Fate of the Soma and Dendrites of Cord-Projection Central Neurons after Proximal and Distal Spinal Axotomy: An Intracellular Dye Injection Study

YUEH-JAN WANG,1 JENG-RUNG CHEN,2 and GUO-FANG TSENG2

ABSTRACT

We used rat rubrospinal neurons as a model to study the soma-dendritic morphology of cord-projection neurons following spinal axonal injury. We examined lumbar-projection neurons following both upper cervical and lower thoracic axotomy to find out whether changes were dependent on the proximity of the lesion to the cell body. Axotomized neurons were marked with retrograde tracer and studied 4 and 8 weeks later with intracellular dye injection technique. Axotomy resulted in prominent shrinkage of their soma and relatively minor reduction of their dendritic spreads. The degree of soma shrinkage depended on both the duration of survival and the proximity of lesion. In addition, dendritic modification peaked 4 weeks following proximal lesion, which was also achieved 8 weeks following distal axotomy. Tractotomy at upper cervical and lower thoracic levels also allowed us to compare the effect of distal axotomy on cervical and lumbar-projection neurons. Results show that although cervical-projection neurons responded more quickly than lumbar-projecting ones, they however showed a similar degree of alteration in both their soma and dendrites 8 weeks following distal axotomy. In summary, cord-projection neurons survived 8 weeks following either upper cervical or lower thoracic axotomy with relatively intact dendritic features. Taken together, our data thus far suggest that cord-projection central neurons continue to integrate inputs and control supraspinal targets following spinal axotomy. The minor dendritic shrinkage within two months of spinal axotomy rejuvenates hopes for functional recovery if regeneration of their spinal axons can be achieved at least within this time frame.

Key words: cord injury; intracellular dye injection; red nucleus; rubrospinal; trauma

INTRODUCTION

Spinal trauma usually damages the axons of cord-projection central neurons. Many such injured neurons are known to survive an extended period of time although unable to successfully regenerate their severed axons (Tseng et al., 1991, 1995; Wang and Tseng, 2002). In our laboratory, we use rat rubrospinal (RS) neurons as model to study the fate of cord-projection central neurons following spinal axonal injury for three rationales. First, their cell bodies concentrate in a small nuclear area, the magnocellular part of the red nucleus that can be easily studied. Second, their axons travel in a compact tract in the spinal cord, which can be easily transected. Third, spinal

1Department of Anatomy, College of Medicine, Tzu-Chi University, Hualien, Taiwan.
2Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan.
tractotomy causes no direct deafferentation to these neurons, which may confound the interpretation of results. We learned from previous studies that spinally axotomized rubrospinal neurons not only retained excitatory afferent connections (Wang and Tseng, 2002) but also innervated supraspinal targets (Wang et al., 2000). In addition, injured neurons retained active electrophysiological membrane properties (Chen and Tseng, 1997). However, we do not know how injury affects the morphology of the soma-dendritic compartment; that is, the functional receiving part of these neurons remains largely unexplored. In the past, we had used Rapid Golgi impregnation technique and attempted to study the soma-dendritic morphology of rat lumbar-cord-projection RS (L-RS) neurons following the transection of their axons at low thoracic level (Tseng and Hu, 1996). However, the incompatibility of Golgi impregnation with retrograde tracing impeded us from identifying injured neurons. Thereby L-RS neurons were identified based on their topographical localization, which could be controversial since their distribution in the red nucleus varied between rats (Tseng et al., 1995). In addition, Golgi impregnation was capricious, it might not stain all types of cells randomly (Tseng and Royce, 1986), nor would it reveal the entire extent of all neuronal processes of an impregnated cell (Tseng and Hu, 1996). Another problem we faced before was that impregnated cells were often too close to each other for reconstructing the entire extent of their dendritic processes across sections (Tseng and Hu, 1996). These problems seriously affected our estimation of their dendrites and might have compromised our interpretation. To resolve this, here we used intracellular injection of the fluorescence dye Lucifer yellow (LY) to reveal the entire extent of the soma-dendritic spread of identified axotomized neurons. This method is compatible with retrograde tracing using fluorescence tracer such as Fast blue (FB, Sigma), and FB-labeled injured neurons can be visually selected for intracellular dye injection. This not only enabled us to study injured neurons in isolation but also allowed us to choose neurons distantly apart to avoid the overlapping of their dendritic arbors, thus facilitated the reconstruction of their complete dendritic extent. In addition, we performed the intracellular dye injection on under-fixed brainstem slices. This allowed us to fill many selected cells with ease as compared to intracellular dye injection in live slices or animals, which usually had very low success rate (Tseng and Haberly, 1988; Tseng and Price, 1993). Another important advantage of the present approach is the availability of antibodies to transform the injected fluorescence dye LY to non-fading reaction product, a necessity for subsequent reconstruction of dendritic arbors, which required prolonged observation of sections with microscope.

