Axonal Regeneration of Retinal Ganglion Cells Depending on the Distance of Axotomy in Adult Hamsters

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PURPOSE. To examine the relationship between the distance of axotomy and axonal regeneration of injured retinal ganglion cells (RGCs) systematically and the effect of a predegenerated (pretransected or precrushed) peripheral nerve (PN) graft on axonal regeneration of RGCs axotomized at a definite distance (0.5 mm from the optic disc) in comparison with a normal PN graft.

METHODS. The optic nerve (ON) was transected intraorbitally at 0.5, 1, 1.5, 2, or 3 mm or intracranially at 6 to 8 mm from the optic disc, and a PN graft was transplanted onto the ocular ON stump in adult hamsters. Four weeks after grafting, the number of RGCs regenerating their injured axons into the PN graft was investigated in all animals.

RESULTS. The number of regenerating RGCs decreased significantly when the distance of axotomy increased from 0.5 to 7 mm. A precrushed PN graft was shown to enhance more injured RGCs to regenerate axons than a normal or pretransected PN graft.

CONCLUSIONS. The distance of axotomy on the ON of adult hamsters is critical in determining the number of regenerating RGCs. Thus, experimental strategies to repair the damaged ON by PN transplantation is to attach a precrushed PN graft as close to the optic disc as possible to obtain optimal axonal regeneration of the axotomized RGCs. (Invest Ophthalmol Vis Sci. 2000;41: 3165–3170)

The effect of axotomy on the survival and regenerative capacity of injured retinal ganglion cells (RGCs) depends on several factors. In adult mammals, the nature and site of axonal injury are crucial determinants of RGC survival and regenerative capacity. More RGCs are lost after transection than after crush of the optic nerve (ON), and more RGCs survive after an intracranial lesion than after an intraorbital lesion of the ON. However, no or very few RGCs regrow their injured axons into a peripheral nerve (PN) graft apposed to the ocular ON stump after intracranial axotomy; in contrast regrowth of axons into PN grafts is more prolific from those RGCs that survive axotomy close to their cell bodies.

In order to examine systematically the relationship between the extent of axonal regeneration and the distance between site of axotomy and the cell body and to develop hypotheses concerning factors affecting the responses of central neurons axotomy, we have observed the number of RGCs regenerating axons into PN grafts apposed to ONs cut at various distances intraorbitally and at approximately 7 mm intracranially from the optic disc.

There has been some controversy concerning the possible enhanced ability of predegenerated PN grafts to support regeneration. For this reason we also compared the ability to support regeneration of freshly harvested and predegenerated (pretransected or precrushed) PN grafts. For these experiments the PN grafting was performed at a fixed distance from the optic disc. Some of these results have been presented in abstract.

METHODS

Forty-eight 8-week-old adult female hamsters (Mesocricetus auratus; The Laboratory Animal Unit of Faculty of Medicine, The University of Hong Kong) were used in the present study, and these animals were divided into 2 groups (group I and group II). Group I consisted of 6 subgroups with 6 different transection sites on the ON. Another 2 subgroups were set up in group II to investigate the effects of pretransected and precrushed PN grafts on axonal regeneration of RGCs. Similar lengths of the ON were expected in animals with similar body weights (90–100 g). All animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, 60 mg/kg body wt.; Rhone Merieux Australia Pty Ltd., Pinkenbo, QLD, Australia), and all operations were performed with the use of an operating microscope (Olympus OME, Tokyo, Japan). This investigation adhered to the tenets of the ARVO Statements for the Use of Animals in Ophthalmic and Vision Research.

