ) and it has been shown to facilitate survival of axotomized neurons in the red nucleus of neonatal rats (Kunkel-Bagden and Bregman,'89). It is possible, therefore, that most rubral neurons degenerate after axotomy during early stages of development because they have not established synaptic contacts rostral to the lesion which provide access to trophic factor(s) necessary for their survival. At later stages of development, as well as in the adult animal, rubral neurons have either established such contacts or they have become less dependent upon trophic
factors.

It is possible that naturally occurring cell death also contributed to the loss of rubrospinal neurons observed in our experiments. Indeed, naturally occurring cell death is widespread during embryonic and fetal life (Cowan et al., '84; Hamburger and Oppenheim, '82). In a preliminary attempt to address that issue we searched Nissl stained sections for evidence of dead or dying cells in the red nucleus of developing opossums and observed them as early as EPD15. The number of pyknotic cells peaked between EPD20 and 26 and such cells were no longer present by EPD47. Even when naturally occurring cell death peaked, however, only 10 to 20 pyknotic cells were observed in both red nuclei. Since pyknotic cells were so sparse, we conclude that naturally occurring cell death was not a major contributor to the cell loss observed in our experiments.

Since most rubrospinal neurons die after axotomy during the critical period (Xu and Martin,'89), it is likely that plasticity results primarily from the growth of new axons into the tract. Fig. 31 is a schematic drawing which attempts to explain our results. Four days after the FB injection (Fig. 31, injection), rubral neurons were labeled whose axons were present at the injection site. Since rubral axons grow into the spinal cord over time (Cabana and Martin,'86), we assume that some of these neurons already supported axons with collaterals rostral to the lesion, whereas others did not.
During the 30 day survival after the lesion, it is possible that only neurons with collaterals survived. In the meantime, late growing axons may have reached the lesion and grown around it, accounting for most of the plasticity observed in our previous study (Martin and Xu,'88). The axons which survived transection may have simply healed or, as diagramed in Fig. 31, supported sprouts which grew around the lesion.

Fig. 32 is a similar drawing which attempts to explain why rubrospinal plasticity did not occur at later stages of development. We assume that most, if not all, of the rubral axons destined to innervate the lumbosacral cord had done so by the time of the injection (Fig. 32, FB injection) and that most of them had established collaterals rostral to the lesion (Fig. 32, lesion). Most rubrospinal neurons survived axotomy, but those with insufficient collaterals did not (Fig. 32, observations). Since growth of rubral axons into the lumbosacral cord was essentially over, rubral axons no longer grew caudal to the lesion. If regeneration of cut axons contributed to rubrospinal plasticity during the critical period, it no longer does so at this stage.

In summary, we have shown that: (1) most rubral neurons degenerate after axotomy during the critical period for rubrospinal plasticity, (2) neuronal death after axotomy is age related, i.e. the earlier the lesion, the more cells degenerate, (3) some rubral neurons survive axotomy even at early stages of development, and (4) the rubral neurons which
survive axotomy are smaller and less fluorescent than normal. These results suggest that growth of new axons is a major factor in rubrospinal plasticity. A small number of rubral neurons survive axotomy, however, indicating that true regeneration of cut axons may contribute to some degree.
Fig 23: Schematic drawing of the method employed for the present study. FB injections were made into the caudal thoracic or rostral lumbar cord of developing opossums at different ages (FB injection). Four days later, the neurons projecting to the injection site or beyond had been labeled by FB and a lesion was made several segments rostral to the injection. The animals were maintained for another 30 days before being sacrificed so that the red nucleus could be examined for labeled neurons (observation).
FB INJECTION

4 DAYS

LESION

Fig. 23

30 DAYS

OBSERVATION
Fig. 24: Fluorescence photomicrographs (excitation wavelength equals 360 nm) of a unilateral injection of FB (inj., A) and the lesion (les., B) several segments rostral and ipsilateral to it in a case lesioned at EPD 22 and sacrificed one week later. Since the rubrospinal tract is located in the dorsal part of lateral funiculus, it was completely transected on the lesion side (les., B). The bar in B can be used for A.