With this better defined technique, we first asked what are the dendritic modifications associated with axonal injury? Then we try to find out whether axotomy-induced soma-dendritic modification is dependent on the distance of the lesion to their cell bodies since upper cervical and lower thoracic axotomy removed different amounts of the axoplasm of L-RS neurons. The proximity of axotomy to soma is known to affect retrograde neuronal responses (Lieberman, 1974; Hall and Cohen, 1988). In RS neurons, upper cervical, but not lower thoracic tractotomy of RS neurons were found to trigger their axonal regrowth into sciatic nerve graft (Richardson et al., 1984; Fernandez et al., 1999). This raises the possibility that the phenomenon could be attributed to differences in the responses of neurons to close versus distant axotomy. The other possibility is that cervical-cord-projection RS (C-RS) neurons could respond differently to spinal axonal injury from L-RS neurons since the former are the only additional neurons axotomized following upper cervical tractotomy as compared to lower thoracic tractotomy. In this regard, the fact that the proximal spinal axons of C-, but not L-RS neurons retained the ability to transport retrograde tracer following the removal of their distal segments (Tseng et al., 1996) suggests that the two groups of neurons may survive in different status following axotomy. This prompted us to compare the responses of C- and L-RS neurons following the removal of their distal spinal axons, that is, L-RS neurons following lower thoracic and C-RS neurons following upper cervical axotomy. Thus this study will not only provide information on the soma-dendritic morphology of spinally axotomized cord-projection central neurons, which is relevant to understanding the pathophysiology of cord injury but also enable us to evaluate the responses of cord-projection central neurons following upper cervical and lower thoracic spinal trauma.

**MATERIALS AND METHODS**

Data presented were from 21 young female Wistar rats (100–150 g) in which 12 rats received unilateral spinal tractotomy and the remaining nine rats were control animals. Animals were housed and cared for according to guidelines of the Animal Care and Use Committee of the medical school, and all efforts were made to minimize the suffering of animals.

**RS Tractotomy**

Unilateral RS tractotomy was performed on experimental rats at the C2 (n = 6) or T10 vertebral level (n = 6) as described previously (Tseng et al., 1995, 1996). Briefly, animals were anesthetized intraperitoneally with 7% chloral hydrate (Merck, 0.315 g/kg
A midline skin incision was made over the spinous processes of the C1-3 vertebrae or T9–11 vertebrae for the C2 and T10 tractotomy group, respectively. The lamina of the C2 and T10 vertebra was removed for each group respectively. After opening the spinal dura mater, the right lateral funiculus was transected with a 25-gauge needle. A small crystal of the retrograde tracer FB was placed into the lesion site to label axotomized RS neurons. Animals were then sutured and allowed to survive for 4 ($n = 3$ for both C2 and T10 groups) and 8 weeks ($n = 3$ for each tractotomy group). The spinal tractotomy sites of all animals reported here had been confirmed following the examination of corresponding spinal cord sections (Wang et al., 2000).

In order to fill identified normal RS neurons, in three control rats RS neurons were labeled from the C2, while in another three rats from the T10 vertebral level by inserting a small crystal of FB near the RS tract but avoiding damaging it (Wang et al., 2000). The procedure for opening the vertebral canal was the same as that for the C2 or T10 tractotomy respectively. Although our dye-injection method had a relatively high yield, it was still limited by the time required to fill neurons and the duration of tissue slices that could be maintained following their preparation. Therefore, we performed intracellular dye injection only on neurons of the lesioned nucleus of axotomized animals to ensure that we could sample as many axotomized neurons as possible.

**Fixed Tissue Intracellular Dye Injection**

General principles of fixed tissue intracellular dye injection technique had been described before (Buhl, 1992). In our laboratory, we modified especially the method of fixation to suit our brainstem tissue (Wang et al., 1996).

**FIG. 1.** Intracellular dye injection of tracer-identified rubrospinal neurons in fixed slices. (A) The injection of a Fast blue-marked rubrospinal neuron from an animal 8 weeks following T10 tractotomy. The cell body (arrow) was impaled with a glass micropipette containing 4% LY (arrowhead). Tracer injected permeated into dendrites. The photograph was taken with 20× long-working distance air lens with the slice submerged in PB, and the field diaphragm was also stopped down to improve image. (B,C) Paired photographs of the same red nucleus before and after intracellular dye injection. In B, FB-labeled injured neurons concentrated in the ventrolateral but not the dorsomedial part (*) of the red nucleus. In C, some selected tracer-marked injured neurons were filled with LY. Pairs of arrows of the same directions in B and C point to the same cell. B and C were taken with 10× objective lens and the slice was covered with PB without coverglass, thus limited the resolution. Bar = 50 μm (A), 100 μm (B,C).
red nucleus; two such slices could be obtained from each animal. Slice ready to inject was placed in a dish and covered with thin layer of 0.1 M PB on the stage of a fixed-staged, upright epifluorescence microscope (Zeiss Axioskop). Axotomized neurons and control neurons from normal animals that had subjected to FB retrograde labeling can be identified by the retrograde fluorescence dye they contained. However, prelabeling normal C- and L-RS neurons from the spinal cord might accidentally damage their axons or the retrograde tracer FB might alter neuronal properties, we also used an alternative method to sample C- and L-RS neurons without retrograde labeling. Slices from three normal rats that received no retrograde tracer application were soaked in 10^{-7} M 4',6-diamidino-2-phenyl-indole (DAPI, Sigma) solution in 0.1 M PB for 30 min to label neuronal nuclei before conducting intracellular injection. We used LY (lithium salt, Molecular Probes) as the intracellular dye. Since FB, DAPI and LY could be visualized simultaneously under the same fluorescence filter set (390–420, FT425, LP450), intracellular dye injection can be visually monitored (Fig. 1).