Surgical Procedures

The left ON of group I animals was transected intraorbitally 0.5, 1, 1.5, 2, or 3 mm (n = 30, 6 for each subgroup)
intracranially approximately 7 mm from the optic disc (n = 6). After exposure of the posterior pole of the left eye and the origin of the left ON through a superior temporal intraorbital approach, the dural sheath was longitudinally excised, taking care to avoid damage to the ophthalmic artery located on the inferomedial dural sheath of the ON. The ON was then gently separated from the dorsal aspect of the sheath and completely transected at 0.5, 1, 1.5, 2, or 3 mm from the optic disc using a pair of small iris scissors (Adler, Germany). The distance was accurately measured with the aid of a small ruler. Intraorbital grafting was achieved immediately after ON transection by attaching the proximal tip of a segment of an autologous normal PN (the sciatic nerve) dissected from the left leg to the ocular stump of the transected ON (Fig. 1A). The two nerve ends were reconnected with a 10–0 suture (Ethilon, W2814; Ethicon Ltd., UK). The remaining part of the graft was laid along the midsagittal line, and the left frontal bone was exposed at approximately 7 mm from the optic disc (ca. 2 mm²) was performed using a dental drill (Dremel, USA) in the left frontal bone just rostral to bregma, and a portion of the left frontal lobe below the craniotomy was sucked away until the left ON was exposed. A hole was then made in the meningeal membrane of the inferior surface of the frontal lobe that had not been disrupted during aspiration of the brain tissue. The hole was gently enlarged, and the underlying ON with the dural sheath was completely transected at approximately 7 mm from the optic disc using a pair of small iris scissors (Adler) without any bleeding from the adjacent blood vessels. The distance of the intracranial axotomy has been measured in a pilot study of animals by dissecting the ON at other distances (1, 1.5, 2, or 3 mm) from the optic disc is also shown. RGCs that regenerate their axons into the PN graft are labeled retrogradely with FG applied to the distal end of the graft (lower). (B) Schematic diagrams illustrating intracranial ON transection and the transplantation of a normal PN graft to the ocular ON stump (upper). RGCs that regenerate their axons into the PN graft are labeled retrogradely with FG applied to the distal end of the graft (lower).

In group II animals (n = 12), the left ON was transected intracranially at 0.5 mm from the optic disc with surgical procedures similar to those described above. However, a different transplantation procedure was used in which a predegenerated (pretransected or precrushed) PN graft was apposed to the ocular ON stump (Fig. 1A). The left sciatic nerve was completely transected at mid-thigh level in 6 animals 8 days before transplantation, with the cut end of the distal portion of the transected PN being ligated with a 5–0 suture (Mersilk, W595; Ethicon Ltd.) and displaced in the surrounding muscles to ensure that no regenerating axons from the proximal PN stump were able to grow into the distal nerve stump. In the other 6 animals, the left sciatic nerve was crushed with a pair of fine forceps (Aesculap, Germany) at the same mid-thigh level to test the effect of a precrushed PN graft on axonal regeneration of RGCs. The interval of 8 days was selected because optimal neurite outgrowth-supporting activities were detected in 5 to 8 day pre-degenerated sciatic nerve.15,16

Labeling and Counting of Regenerating RGCs

After the surgery, the animals with intraorbital grafting were allowed to survive for 4 weeks, while the animals undergoing intracranial grafting procedures survived for 8 weeks to ensure sufficient time for axonal regeneration to occur based on the maximum rate of growing optic axons in a PN graft (approximately 1–2 mm/d).17,18 A longer survival time was used for the intracranial group of animals because, compared with the intraorbital group, the distance of axotomy was at least 4 mm longer, and the site of FluoroGold application was further away from the attachment of the PN graft (see below). Three days before they were killed, the PN graft in the animals with intraorbital grafting was exposed and severed at approximately 0.5 cm from the attach-
ment site. For the animals with intracranial grafting, the PN graft was exposed and severed at the site of craniotomy at approximately 1 cm from the attachment site. A small piece of Gelfoam soaked in 6% FG was applied to the cut end of the graft to retrogradely label the regenerating RGCs (Fig. 1). No transcardial perfusion was carried out. The left eye was enucleated immediately after the animal was killed with an overdose of sodium pentobarbital. The retina was dissected in 4% paraformaldehyde (Merck, Germany) in phosphate buffer (0.1 M, pH 7.4; Sigma), post-fixed in the same fixative for 1 hour, and rinsed in phosphate-buffered saline (0.1 M, pH 7.4; Sigma) for three times. It was then flatmounted in glycerol (Merck) on a glass slide and coverslipped. The FG-labeled regenerating RGCs were counted in all the retinas under a fluorescence microscope (model MM-11; Nikon, Japan) equipped with an interchangeable filter (400 nm for FG).