Fig. 25: Fluorescence photomicrographs of FB labeled neurons in the red nucleus contralateral (contra., A) and ipsilateral (ipsi., B) to the lesion from the case shown in Fig. 24. Some of the labeled neurons contralateral to the lesion appear to be degenerating (arrow heads), whereas all of the ones on the ipsilateral side were normal in appearance. The bar in A can be used for B.
Fig. 26: Fluorescence photomicrographs of FB labeled neurons in the red nucleus contralateral (contra., left column) and ipsilateral (ipsi., right column) to a lesion made at EPD 22. The animal was sacrificed 30 days later. The photomicrographs were taken from every 4th section through the red nucleus from its rostral (top) to caudal (bottom) pole. Note that labeled neurons on the side contralateral to the lesion were much fewer than those on the ipsilateral side. The bar in A can be used for B-L.
Fig. 27: Fluorescence photomicrographs of FB labeled neurons in the red nucleus contralateral (contra., A and C) and ipsilateral (ipsi., B and D) to a lesion made at EPD 22. The animal was sacrificed 30 days later. C and D are higher magnifications of A and B, respectively. The number of labeled neurons on the side contralateral to the lesion was much less than on the ipsilateral side. The bars in A and C can be used for B and D.
Fig. 27
Fig. 28: Fluorescence photomicrographs of labeled neurons in the red nucleus contralateral (contra., left column) and ipsilateral (ipsi., right column) to the lesion in 4 cases lesioned at EPD 22 (A and B), EPD 30 (C and D), EPD 40 (E and F), and EPD 54 (G and H). Notice that the number of the labeled neurons contralateral to the lesion was much less than that on the ipsilateral side in all cases. Moreover, the number of labeled neurons on the contralateral side increased with age (E and G). Heavily labeled, small profiles were present in the contralateral red nucleus (arrows in E). The arrow heads in G indicate small labeled neurons. The bars in A, C, E, G indicate 100um and can be used for B, D, F, H, respectively.
Fig. 28
Fig. 29: Plots of labeled rubrospinal neurons (dots) from 3 cases lesioned at EPD 26, EPD 40, and EPD 54. The levels of the red nucleus plotted were drawn on the left. Note that the number of labeled neurons in the red nucleus contralateral to the lesion was much less than on the ipsilateral side. Moreover, the number of neurons on the contralateral side increased with age.
Fig. 29
Fig. 30: Histograms showing the mean number of labeled neurons (upper histogram) and Nissl stained neurons (lower histogram) in the red nucleus contralateral (contra.) and ipsilateral (ipsi.) to the lesion in all of the cases with bilateral injections. The counts of Nissl stained neurons were made from the same sections used to count labeled cells, but only neurons with clear nuclei and nucleoli were included. The error bars indicate standard deviation.
Fig. 30

Number Of FB Prelabeled RN Neurons

Number of Nissl Stained RN Neurons

EPD22  EPD30  EPD40  EPD54

Contra.  Ipsl.
Fig. 31. Schematic drawing which attempts to explain rubrospinal plasticity during the critical period. When the injection was made, only those neurons whose axons had reached the injection site incorporated FB (FB injection). Since the development of the rubrospinal tract occurs over time, many axons were still growing into the spinal cord. Four days later, a lesion of rubrospinal tract was made several segments rostral to the injection (lesion), and thirty days later, late growing axons had grown around the lesion. The rubral neuron(s) which degenerated after axotomy are indicated by a small solid dot in the red nucleus and a dotted line in the spinal cord (observations). Some rubral neurons survived, however, and it is possible that collaterals from their axons contributed to rubrospinal plasticity (observation).
Fig. 31
Fig. 32: Schematic drawing which attempts to explain lack of rubrospinal plasticity after the critical period. The injection and lesion were made as described in Fig. 31. At this age, however, most rubral axons had arrived at their destination so most of them incorporated the label and were cut. Thirty days after lesion, more rubrospinal neurons survived axotomy because they had established collaterals rostral to the lesion. Since rubral axons no longer grow into the spinal cord, plasticity does not occur.
Fig. 32
LITERATURE CITED


Chapter III.