RS neurons are topographically organized, tracer application at T10 labeled only L-RS neurons located mainly in the ventrolateral (VL; Fig. 1B), while C2 application labeled additional C-RS neurons in the dorsomedial (DM) portion of the caudal red nucleus (Tseng et al., 1995, 1996). Since the main part of the present study is to compare the effect of the distance of axonal lesion site to cell bodies, that is, the effect of C2 and T10 axotomy of L-RS neurons, we concentrated on filling these neurons following both treatments. In the T10 tractotomy group, all FB-labeled rubral neurons can be selected, however only those in the VL part of the caudal nucleus of the C2 tractotomy group can be selected for this purpose (Fig. 2). The criteria for sampling control neurons using either FB- or DAPI-labeling method from normal animals followed the same principles (Fig. 2A).

Another part of the present study is to compare the effect of distal axotomy on L- and C-RS neurons. Neurons in the DM area of the caudal red nucleus of both C2 tractotomized and control normal animals were sampled for this purpose.

For injection, a 20× long-working-distance lens was used. Micropipette was filled with 4% LY (Sigma) in water and mounted on a three-axial hydraulic micromanipulator (Narishige). To avoid cells with truncated dendritic trees, selective cells deep to the surface of the slice were impaled and filled with LY using constant negative current till their terminal dendrites brightly fluoresced (Fig. 1A). Intense filling of each neuron took about 10–15 min. Slices prepared from animals perfused with our modified

**FIG. 2.** Low-power photographs of the left caudal red nucleus from C2-FB–prelabeled normal (A) and unilaterally (right side) tractotomized rats 4 (B) and 8 weeks (C) following surgery. Pictures were taken from 60 μm sections of the corresponding 300-μm-thick slices. LY injected in these sections had been converted to DAB reaction product using immunohistochemical procedures. A dashed line was drawn arbitrarily in each nucleus to separate the dorsomedial from the ventrolateral area, which contains C- and L-RS neurons, respectively. Bar = 100 μm.
method were good for intracellular injection within about 8 hours of their preparation if stored in a refrigerator at 4°C. Several neurons were usually filled from each surface of the selected rubral area of each slice (Fig. 1C). Slice was then removed, postfixed in 4% paraformaldehyde in 0.1 M PB for 3 days, rinsed thoroughly in 0.1 M PB, cryoprotected and sectioned into 60-μm-thick sections with a sliding microtome equipped with a freezing stage (Nippon Optical Works). Sections were collected in 0.1 M PB for subsequent histochemical processing.

**Histochemical Processing of LY-Injected Cells**

Sections were treated in 1% H2O2 and 1% Triton X-100 in 0.1 M phosphate-buffered saline (PBS), pH 7.3 for 1 h to remove endogenous peroxidase activity. After washing three times in 0.1 M PBS, sections were incubated with 1:200 anti-LY (peroxidase conjugated, Molecular Probes) for 18 h at 4°C. Finally, sections were reacted with 0.05% DAB and 0.003% H2O2 in 0.05M Tris buffer. Sections were then mounted on slide, air-dried, dehydrated in ethanol, cleared in xylene, and cover-slipped with Permount.

**Camera Lucida Reconstruction and Data Analysis**

The soma-dendritic morphology of reacted neurons was reconstructed through serial sections using Camera Lucida drawing tube and 40× objective lens in the two-dimensional plane. Since cells can be selectively injected at different location of the interested area, the reconstruction of their dendritic arbor through serial sections was straightforward. The beginning of a dendrite from soma was taken as the point where the convex curvature of the soma became concave (Tseng and Royce, 1986). Sholl's analysis was performed on the two-dimensional reconstruction by counting dendritic crossings with concentric circles of increasing radius at 25-μm increments from the center of soma (Tseng and Prince, 1993). Soma area and total dendritic length were determined from the two-dimensional reconstruction using the PC software Image-Pro Plus (Media Cybernetics). Since all tissues were processed using the same histochemical protocols, they were subjected to the same degree of tissue shrinkage, which was likely to be comparable to those of our earlier study (Tseng and Hu, 1996) for they had almost identical mean cell body sizes. No attempt was made to correct the tissue shrinkage in this study. Two-tailed Student’s t tests were used to compare between groups of neurons to find out whether any of the parameters was altered following treatment.