**Statistical Analysis**

For statistical analysis, Mann–Whitney U test\(^1\) was used to compare the mean numbers of regenerating RGCs between every two subgroups. These comparisons were conducted to take into account the relationship between the number of regenerating RGCs and the increased distance of axotomy from the optic disc; a possible critical distance of ON transection for axotomized RGCs to regenerate into a PN graft; and the different effects of a normal or predegenerated PN graft on the number of regenerating RGCs at the same distance of ON transection. All statistical analyses were performed on a computer using SPSS for Windows statistical package (version 6.0, 1989–1993; SPSS, Cary, NC.).

**RESULTS**

The number of regenerating RGCs was determined by counting retrogradely FG-labeled RGCs in all retinas. In group I, the labeled RGCs were consistently distributed over the whole retinal eccentricity and covered all quadrants, indicating that RGCs from the entire retina contributed to the regeneration of axons in the PN graft. RGC density was higher in the central retinal regions, decreasing toward the peripheral retina. The most intensive distributions of RGCs were located in the superotemporal quadrants, and the lowest RGC densities were found in the inferior nasal quadrants. The general morphology of regenerating RGCs (Fig. 2A) in all subgroups was similar to that of regenerating RGCs described previously.\(^2\) They usually had enlarged cell bodies with simplified dendrites.

The number of RGCs regenerating axons into the normal PN grafted onto the ocular stump of the ON transected at 0.5 (Fig. 2C), 1, 1.5, 2 (Fig. 2D), 3, or 7 mm from the optic disc was 1644 ± 621, 1146 ± 163, 618 ± 56, 352 ± 45, 52 ± 16, or 0.33 ± 0.52, respectively (mean ± SD). The raw data for each animal in all the subgroups (n = 6) are listed in Table 1. It was shown that there was a significant decrease (P = 0.0104) in the number of regenerating RGCs with an increase in distance of axotomy and grafting (Fig. 3). Significant differences were observed between every two adjacent transection sites (i.e., 0.5–1, 1–1.5, 1.5–2, 2–3, and 3–7 mm distance points with P = 0.0104, 0.0039, 0.0039, 0.0039, and 0.0033, respectively) and maintained when two distant transection sites were compared (i.e., 0.5–1, 1.5, 2, 3, or 7 mm and 1–1.5, 2, 3, or 7 mm; 1.5–2, 3, or 7 mm; and 2–3 or 7 mm). Thus, all distances equal to or longer than 0.5 mm between two transection points were significantly different. A nonparametric test was used (Mann–Whitney U test), and the figures for the median and range of all groups of animals are listed in Table 1.

The distribution and morphology (Fig. 2B) of the regenerating RGCs in group II animals with a predegenerated PN graft were similar to those in group I. Increased number of regenerating RGCs (P = 0.0131) was detected in the animals with the precrushed PN graft (2465 ± 568; Fig. 2F) when
compared with those with the normal (1644 ± 212; Fig. 2C) or pretransected (1628 ± 579; Fig. 2E) PN graft (Fig. 4). No significant difference was shown between the animals with the normal and pretransected PN grafts (P > 0.05; Fig. 4).

DISCUSSION

The effects of distance of axotomy on RGC survival and regeneration reported previously might be due to different types of intraorbital and intracranial ON lesions. The present study provides a systematic attempt with the same type of intraorbital ON lesion to elucidate how the number of regenerating RGCs decreases with an increase in the distance of axotomy from the optic disc. We have shown that there is a tight correlation between distance of axotomy on the ON of adult hamsters and the number of RGCs regenerating into a PN graft, and a precrushed PN graft can enhance more injured RGCs to regenerate than a normal or pretransected PN graft. Therefore, experimental manipulations using a PN graft to repair the damaged ON should use a precrushed PN transplanted as close to the optic disc as possible to obtain optimal axonal regeneration of the axotomized RGCs.