FURTHER EVIDENCE THAT RUBROSPINAL PLASTICITY IN THE DEVELOPING OPOSSUM RESULTS PRIMARILY FROM THE GROWTH OF NEW AXONS

ABSTRACT

We have shown previously that rubral axons can grow around a lesion of their pathway in the developing opossum and that a critical period exists for that plasticity (Martin and Xu, Dev. Brain Res., 39:303-308). Since most rubrospinal neurons degenerate after axotomy during the critical period, we have proposed that this plasticity results primarily from the growth of new axons into the spinal cord and not regeneration of cut axons (Xu and Martin, '89 and part II of dissertation). In the present study, we used a double labeling paradigm to test that hypothesis.

Four groups of pouch young opossums received bilateral or unilateral injections of Fast Blue (FB) into the caudal thoracic or upper lumbar cord (T12-L2) at different ages in order to label rubrospinal neurons. Three to four days later, the rubrospinal tract was transected unilaterally, 4-5 segments rostral to the injection(s). If the injection was unilateral, the lesion was made ipsilateral to it. The animals were maintained for about 1 month before a second
marker, Diamidino Yellow (DY), was injected, usually bilaterally, between the FB injection and the lesion. The animals were maintained for 5 days before being sacrificed so that sections through the red nucleus and spinal cord could be examined with a fluorescence microscope.

During the critical period for plasticity, only a few rubral neurons contralateral to the lesion were labeled by FB alone, supporting our previous suggestion that most axotomized neurons degenerated. In contrast, many neurons were labeled by DY alone, indicating that their axons were not present in the caudal cord at the time of the FB injection, but that they had grown around the lesion during the one month survival to incorporate DY. A few double labeled neurons were also found, however. One interpretation of such neurons is that they survived axotomy, as evidenced by the presence of FB, and that they supported axons which grew around the lesion to incorporate DY. After the critical period, more rubral neurons were labeled by FB, but none were labeled by DY.

The results of these experiments confirm our previous suggestion that developmental plasticity of the rubrospinal tract results primarily from the growth of new axons around the lesion and that a critical period exists. Our results also suggest that regeneration of cut axons may contribute to rubrospinal plasticity.
INTRODUCTION

In a previous study, we showed that rubral axons can grow around a lesion of their pathway to innervate appropriate areas of the spinal cord caudal to it and that a critical period exists for that plasticity (Martin and Xu, '88; Xu and Martin, '89; part I of dissertation). Since most rubral neurons die after axotomy, we have suggested that rubrospinal plasticity results primarily from growth of new axons into the tract (Xu and Martin, '89; part II of dissertation).

In order to provide direct evidence for the role of late growing axons in rubrospinal plasticity and to gain additional insight into the possibility of true regeneration, we undertook the following experiments. Pouch young opossums of different ages received unilateral or bilateral injections of Fast Blue (FB) into the caudal thoracic or rostral lumbar cord to label rubrospinal neurons. Three to four days later, the rubrospinal tract was transected unilaterally 4-5 segments rostral to the injection. For animals with a unilateral injection, the lesion was made ipsilateral to it. All animals were allowed to survive for approximately one month before a second marker, Diamidino Yellow (DY), was injected into the cord, usually bilaterally, between the FB injection and lesion. The intent of the second injection was to label any rubral neurons whose axon had grown around the lesion during the one month survival. Five days later, the animals were
sacrificed and the tissues prepared for microscopic examination. We observed that, during the critical period: (1) only a few rubral neurons were labeled by FB contralateral to the lesion, further confirming that most axotomized neurons degenerate, (2) many rubral neurons were labeled by DY alone, indicating that their axons were not present in the caudal cord when FB was injected, but that they had grown around the lesion to incorporate DY, and (3) a few double labeled neurons were present, suggesting that some of the rubrospinal neurons which survived axotomy supported axons which grew around the lesion. We conclude, therefore, that the growth of new axons around the lesion is, indeed, a major factor in rubrospinal plasticity, although true regeneration may also contribute.
MATERIALS AND METHODS