In this study, we found that the soma sizes of normal C- and L-RS neurons sampled with the DAPI method was 779 ± 306 μm² (range 448–1,201 μm², n = 6) and 954 ± 311 μm² (range 440–1,572 μm², n = 22), respectively, that is, 15% and 21% larger than the corresponding FB-prelabeled normal neurons (Table 1). The differences in soma sizes could be resulted from sampling bias since DAPI marks nucleus while FB marks the whole cell body. Micropipette directing toward the immediate vicinity of a DAPI-labeled nucleus was more effective than FB marks the whole cell body. Micropipette directing toward the immediate vicinity of a DAPI-labeled nucleus was more effective than FB marks the whole cell body. Micropipette directing toward the immediate vicinity of a DAPI-labeled nucleus was more effective than FB marks the whole cell body.

<table>
<thead>
<tr>
<th>Table 1. Soma-Dendritic Properties of Lumbar Cord-Projection Rubrospinal Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters</td>
</tr>
<tr>
<td>Soma area (μm²)</td>
</tr>
<tr>
<td>(239–1,944)</td>
</tr>
<tr>
<td>Total dendritic length (μm)</td>
</tr>
<tr>
<td>(1,366–5,767)</td>
</tr>
<tr>
<td>Total no. of terminal dendrites</td>
</tr>
<tr>
<td>Total no. of dendritic trunks</td>
</tr>
<tr>
<td>(3–8)</td>
</tr>
</tbody>
</table>

<sup>a</sup> t < 0.005 between prelabeled normal and the indicated.
<sup>b</sup> t < 0.005 between T10-4w and the indicated.
<sup>c</sup> t < 0.05 between T10-8W and the indicated.
<sup>d</sup> t < 0.05 between prelabeled normal and the indicated.

Values presented are mean ± SE (range). Two-tailed t test was conducted between groups and statistically significant difference was marked with superscript.

Prelabeled normal represents L-RS neurons identified by retrograde tracer labeling from the lumbar spinal cord.
likely to impale cells with larger cell bodies. On the other hand, FB labeling revealed the exact shape of the cell and even its proximal dendrites, thus easier for one to insert a micropipette into the soma of the cell selected for dye injection, therefore good at revealing cells of various sizes. Since all other soma-dendritic parameters of normal C- and L-RS neurons sampled with the DAPI-labeling method (data not shown) were comparable to those of the corresponding normal neurons prelabeled with FB (Tables 1 and 2), they are likely to be the same population of neurons. In this study, all axotomized neurons were labeled with FB, we therefore chose to compare them to normal neurons sampled using the FB-labeling method in all analyses throughout this paper.

RESULTS

Normal Neurons

In normal neurons, L- and C-RS neurons labeled simultaneously from C2 were distinguished arbitrarily based on their topographic localization in the VL and DM part of the caudal red nucleus, respectively (Fig. 2A); however, prelabeling from T10 marked only L-RS neurons. Normal L-RS neurons had typical multipolar morphology (Figs. 1A-C, 2A, 3A,B, and 4A,B). Their dendrites were usually smooth (Fig. 3A,B) with only occasional spines or dendritic appendages (Fig. 5A,B, arrows). Dendritic appendages were often complex with numerous fine tortuous processes (e.g., arrows in Fig. 5A). The soma area, total dendritic length, total number of terminal dendrites, and total number of dendritic trunk of prelabeled normal L-RS neurons are listed in Table 1.

Normal C-RS neurons were also multipolar (Fig. 2A) with dendritic features resembling those of L-RS neurons (Table 2). Although their mean cell body size and number of terminal dendritic branches appeared to be smaller than those of normal L-RS neurons (compare Table 1 with Table 2), none of these were statistically significant.

Time-Dependent Changes of L-RS Neurons following T10 Tractotomy

Tractotomy at T10 removed the distal spinal axons of L-RS neurons, which were located mainly in the VL area of the caudal red nucleus (Fig. 1B). Four weeks following such a lesion, axotomized L-RS neurons remained multipolar in shape (Figs. 3C and 4C) however with reduced cell body sizes, which nevertheless was statistically insignificant (Table 1). By 8-week-post-lesion, their cell bodies had shrunken further (Figs. 3D, 4D, 6A, and 7A, Table 1) and in addition, the total number of terminal dendrites per cell had also become significantly reduced (Table 1). The reduction in terminal dendrites was not accompanied by trimming of their dendritic trunks while a small and statistically insignificant decrease of total dendritic length was also observed (Table 1). At this time, occasional neurons with extensive dendritic arbors could still be encountered (Fig. 6A) although most of them appeared to have reduced dendritic spread. Closer examination revealed that dendritic appendages of various complexities (Fig. 5C,D, arrows) resembling those of normal neurons were present on L-RS neurons 4 and 8 weeks following T10 tractotomy.