In the present study, the number of regenerating RGCs decreased significantly when the distance of axotomy increased from 0.5 to 7 mm, with a sharp decline between 0.5 and 3 mm. Our data do not seem to relate to the increased survival of RGCs because Villegas-Pérez and colleagues have shown in the rat that after an intraorbital ON cut at 0.5 or 3 mm or an intracranial ON crush at 8 or 10 mm, the number of surviving RGCs decreases as the distance of axotomy increases. Thus, we have formulated the following two hypotheses to explain our result. First, regarding neurotrophic effect of the PN graft, in order for neurons to initiate an extensive regenerating response, stimulatory signals initiated by the neurotrophic factors (NTFs) released from the PN graft, which is attached to the ON stump, might be required. Schwann cells have been shown to secrete many different types of NTFs, and some may be important for axonal regeneration (nerve growth factor, fibroblast growth factor, platelet-derived growth factor, ciliary neurotrophic factor [CNTF], glia-derived growth factor, and brain-derived neurotrophic factor). However, the NTFs contained in the PN graft might not be effective in inducing regeneration after distal axotomy because

TABLE 1. Raw Data, Mean Number, SD, Median, and Range of Regenerating RGCs in Different Subgroups

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>No. of Regenerating RGCs (n = 6 For Each Subgroup)</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal PN</td>
<td>0.5 mm</td>
<td>1889 ± 1820</td>
<td>1668 ± 1636</td>
<td>1567 ± 1287</td>
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<tr>
<td></td>
<td>1.0 mm</td>
<td>1382 ± 1298</td>
<td>1159 ± 1037</td>
<td>1005 ± 999</td>
</tr>
<tr>
<td></td>
<td>1.5 mm</td>
<td>707 ± 641</td>
<td>623 ± 609</td>
<td>596 ± 536</td>
</tr>
<tr>
<td></td>
<td>2.0 mm</td>
<td>400 ± 390</td>
<td>367 ± 352</td>
<td>332 ± 276</td>
</tr>
<tr>
<td></td>
<td>3.0 mm</td>
<td>76 ± 67</td>
<td>52 ± 47</td>
<td>40 ± 33</td>
</tr>
<tr>
<td></td>
<td>7.0 mm</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Pretransected PN</td>
<td></td>
<td>2568 ± 2118</td>
<td>1409 ± 1309</td>
<td>1246 ± 1124</td>
</tr>
<tr>
<td>Precrushed PN</td>
<td></td>
<td>3135 ± 2922</td>
<td>2730 ± 2374</td>
<td>1934 ± 1695</td>
</tr>
</tbody>
</table>

FIGURE 3. The line graph shows that 4 weeks after intraorbital or 8 weeks after intracranial ON transection and PN transplantation, the number of regenerating RGCs decreased significantly when the distance of axotomy increased from 0.5 to 7 mm from the optic disc. There were six animals in each of the experimental subgroups. Error bar, SD.

FIGURE 4. The histogram illustrates the mean numbers of RGCs regenerating their axons into a normal or predegenerated (pretransected or precrushed) PN graft. The black column with an asterisk at the 0.5-mm distance point represents the mean number of regenerating RGCs in the precrushed PN graft subgroup (n = 6), which is significantly different (P = 0.0374) from those in the normal (n = 6) or pretransected PN graft (n = 6) subgroups. Error bar, SD.
the signals might have progressively dissipated with increasing length of the optic axons or it might take too long for the stimulatory signals to reach the cell bodies to have any significant effect.\textsuperscript{7} Intravitreal PN grafts have been shown to enhance the regeneration of axotomized RGCs,\textsuperscript{5,27} and we now know that CNTF might be responsible for such a promoting effect.\textsuperscript{28,29} Second, regarding the inhibitory effect of the ON, there are neurite growth inhibitory components\textsuperscript{30} such as NI-35 or NI-250(Nogo-A)\textsuperscript{31,32} present in myelin and oligodendrocyte membranes in the central nervous system (CNS), which might generate some retrograde signals to the cell bodies to suppress the growth potential of the axons by preventing or delaying axotomized neurons from switching to a growth mode. Thus, proximal axotomy would remove most of these nonneuronal components and also eliminate most of the retrograde inhibitory signals, leading to vigorous regeneration of axotomized RGCs. However, very few RGCs can regenerate after distal axotomy because a large number of the inhibitory components are still interacting on the ON, and they could send sufficient retrograde inhibitory signals to suppress axonal regeneration of axotomized RGCs. It could be observed during the surgery that the very proximal part (approximately 0.5 mm from the optic disc) of the ON is thinner, whereas the part longer than 0.5 mm from the optic disc is much thicker because myelinated fibers are scarce or even absent in the proximal part of the ON.\textsuperscript{53} The lack of myelin and oligodendrocytes in the beginning part of the ON suggests the absence of neurite growth inhibitors and, therefore, allows more axotomized RGCs to regenerate into a PN graft. In the present study, the highest number of regenerating RGCs has been obtained with the axotomy at 0.5 mm from the optic disc, thus providing some support for this hypothesis.