A total of 59 pouch young opossums was used for this study (Table III.). They were obtained from females captured in the wild or bred in captivity at The Ohio State University. The snout-rump length (SRL) of each animal was measured so that its estimated postnatal day (EPD) could be established by using the growth curve of Cutts et al. ('78). The birth of opossums takes place 12-13 days after conception (McCready, '38; Cutts et al., '78), which was considered to be postnatal day one. The animals were subjected first to injections of FB into the caudal spinal cord at EPD 18 (range, n=21), EPD 26 (range, n=21), EPD 36 (range, n=12), and EPD 50 (range, n=5) while they were still in the mother's pouch. The mother was anesthetized by a 1.2 ml intramuscular injection of Ketamine (100mg/ml) followed by inhalation of Metofane. The pouch-young opossums, while still attached to the nipples, were anesthetized individually by hypothermia or Metofane inhalation so that the lower thoracic or upper lumbar cord (T12-L2) could be exposed for bilateral or unilateral injection of 0.20 - 0.25 ul of 3% FB (Fig. 33, Injection). After the incisions were closed, the animals were returned with their mother to the vivarium. Four days later, they and the mother were reanesthetized as described above so that the rubrospinal tract could be cut unilaterally, 4-5 segments rostral to the injection(s) (Fig. 33, Lesion). For animals
Table III. Summary of Animals Employed

<table>
<thead>
<tr>
<th>FB Inj. Age</th>
<th>Lesion Age</th>
<th>Survival in Days Before DY Inj.</th>
<th>No. of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPD18 (EPD16-19)</td>
<td>EPD22 (EPD19-23)</td>
<td>29-32</td>
<td>21</td>
</tr>
<tr>
<td>EPD26 (EPD24-29)</td>
<td>EPD30 (EPD28-33)</td>
<td>30-34</td>
<td>21</td>
</tr>
<tr>
<td>EPD36 (EPD30-38)</td>
<td>EPD40 (EPD35-43)</td>
<td>29-33</td>
<td>12</td>
</tr>
<tr>
<td>EPD50</td>
<td>EPD54</td>
<td>33</td>
<td>5</td>
</tr>
</tbody>
</table>
with a unilateral injection, the lesion was made ipsilateral to it. All animals were maintained in the pouch for about 1 month before 0.20 -0.25ul of DY were injected, usually bilaterally, between the FB injection and the lesion (Fig. 33, DY Injection). Five days later, the animals were sacrificed by an overdose of the anesthetic and perfused transcardially with hypertonic saline followed by a 0.2 M citrate buffer - 20% formaldehyde solution. The brain and spinal cord were dissected out and immersed in 30% sucrose citrate buffer for approximately 24 hours at 4°C. The brains were scored by a shallow cut on the side of the injection and lesion so that laterality of the tissue sections could be determined after mounting. Frozen sections of the injected segments of the spinal cord, the lesion site and the brainstem were cut in the coronal plane at 40 µm. The sections were mounted immediately and coverslipped with Entellan (Merck) for viewing and photography with a Leitz (Orthoplan) fluorescence microscope using the A cube of the Ploem illumination system (excitation wavelength = 340-380 nm).