Table 2. Soma-Dendritic Properties of Cervical Cord–Projection Rubrospinal Neurons

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Prelabeled normal identified by retrograde tracer FB (n = 15)</th>
<th>C2-4w (n = 18)</th>
<th>C2-8w (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soma area (μm²)</td>
<td>675 ± 346 (237–1,382)</td>
<td>433 ± 131a (222–713)</td>
<td>463 ± 161a (241–775)</td>
</tr>
<tr>
<td>Total dendritic length (μm)</td>
<td>2,585 ± 946 (1,352–4,424)</td>
<td>2,147 ± 552 (1,520–3,477)</td>
<td>2,249 ± 608 (1,341–3,139)</td>
</tr>
<tr>
<td>Total no. of terminal dendrites</td>
<td>14.3 ± 5.5 (9–31)</td>
<td>11.9 ± 2.9 (8–16)</td>
<td>12.2 ± 4.2 (6–19)</td>
</tr>
<tr>
<td>Total no. of dendritic trunks</td>
<td>4.9 ± 0.9 (4–7)</td>
<td>4.6 ± 0.9 (3–6)</td>
<td>4.9 ± 1.4 (3–8)</td>
</tr>
</tbody>
</table>

aP < 0.05 between prelabeled normal and the indicated.
Values given are mean ± SE (range). Two-tailed t test was conducted and statistically significant difference marked with superscript.
Prelabeled normal are C-RS neurons identified topographically from the population of rubrospinal neurons labeled retrogradely from the cervical spinal cord.
FIG. 3. Dye-filled L-RS neurons except the one marked with an arrow in the upper right corner of panel E, from normal and unilaterally tractotomized rats. (A,B) Normal neurons sampled with DAPI-labeling technique (N) and those sampled by Fast blue-retrograde labeling from the T10 (prelabeled-N), respectively. (C,D) Neurons 4 and 8 weeks following T10 tractotomy while (E) and (F) are neurons surviving 4 and 8 weeks following C2 tractotomy. Many of the neuronal processes were truncated in these photographs because they were taken from 60-μm-thick sections of the originally 300-μm-thick slices used for intracellular dye injection. Bar = 50 μm.
In C2-tractotomized animals, we sampled L-RS neurons based on their topographical localization (Fig. 2B,C). In contrast to T10 lesion, axotomy at C2 removed long segments of the spinal axons of L-RS neurons. Four weeks following C2 lesion injured L-RS neurons had shrunk cell bodies but remained multipolar in shape (Figs. 2B, 3E, 4E, and 7B). In addition, their total numbers of terminal dendrites and total dendritic length were both reduced (Fig. 7B, Table 1). The degree of these changes were comparable to that occurred 8 weeks following T10 axotomy of the same neurons (Fig. 8A-C, Table 1). Prolonged survival to 8 weeks reduced the soma sizes of injured cells further (Figs. 3F, 4F, 7B, and 8A). Their soma sizes at this point were also statistically smaller than those of L-

**FIG. 4.** Representative Camera-lucida reconstructed normal (A,B) and axotomized L-RS neurons (C–F) revealed by intracellular dye injection. Cell bodies of L-RS neurons shrank considerably following spinal axotomy. The degree of soma shrinkage was dependent on the closeness of the injury to their cell bodies and the duration of the survival. However, the profuseness of their dendritic arbors was only mildly reduced. Abbreviations are the same as in previous figures. Bar = 100 μm.
RS neurons 4 and 8 weeks following distal axotomy (Table 1, Fig. 8A). Despite the continuing shrinkage of their cell bodies, dendritic parameters including the already reduced total number of terminal dendrites and total dendritic length remained more or less the same (Figs. 7B and 8B,C, Table 1). Despite all these changes, proximal axotomy failed to reduce the number of dendritic trunks of injured neurons (Table 1).

At closer examination, complex dendritic appendages resembling those of normal animals were also present on neurons 4 and 8 weeks following C2 tractotomy (Fig. 5E,F, arrows). Although most injured cells had reduced dendritic arbors, occasional cells with rather profuse dendritic spread could still be identified 8 weeks following C2 tractotomy (Fig. 6B). To examine how the profuseness of the dendritic branches of L-RS
neurons was altered, we counted and plotted the number of their dendritic branches at 25-μm steps from the center of their cell bodies peripherally (Fig. 9). Those of axotomized neurons (empty circles) started to deviate from normal neurons (filled circles), reflecting a reduction in the number of their dendritic branches, about 100 μm from the center of their cell bodies. If we took the shrinkage of their cell bodies into account by shifting the curve of the axotomized neurons toward the right by 25 μm, the substantial reduction in dendritic branches appeared to occur at about 150 μm from the center of their cell bodies.