Although the extrinsic influences exerted by nonneuronal cells are important for CNS axonal regeneration, we cannot exclude the possibility that some CNS neurons lose intrinsic regenerative capability. Recent studies have shown that most RGCs lose their regenerative capacity once the retinal axons shift from an elongation to an arborization growth mode.\textsuperscript{34} GAP-43 is not detected in normal RGCs of adult rats or hamsters. Very few damaged RGCs express GAP-43 after distal axotomy, but a lot more express this protein when axotomy is close to the cell bodies.\textsuperscript{27,55,56} In addition, after proximal axotomy, the expression level of GAP-43 messenger RNA in the axotomized retinas was shown to be increased.\textsuperscript{59} The expression of c-Jun begins later and declines faster after distal than proximal ON transection.\textsuperscript{57} Axotomized RGCs regenerating through a PN graft coexpress c-Jun and GAP-43 for several weeks.\textsuperscript{38,59} Deprivation of NTFs or removal of inhibitory factors, as a result of ON transection, could be part of the explanation for the expression of growth-related genes such as GAP-43. It is possible that the distance of axotomy can affect the intrinsic growth potential in RGCs and thus influence the regenerative capacity of RGCs. In addition to the effect of the distance of axotomy, the interruption of blood supply to the intracranial portion of the ON might be an additional factor to explain why very few RGCs were able to regrow into the PN after intracranial ON transection. The blood supply to the retina was not affected when the ON was transected intraorbitally (0.5–3 mm from the optic disc), because the ON was cut after opening the dorsal part of the dura. However, the entire ON and surrounding meninges were severed simultaneously when we performed intracranial ON transection. This procedure might disrupt some of the blood supply to the ON, resulting in ischemic necrosis in the distal part of the ocular ON stump.\textsuperscript{5,40} However, such ischemic damage could not be found after intraorbital ON transection.\textsuperscript{41} Thus, the ischemic necrosis might result in the formation of an extensive scar, which could limit retrograde axonal transport of NTFs toward the cell bodies of RGCs and prevent axons from regenerating into the PN graft after intracranial ON transection.

The evidence for the enhancing effect of predegenerated PN segments on the regeneration and survival of either peripheral nervous system or CNS axons remains inconclusive and controversial\textsuperscript{12,13,42,43} since Cajal’s early studies. Bahr and co-workers observed that a PN graft precrushed 1 week before grafting significantly enhances more RGCs to survive and regenerate compared with a normal PN graft 3 months, but not 6 months, after transplantation.\textsuperscript{12} However, Thanos and Mey\textsuperscript{13} revealed that a similar precrushed PN cannot enhance the number of regenerating RGCs 2 months after grafting. These opposing results were interpreted by Thanos as different labeling methods used to detect RGCs in the two research groups. In the present study, the precrushed PN graft induced more RGCs to regenerate than the normal and pretransected PN graft at the 0.5 mm distance point 4 weeks after grafting. As compared with the pretransected PN graft, the continuity of the nerve sheaths and the basal lamina is preserved in the precrushed PN graft before grafting. Axonal degeneration in the PN is slower after a crush than after a cut.\textsuperscript{44} There is normally a second phase of Schwann cell proliferation in which regenerating axons grow into the distal nerve stump.\textsuperscript{45} More NTFs produced by a greater number of Schwann cells in the precrushed PN graft may contribute to the greater number of RGCs regenerating into the precrushed PN graft observed in the present study. In addition, various extracellular molecules have been suggested to be important for underlying the ability of Schwann cells to foster axonal regeneration. These include L1,\textsuperscript{56} L2,\textsuperscript{47} N-cadherin,\textsuperscript{48} neural cell adhesion molecules (N-CAM),\textsuperscript{49} and heparan sulfate proteoglycan complex.\textsuperscript{50} Whether any of these molecules are differentially upregulated after a crush or cut lesion of the PN remains to be investigated.

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References


34. Chen DF, Jhaveri S, Schneider GE. Intrinsic changes in developing retinal neurons result in regenerative failure of their axons. Proc Natl Acad Sci USA. 1995;92:7287–7291.