In the cases with bilateral injections of FB and DY, the positions of FB and DY single labeled as well as double labeled neurons were recorded from every 3rd section through both red nuclei using an X-Y plotter attached to the microscope stage by position transducers. All labeled neurons were recorded regardless of their labeling intensity, including those not sectioned through the nucleus. Counts of
the neurons labeled by one or both markers were made from the plots. Since 99-99.5% of the rubral neurons which innervate caudal thoracic or rostral lumbar levels of the cord project contralaterally in developing and adult opossums (Xu and Martin,'90), most of the rubral neurons ipsilateral to the lesion were not axotomized. For that reason, they were used as controls. Statistical analysis was carried out in the opossums lesioned at EPD 22 because they were within the critical period for rubrospinal plasticity. A paired student t-test was used to determine if the difference in the number of labeled neurons on the two sides was statistically significant. In some of the cases with unilateral injections, FB diffused across the midline labeling rubrospinal neurons ipsilateral as well as contralateral to the lesion. In such cases we plotted the positions of labeled neurons in the red nucleus on both sides.
RESULTS

A total of 21 animals was operated during the critical period for rubrospinal plasticity and Fig. 34 shows the lesion (A), the DY injections (B), and the FB injections (C) from a case lesioned at EPD 22. The rubrospinal tract is located within the dorsal part of the lateral funiculus (Cabana and Martin,'86; Martin and Dom,'70), so it was completely transected by the lesion. As can be seen in Fig. 34A, there was little evidence for the spread of either dye to the lesion site. Since the FB injections were made 38 days before sacrifice, they were small and shrunken in appearance (C). Fig. 35 shows the labeled neurons in the red nucleus contralateral (A and C) and ipsilateral (B and D) to the lesion. In the contralateral red nucleus, the total number of labeled neurons was much less than on the ipsilateral side. Most of the neurons labeled on the contralateral side were labeled by DY alone (A and C, solid block arrows), although neurons labeled by FB alone or by FB and DY (A and C, arrow heads) were present. Figs. 35B and 35D show labeled neurons in the red nucleus ipsilateral to the lesion, most of which were double-labeled (open block arrows).

Fig. 36 illustrates results from a second (Fig. 36A and 36B) and a third (Fig. 36C) animal lesioned at EPD22. As in the previous case, most of the labeled neurons in the red nucleus contralateral to the lesion contained DY alone (Fig.
36A, open block arrows), although neurons labeled by FB alone (Fig. 36C, arrows) and double-labeled neurons (Fig. 36B, solid block arrows) were observed. In some cases, a few of the labeled neurons appeared to be degenerating (Fig. 36C, arrow head). All of the labeled neurons were found within areas of the red nucleus which normally project to the lower thoracic or upper lumbar cord (Martin et al.'81).

A plot of labeled neurons in the red nucleus contralateral and ipsilateral to the lesion is illustrated in Fig. 37 from a fourth case subjected to bilateral injections of FB on EPD 18 and a lesion 4 days later (EPD 22). All three types of labeled neurons were observed on both sides, but the number and percentage of each type was different. In the contralateral red nucleus, the total number of labeled neurons was much less than on the ipsilateral side and most of them were labeled by DY alone. Fig. 38 is a histogram showing the number of labelled neurons contralateral (contra.) and ipsilateral (ipsi.) to a lesion of the rubrospinal tract made at EPD22 in the animals with bilateral injections of FB (n=9). The results show that: (1) the total number of labeled neurons contralateral to the lesion (mean=128) was less than on the ipsilateral side (mean=301), and the difference between the two sides is statistically significant (P<0.01), (2) many DY single labeled neurons (mean=65, 54% of those on the ipsilateral side) were observed in the contralateral red nucleus, (3) neurons labeled by FB alone were fewer on both
sides than those labeled by DY alone, and (4) a few double labeled neurons (mean=14, 18% of those on the ipsilateral side) were found in the contralateral red nucleus.

In some cases, a unilateral injection of FB was made. Since the dye crossed the midline, however, labeled rubral neurons were found bilaterally and the intensity of labeling almost equaled that produced by bilateral injections. Figs. 39A and 39B are photomicrographs showing labeled neurons on the side contralateral (contra.) and ipsilateral (ipsi.) to the lesion from a representative case subjected to a unilateral injection of FB (39E) and a lesion at EPD22. As in the cases described above, most of the labeled neurons in the contralateral red nucleus were labeled by DY alone (Fig. 39A, solid block arrows), while a considerable number of the labeled neurons on the ipsilateral side were double labeled (Fig. 39B, open block arrows). Figs. 39C, D and E show sections through the lesion, the DY injection and the FB injection, respectively.