**C-RS Neurons following C2 Tractotomy**

C2 tractotomy removed the distal spinal axons of C-RS neurons that reside in the DM area of the caudal red nucleus. Neurons were sampled based on their topography (Fig. 2B,C). Four weeks following such a lesion, their cell bodies shrunk significantly, which remained at about the same reduced level by 8 weeks following lesion (Table 2, Fig. 7C). In fact, the degree of the soma shrinkage of C-RS neurons 4 weeks following C2 lesion was already comparable to that of L-RS neurons 8 weeks following T10 axotomy (Fig. 10A). Despite this, den-
Dendrites of C-RS neurons showed minimal alterations (Fig. 2B,C) including small and statistically insignificant reductions of total dendritic length and number of terminal dendrites while the number of dendritic trunks remained unaltered 4 and 8 weeks following axotomy (Table 2, Fig. 7C). In addition, dendritic appendages of various complexities resembling those observed in normal neurons were also present on these neurons 4 and 8 weeks following C2 axotomy (not shown). Detailed analysis of their dendritic branching pattern 8 weeks following injury show that the number of their dendritic branches at various distances from the center of their soma remained almost the same as those of control neurons (Fig. 9, compare filled with empty triangles).

**DISCUSSION**

**Fixed Tissue Intracellular Dye Injection versus Golgi Impregnation**

In this study we used fluorescent retrograde tracer to mark axotomized neurons and in the same time per-
formed intracellular dye injection in fixed tissue with epi-fluorescence illumination. This enabled us to reveal the morphology of selective identified cell with, relatively speaking, no constraint on time. A technical modification worth mentioning is that we performed intracellular dye injection on 300-μm tissue slices obtained fresh from animal perfused with a fixative containing 2% paraformaldehyde in 0.1 M PB. We found in a series of trial experiments that the cell membrane of well-fixed rubral neurons, for instance those fixed with 4% paraformaldehyde was leaky to the intracellular dye LY, it leaked out of the cell instantaneously. Perfusion with half usual-strength fixative yielded slices with neurons that retained LY well thus allowed us to study the morphology of tracer-marked injured RS neurons at different time points.

With the present method, the general features of RS neurons we obtained resembled those of earlier Golgi studies (Reid et al., 1975; Tseng and Hu, 1996). In addition, the mean soma size of normal L-RS neurons we sampled with the DAPI method was almost identical to that of our previous Golgi studies (Tseng and Hu, 1996). The biggest discrepancy is in the dendritic spread, total dendritic length in the present study was approximately two and a half times that of our previous Golgi study although the number of dendritic trunks and terminals were about the same (Tseng and Hu, 1996). This large difference could have resulted, in part, from the difficulties associated with reconstructing Golgi-impregnated dendrites, especially across section. However the methodological difference in how the dendritic length was measured between this and the previous studies could play an even more important role. Previous study measured length as the linear distance from the beginning to the end of the dendrites whereas the present study used computer software which taken into account the tortuous course of the processes. Thus, the methods we used in the present study not only allowed us to study identified axotomized neurons but also provided more accurate mea-

FIG. 9. Plots of the Sholl’s analysis result of normal and injured L-RS and C-RS neurons 8 weeks following upper cervical axotomy. Concentric circles with radii incrementing at 25-μm steps were used to count dendritic intersections from the center of the soma toward periphery. These plots reveal the profuseness of the dendritic trees of each group of neurons. The profuseness of L-RS neurons was reduced while that of C-RS neurons was unaltered.

Distal axotomy:
L-RS vs C-RS neurons

FIG. 10. Plots comparing the effect of distal axotomy on the soma sizes (A), total dendritic length (B), and total number of terminal dendrites (C) between L- and C-RS neurons over time. Data presented were normalized to corresponding normal neurons and expressed as ratios of those of normal.
sure of their dendritic spread. Regarding dendritic appendages, although we could not rule out the possibility that the injected dye or the antibody used for its subsequent conversion to non-fading reaction product might not enter these fine protuberances sufficiently, they appeared to be similar to those revealed by Golgi impregnation method (Tseng and Hu, 1996). They occurred occasionally on the otherwise rather smooth dendrites of both control and axotomized neurons, which didn’t seem to have changed following axotomy. This argues against the possibility that they might resemble the dendritic expanded structures reported in neck motoneurons following axotomy (Rose and Odlozinski, 1998). The persistence of these dendritic appendages, which presumably are areas of intensive synaptic contacts (Wilson et al., 1987) is in accord with our recent finding that spinally axotomized RS neurons continue to receive excitatory inputs (Wang and Tseng, 2002). Despite all these, the possibility that dendritic appendages were largely eliminated in some severely reactive injured neurons, which might have escaped our examination can not be ruled out completely.

Another difference is that previous Golgi impregnation study revealed some presumed injured L-RS neurons with severely truncated dendrites following T10 axotomy (Tseng and Hu, 1996), while no such neurons was identified in the present study. A plausible explanation for this discrepancy is that these severely affected neurons might have leaky membrane so that they cannot be sampled using the retrograde tracer marking and intracellular dye injection technique we employed here. Since we could successfully fill almost all FB-marked neurons that we chose to inject in the slices that we were working with, these severely reactive cells if present were likely to be so leaky that they were unable to retain the retrograde tracer FB thereby escaped from been sampled. This potential problem might lead to our underestimation of the dendritic shrinkage and also changes of the dendritic appendages in axotomized neurons. On the other hand, Golgi impregnation could be good at staining these severely injured neurons since their variations were often used to stain degrading neurons.