In animals operated after the critical period, DY single labelled and double labeled neurons were not found contralateral to the lesion. Fig. 40A shows many single labeled DY neurons (solid block arrows) as well as one double labeled neuron (open block arrow) in the red nucleus contralateral to a lesion made during the critical period. After the critical period, a few neurons were labeled by FB alone in all animals (Figs. 40C, E, and G, long arrows); the
older the animal, the more FB labeled neurons were found. At some ages, small heavily labeled cells (Figs. 40C, E and G, arrow heads) were present contralateral to the lesion. Such cells were probably macrophages which had engulfed degenerated neurons labeled by FB. In the red nucleus ipsilateral to the injection, all types of labeled neurons could be seen at each of the ages studied (Fig. 40B, D, F, and H) and the total number of labeled neurons was far greater than on the contralateral side. Small labeled profiles were sparse to absent in the ipsilateral red nucleus.
The fluorescent dyes FB and DY worked well for this study because they transport rapidly, they remain in labeled neurons for a relatively long time, and they are not overly toxic to labeled neurons (Skirboll et al.,'84). Moreover, the two dyes can be distinguished from one another at the same wavelength (360 nm). Both FB and DY produced necrosis at the injection site, but it had little effect on the survival of labeled neurons in the red nucleus. This conclusion is based on the large number of neurons labeled ipsilateral to the lesion 38-40 days after FB injections and 5 days after DY injections.

Corticospinal plasticity has been demonstrated in neonatal hamsters (Kalil and Reh,'79, '82; Kalil,'84), rats (Bates et al.,'89; Bernstein and Stelzner,'83; Bregman et al.'89; Donatelle,'77; Schreyer and Jones,'82,'88), and cats (Bregman and Goldberger,'82,'83; Tolbert and Der,'87). Kalil and Reh ('79, '82) originally claimed in the hamster that corticospinal plasticity resulted from regeneration of cut axons, but others have concluded that it results from growth of late arriving axons which circumvent the lesion (Bregman and Golderber,'82,'83; Bryz-Gornia and Stelzner,'86; Schreyer and Jones,'83; Tolbert and Der,'87). The most direct evidence for the latter conclusion was provided by Tolbert and Der ('87) who showed in the kitten that immature pyramidal tract neurons degenerate after axotomy and, thus, could not support
regenerating axons. Merline and Kalil ('90) have restudied this issue in the hamster using a prelabeling paradigm similar to that employed by Tolbert and Der ('87) and come to the same conclusion. The relative paucity of FB labeled neurons in our experiments supports our previous contention that most rubrospinal neurons, like cortical neurons (Tolbert and Der, '87; Merline and Kalil, '90), die after axotomy during the critical period for plasticity (part II of dissertation), suggesting that new growth is a major factor in rubrospinal plasticity. More direct evidence for that suggestion is provided by the relatively large number of neurons labeled by DY alone. It is reasonable to assume that the axons of such neurons reached the lesion during the 30 days survival and simply grew around it (Fig. 41).