L-RS Neurons: Effects of Survival Duration and the Proximity of Axotomy to Cell Bodies

Our key finding is that spinal axotomy induced a time-dependent modification of the soma-dendritic spread of L-RS neurons and the extent of this modification was also dependent on the proximity of the lesion to the cell bodies. Since cell bodies contain the protein synthesis machinery for axons and support their metabolism, trimming axons is expected to reduce the metabolic load of their parent cell bodies and may thus lead to their shrinkage. Two factors could affect the speed and extent of the somatic shrinkage, first, the amount of axoplasm removed by the lesion, which was instantaneous; second, the subsequent retrograde degeneration of the axons proximal to the lesion, which however was extremely slow in the CNS (Tseng et al., 1995). Thus, the first factor is likely to dominate the response of the injured neurons. This rationale could easily explain the larger and precipitous shrinkage of the L-RS cell bodies following proximal than distal axotomy since the former removed a much longer segment of axons, at least 4–5 cm longer than the latter instantaneously (Tseng et al., 1995).

Regarding dendrites, neuronal interaction with targets determines their configuration and the depletion of this interaction causes retraction of dendrites (Sumner and Watson, 1971; Standler and Bernstein, 1982; Yawo, 1987; Naumann et al., 1992; Brännström et al., 1992a,b; O’Hanlon and Lowrie, 1995); however, cat neck motoneurons on the contrary expand their dendritic trees following axotomy (Rose and Odlozinski, 1998). In the present model, spinal axotomy, which removed the interaction of these neurons with their spinal targets, caused the injured neurons to retract their dendrites near terminations (Tseng and Hu, 1996). This in effect might have resulted in the elimination of short terminal dendrites thereby reducing the total numbers of terminal dendrites without shortening their total dendritic length dramatically or changing the number of their dendritic trunks. Another observation was that shrinkage of dendritic arbors in L-RS neurons was in general much less than the alterations of their cell bodies and it leveled off at about 85% of that of control neurons, which in the case of proximal axotomy occurred within 4 weeks after axotomy (Fig. 7B). This relatively minor dendritic reduction could have resulted from two counteracting influences. On the one hand, axonal injury and the subsequent reduction of cell body sizes could induce shrinkage. On the other hand, the persistence of excitatory afferent contacts (Wang and Tseng, 2002) would do the opposite since synapticca I released glutamate could exert a trophic effect sufficient to maintain dendritic structures via AMPA receptor activation (McKinney et al., 1999). The dominance of the latter factor in the present model is supported by the fact that these neurons continue to express normal levels of AMPA receptor subunits following spinal axonal injury (Wang and Tseng, 2002). These two rivaling factors might reach a quasi-balance not long after injury and remain more or less the same at least until about 8 weeks following injury.

L- Versus C-RS Neurons: Normal and following Distal Axotomy

In this study, we found that although they both project to the spinal cord and are multipolar in shape, L-RS neu-
rons in general had larger cell bodies, slightly longer total dendritic length and higher total number of terminal dendrites than C-RS neurons. However, none of these differences was statistically significant perhaps due to the large variability between neurons and the relatively small sample size of C-RS neurons. To find out whether these two groups of neurons responded differently to distal axotomy, C2-tractotomized C-RS neurons were compared with T10-tractotomized L-RS neurons. Both neurons reduced their soma sizes significantly 4 weeks following axonal injury, however C-RS neurons seemed to be more severely affected than L-RS neurons (Fig. 10A). This could be reasoned based on the assumption that injured cell’s reaction is proportional to the fraction of their cytoplasam removed. In this regard, the spinal axons of C-RS neurons are at least 4–5 cm shorter than those of L-RS neurons (Tseng et al., 1995), therefore the total amount of cytoplasam of C-RS neurons is likely to be less than that of L-RS neurons. Assuming that the removed distal axons contained about the same amount of cytoplasam in both groups of neurons, this amount would account for a larger fraction of the total cytoplasam of C-RS neurons. Thus, distal axotomy would reduce the metabolic load of C-RS neurons more, thereby a greater instantaneous reduction of their soma sizes. Another observation is that distal axotomy caused no slow progressive reduction of the soma sizes of C-RS neurons from 4 to 8 weeks following injury (Figs. 7C and 10A). This perhaps reflected our earlier observation that the proximal spinal axonal stumps of C-RS, but not L-RS, neurons retained the ability to transport retrograde tracer following axonal transection (Tseng et al., 1996). If axonal transport capability reflects the functional intactness of axons, this would suggest the absence of retrograde axonal degeneration, that is, the lack of a slow removal of the cytoplasam from the proximal axonal stump of C-RS but not L-RS neurons following injury. This is expected to cause no slow decrease in the metabolic demand of the parent cell bodies of C-RS neurons following their initial drop. Interestingly, our results also showed that distal axotomy-induced alterations of the total dendritic length and number of terminal dendrites of C-RS neurons also displayed no late reduction component like that occurred to their cell bodies. These trivial differences between the responses of C- and L-RS neurons to distal axotomy (Fig. 10B,C) could also be explained by the same reasoning related to our observation that the proximal stumps of the C- but not L-RS axons retained retrograde transport capability following distal axotomy (Tseng et al., 1996).

Thus, C-RS neurons seems to respond to axotomy with a different time course from that of L-RS neurons. This suggests that upper cervical spinal trauma could cause a condition different from that following lower thoracic spinal trauma since C-RS neurons could only be axotomized following the former. On the other hand, upper cervical spinal trauma could also simultaneously damage the spinal axons of L-RS neurons close to their cell bodies. Our present data show that, like C-RS neurons axotomized at this vertebral level proximally axotomized L-RS neurons responded with a large initial drop of their dendritic spread, which also stabilized in 4 weeks although their cell bodies continued to shrink at least until 8–weeks post-injury. Thus, our results suggest that cord-projection central neurons might enter a temporarily stabilized functional status sooner following upper cervical than lower thoracic axotomy although the former did however cause more serious cell body shrinkage than the latter. In rubrospinal neurons, the expression of C-Jun was increased and maintained for about 2 weeks following upper cervical but not lower thoracic axotomy (Jenkin et al., 1993). In addition, their expressions of several cytoskeletal protein mRNAs were increased and maintained for about a week following upper cervical axotomy (Tetzlaff et al., 1991), while lower thoracic axotomy triggered only marginal responses (Fernandes et al., 1999). These cervical axotomy-associated short-lasting responses might play a role in the quick settling of the dendritic modification of rubrospinal neurons following upper cervical axotomy. However, how does this dendritic modification characteristic relate to the ability of these neurons to regenerate their axons following upper cervical axotomy (Richardson et al., 1984; Fernandes et al., 1999) remains to be investigated.

**Significance of Axotomized Neurons to Survival with Relatively Intact Dendritic Arbors**

Our data show that cord-projection central neurons survive spinal axotomy with greatly reduced soma sizes and marginally shortened dendritic spread at least within the first two months of injury. At the same time, injured neurons expressed normal levels of ionotropic glutamate receptors while excitatory afferents continue to contact them at synapses of normal appearances (Wang and Tseng, 2002). However, inhibition from nearby reticular formation, which presumably were activated by cortical excitation was reduced (Fu et al., 1996; Chen and Tseng, 1997). These indicate that surviving injured neurons were dominated by excitatory inputs. On the other hand, axotomized neurons survived with higher input resistance, steeper f-I slope, and higher steady-state spiking frequency than normal neurons (Chen and Tseng, 1997) suggesting that they were more excitable than their normal counterparts. Under these circumstances axotomized cells are expected to activate their supraspinal targets that
remained to be innervated (Wang et al., 2000) more vig- orously than normal neurons. Another consideration is that all our findings suggest that cord-projection central neurons quickly resume the operation of their remaining functions following spinal axotomy. This is in sharp contrast to central neurons projecting to peripheral targets, for example, facial motoneurons, which quickly shielded itself from excitatory inputs (Blinzinger and Kreutzberg, 1968) and entered a ready-to-regenerate mode. The characteristics associated with cord-projection central neurons may thus expose then to excitotoxicity (Choi, 1988; Wang and Tseng, 2002). In fact, our latest studies show that unlike facial motoneurons, cord-projection central neurons failed to upregulate the expression of Mn–superoxide dismutase (Tsai and Tseng, unpublished observations) to cope with the potential oxidative stress resulted from the enhanced neuronal excitability following axonal injury (Gonzalez-Zulueta et al., 1998). Alternatively, the responses of the soma and dendrites - the receiving part of the spinaly axotomized cord-projection central neurons could also be taken optimistically. The relatively intact dendritic morphology, sustained excitatory afferents (Wang and Tseng, 2002) and the maintenance of functional membrane properties (Chen and Tseng, 1997) suggest that injured neurons survived in a reasonably sound functional status. This suggests that the cells may be in a status capable to resume their spinal functions if the regeneration of their spinal axons can be achieved. In this regard, post-trauma treatments that promote and/or sustain the survival of these injured neurons may be warranted since the regeneration of severed spinal axons if achievable may be time-consuming.

ACKNOWLEDGMENTS

This work was supported by research grants from the National Science Council of Taiwan (NSC-89-2320-B002-077 and NSC-89-2320-B320-037) to G.-F.T. and Y.-J.W.

REFERENCES


Address reprint requests to:
Guo-Fang Tseng, Ph.D.
Department of Anatomy and Cell Biology
College of Medicine
National Taiwan University
No. 1, Section 1, Jen-Ai Rd.
Taipei, Taiwan, 100

E-mail: guofang@ha.mc.ntu.edu.tw