Indirect evidence from previous studies also supports our contention that new growth is a major contributor to rubrospinal plasticity. Development of the rubrospinal tract occurs postnatally in the opossum (Cabana and Martin,'85a,'86; Martin et al.,'86a,'86b) and rubral axons appear to grow into the spinal cord over a period of time (Cabana and Martin,'86). When the rubrospinal tract was transected during the critical period (at EPD 22), early arriving axons were cut, but we assume that many axons had not reached the lesion site (Fig. 41). When the lesion was made after the critical period, most of the axons destined for the caudal cord had probably grown past the lesion site (Fig. 42).
Although rubrospinal plasticity appears to result primarily from new growth, we cannot eliminate true regeneration as an additional factor. The presence of double labeled neurons in the contralateral red nucleus during the critical period, suggests that some neurons survived the lesion, as evidenced by the presence of FB, and that they supported axons which subsequently grew around it to pick up DY (Fig. 42). It is likely that the number of such neurons was even greater than indicated by our results because neurons could not be distinguished as double labeled unless they were sectioned through the nucleus. It is possible, however, that some of the late growing axons incorporated residual FB as well as DY and that true regeneration did not occur. Bates and Stelzner ('89) have studied corticospinal plasticity in the rat using an experimental paradigm similar to ours, but they made the lesion by removing the first dye so that it was not available to late growing axons. Since they found a few double labeled neurons, it appears that true regeneration, as well as late growth, contributes to corticospinal plasticity. The presence of double labeled neurons in our experiments might also be explained if a significant number of rubrospinal neurons projected ipsilaterally during the critical period. Although such neurons exist, they only constitute 0.5-1% of the neurons which innervate the caudal cord (Xu and Martin, '90).

It is interesting to speculate on why some neurons
survived axotomy during the critical period for plasticity and why their numbers increase with age. As illustrated in Fig. 41, it is possible that the neurons which survived lesions during the critical period had axons with sustaining collaterals rostral to the lesion, whereas those which failed to survive did not. It is possible that such collaterals have access to trophic factors (Barde,'89; Gage et al.,'88; Hagg et al.,'88,'89; Korsching,'86; Kromer,'87). After the critical period (Fig. 42), more axons probably had such collaterals so the neurons which supported them survived.

Lack of rubrospinal plasticity after the critical period may not simply reflect the lack of new growth. In fact, a few axons may be added to the rubrospinal tract after the critical period. Evidence for that possibility is provided by the presence of a few single labeled DY neurons in the red nucleus ipsilateral to lesions made after EPD 30 (Fig. 40D, F, and H, solid block arrows). It is possible, therefore, that other factors, including the environment encountered by late growing or regenerating axons, play important roles (Aguayo,'85; Gorio et al.,'85; Nieto-Sampedro and Cotman,'86; Olson,'85; Reier et al.,'83). Such factors include the development of an astrocytic response to the lesion (Reier et al., '83) and the appearance of myelin (Schwab, '88).
Fig. 33: Schematic drawing of the method employed for the present study. FB injections were made into the caudal thoracic or rostral lumbar cord of developing opossums at different ages (FB injection). Four days later, the neurons projecting to the injection site or beyond had been labeled by FB and a lesion was made several segments rostral to the injection (lesion). The animals were kept for another 30 days before a second marker, DY, was injected into the cord between the FB injection and lesion (DY injection). Five days later, the animals were sacrificed and the red nucleus was examined for neurons labeled by FB or DY alone and double labeled neurons (observation).
Fig. 34: Fluorescence photomicrographs (excitation wavelength equals 360 nm) from a case subjected to bilateral injections of FB and DY and lesioned at EPD 22. The lesion (A), the DY injections (B) and the FB injections (C) are illustrated. The central canal (c.c.) is indicated in A. The bar in B can be used for C.
Fig. 35: Fluorescence photomicrographs of neurons labeled by FB or DY alone and by both markers in the red nucleus contralateral (contra. RN) and ipsilateral (ipsi. RN) to the lesion from the case shown in Fig. 34. On the contralateral side, most of labeled rubral neurons were labeled by DY alone and two examples are indicated by solid block arrows. One double labeled neuron is present in the field (arrow head). On the ipsilateral side, all types of labeled neurons could be seen, although most were double labeled (open block arrows). C and D are higher magnifications of A and B, respectively. The bars in A and C can be used for B and D.
Fig. 35

Contra. RN

Ipsi. RN

100 µm

100 µm
Fig. 36: Fluorescence photomicrographs of neurons labeled by FB or DY alone or by both markers in the red nucleus contralateral to the lesion (contra. RN) from animals which received bilateral injections of FB and DY and were lesioned at EPD 22. DY single labeled neurons were the most numerous (A, open block arrow), but double labeled neurons could be seen (B, solid block arrows). A few FB single labeled neurons were also present (C, long arrows). Occasionally, degenerating neurons could also be identified (C, arrow head). The bar in A can also be used for B and C.
Fig. 36
Fig. 37: Plot of FB (dots), DY (stars) and double labeled (stars on dots) neurons from a case subjected to bilateral injections of FB and DY and lesioned at EPD 22. The levels of the red nucleus plotted were drawn on the left. Note that the total number of labeled neurons on the side contralateral to the lesion was much less than on the ipsilateral side. In the contralateral red nucleus, most of the labeled neurons were labeled by DY alone.
Fig. 37

Rostral

Caudal

1mm

Axotomized

Normal

110

Labeling:
- Doubt
- FB
- DY

1mm
Fig. 38: Histogram showing the mean numbers of 3 types of labeled rubrospinal neurons contralateral (Contra.) and ipsilateral (Ipsi.) to the lesion in a group of animals subjected to bilateral injections of FB and DY and a unilateral lesion at EPD 22. The counts were made from every 3rd section of the red nucleus. The error bars indicate standard deviation.
Number of Labelled Neurons

Labelling
- Double
- FB
- DY

Number of Cells

Contra.  Ipse.

Fig. 38
Fig. 39: Fluorescence photomicrographs of rubrospinal neurons labeled by FB or DY alone and by both markers contralateral (Contra. RN, A) and ipsilateral (Ipsi. RN, B) to the lesion (C, Lesion) from a case subjected to unilateral injection of FB (E, FB inj.). The lesion was made 4 days later (EPD 22). The injections of DY are shown in D. In the contralateral red nucleus, a few DY single labeled neurons (solid block arrows) are indicated, while in the ipsilateral side, a few double labeled neurons are pointed out (open block arrows). The spared spinal cord at the lesion site is outlined by dots. The bar in A can be used for B. The bars in C, D and E equal 200 μm.
Fig. 39
Fig. 40: Fluorescence photomicrographs of FB labeled neurons contralateral (Contra. RN, left column) and ipsilateral (Ipsi. RN, right column) to the lesion in 4 cases lesioned at EPD 22 (A and B), EPD 30 (C and D), EPD 40 (E and F), and EPD 54 (G and H). During the critical period for plasticity (A), many DY single labeled (solid block arrows) and a few double labeled (open block arrow) neurons were found in the contralateral red nucleus. After the critical period (C, E, and G), DY single labeled and double labeled neurons were not found in the contralateral red nucleus. A few FB single labeled neurons were present, however (C, E, and G, long arrows), as well as many heavily labeled, small cells (C, E, and G, arrow heads). The bar in A can be used for B, C, D, E, and F. The bar in G can be used for H.
Fig. 40
Fig. 41: Schematic drawing which attempts to summarize and explain the results obtained during the critical period for plasticity. The injection of FB was made into the caudal thoracic or rostral lumbar cord to label neurons whose axons were present at the level of the injection (FB injection). The lesion, made 4 days later, transected the axons of FB labeled neurons, but spared those which arrived later (lesion). During the 30 days survival, late arriving axons grew around the lesion putting them in a position to incorporate DY (DY injection). Neurons associated with such axons were thus labeled by DY 4 days later (observation). Most of the neurons whose axons were transected died, so few of them were present at the time of sacrifice. Some of the surviving, FB labeled neurons also contained DY, suggesting that they supported one or more collaterals which grew around the lesion (observation).
Fig. 41
Fig. 42: Schematic drawing which attempts to summarize and explain the results obtained after the critical period for plasticity. We assume that most of the axons destined to reach the caudal cord had done so by the time of FB injection (FB injection) and were cut 4 days later (lesion). Since there were few, if any, late arriving axons, they were not available to incorporate DY. The FB labeled neurons which survived axotomy may have developed sustaining collaterals by the time of lesion.
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