Sprouting of Axon-like Processes from Retinal Ganglion Cells as an Alternative Mode of Axonal Repair after Injury
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Introduction

The pioneering work of Ramón y Cajal (1928) has shown that adult neurons of the mammalian central nervous system (CNS) cannot achieve functional regeneration after injury, in sharp contrast to neurons damaged in the peripheral nervous system (PNS). This pessimistic view has recently begun to change as new experimental data are revealing the mechanisms of regenerative failure of CNS neurons and how they can be stimulated to regain their functions. It is now known that the environment of the adult CNS rather than some intrinsic properties of the neurons which is responsible for their incapability to regenerate their axons. For instance, growth inhibitory molecules have been found to be associated with oligodendrocytes and the myelin they produce (Caroni and Schwab, 1988), and microglial cells actively destroy injured neurons (Thanos et al., 1993). A number of strategies have been used to try to overcome the adverse CNS environment, either by suppressing the activities of the glial cells and their inhibitory factors (Schnell and Schwab, 1990; Thanos et al., 1993), or replacing the CNS environment with one more favourable for growth, such as foetal nervous tissue (Sievers et al., 1989) or peripheral nerve (PN) (Aguayo, 1985). Using the visual system as a model, we and others have transplanted segments of PN into the retina or replaced the optic nerve (ON) with a segment of PN in adult rodents and have demonstrated that retinal ganglion cells (RGCs) can regenerate lengthy axons along the graft (So and Aguayo, 1985; Berry et al., 1986; So et al., 1986; Vidal-Sanz et al., 1987). By connecting the other end of the graft to the superior colliculus, well differentiated synapses have been observed (Carter et al., 1989), and electro-physiological responses evoked by regenerating retinal axons could be recorded in the superior colliculus (Keirstead et al., 1989).

Although it is now known that some damaged RGCs may be able to reconnect with both their afferent and target neurons physiologically, the population of RGCs which are able to regenerate their axons along the PN is only about 10% of the total RGC population at most, and the number of functionally reconnected RGCs is even
smaller. Thus, it raises the question of to what extent can the behaviour of CNS neurons be modified to enhance the degree of functional recovery. Our recent studies indicate that RGCs can display great morphological plasticity under the appropriate stimuli and which may be viewed as an attempt to achieve restoration of function. Specifically, an axotomized RGC can produce *de novo* axon-like processes when the damaged axon cannot regrow, suggesting an alternative mode of repair and reconnection with the target.

**Axonal regeneration and axon-like process formation**

In previous studies in which an autologous PN segment was grafted to the retina of adult rats and hamsters (Fig. 1A), extensive regrowth of the damaged axons of RGCs in the graft could be induced, as shown by labelling the cells with a retrograde tracer, horseradish peroxidase (HRP) applied to the graft (Fig. 1C) (So and Aguayo, 1985; So *et al.*, 1986). This contrasts with the abortive growth of RGC axons when they are injured in the retina (McConnell and Berry, 1982) or ON (Kiernan, 1985) and suggests that the PN provides specific trophic factors and/or substrate necessary for axonal regrowth.

During the course of our study on the axonal regrowth of RGCs using the PN grafting paradigm, a novel type of sprouting was discovered (Cho and So, 1989). When PN grafting to the retina was performed *concurrently* with intraorbital ON crush (Fig. 1B), a small population of RGCs located central to the grafting site became labelled by HRP applied to the graft (Fig. 1D) (Cho and So, 1989). The fact that these cells are retrogradely labelled indicates that they have sent neuronal processes into the graft. Using a reduced silver staining technique modified from the protocol of Leicester and Stone (1967), it was demonstrated that cells of the central population of RGCs elaborated new sprouts from either the soma or dendritic tree to innervate the graft (Fig. 2). Because of the resemblance of these sprouts in external appearance (constant cross-sectional thickness, sparse branching) and behaviour (long distance growth in the PN graft) to axons, the term axon-like processes (ALPs) was coined to describe them. Each RGC only emits one ALP to innervate the graft directly. However, in 31% of the cells studied, a few additional ALPs (ranging from 1 to 8) are produced but they do not extend into the graft. Therefore, some mechanism seems to be in operation to limit the number of ALPs per RGC which is able to innervate the PN.
Factors affecting sprouting of ALPs

Axotomy of RGCs is a pre-requisite for inducing ALPs. This is indicated by the fact that when PN grafting to the retina is performed in the absence of ON crush, the central population of sprouting RGCs (which emit ALPs) do not appear. Thus, intact RGCs or those which suffer damage to the dendritic tree alone do not elaborate ALPs even in the presence of a PN.

The idea that axonal damage provides a specific signal for sprouting is further suggested by the finding that the distance of axotomy from the soma dictates whether ALPs could be elicited. If a PN is grafted to the retina and concurrently the optic pathway is lesioned at distances from the eye greater than that of intraorbital ON crush, the central population of sprouting RGCs becomes greatly diminished (Cho and So, 1993). This decrease of the sprouting stimulus as a result of the distant axotomy stands in contrast to the less severe neuronal cell death seen after similar axonal injury (Lieberman, 1974; Villegas-Perez et al., 1989). Further studies with our model may shed light on the nature of the axotomy signal and how it regulates neuronal sprouting and survival differentially.

The growth status of the damaged axon dictates whether ALPs can be produced. When the proximal stump of the injured axon is in contact with the PN graft, only extensive axonal regeneration into the graft occurs. In contrast, RGCs of the central population whose damaged axons reside in the ON and cannot regenerate because of the unfavourable CNS environment will elaborate ALPs to innervate the graft.

Sprouting of processes which resemble axons have been described in some vertebrate CNS neurons after axotomy (lamprey anterior bulbar neurons: Hall and Cohen, 1988; cat α-motoneurones: Lindā et al., 1985; Havton and Kellerth, 1987) but they do not seem to require any favourable extrinsic stimulus to support their formation. However, the presence of a PN graft placed close to the axotomized RGCs is necessary to stimulate the formation of ALPs. In the above described PN grafting and concurrent ON crush paradigm, the central population of sprouting RGCs is confined at most only to 1.5 mm from the graft, with most cells situated less than 500 μm from it. This suggests that the trophic stimulus arising from the PN takes the form of diffusible factor(s) and exerting its action on the RGCs with a diffusion gradient effect.
Further evidence supporting a role of the active influence of the graft on the growth of ALPs comes from examining the trajectories of ALPs. When the initial trajectory of each ALP (defined as the first 100 μm of length after initiation from the cell) was measured relative to the grafting site (Fig. 3), it was found that greater than 60% of them have very small orientation angles (30° or less) with respect to the PN graft, suggesting that at least during its early phase of formation and growth, the ALP is actively guided by the PN.

**Intravitreal PN grafting stimulates axotomized RGCs to sprout ALPs**

In order to gain further insights into how the PN influence ALP formation and to study the pattern of ALP growth, a new experimental paradigm was developed: the ON was crushed intraorbitally and concurrently a 2 mm long piece of autologous PN was implanted into the vitreous body of the ON-crushed eye (Cho and So 1992). This resulted in the PN being located in the vitreous and separated physically from the retina by a small distance (Fig. 4). Thus, any influence arising from the PN is likely to operate only via a diffusion mode. Silver staining was used to examine the morphology of RGCs at 2 weeks to 2 months post-axotomy.

The presence of an intravitreal PN stimulates on average 323 axotomized RGCs to sprout ALPs at 2 weeks post-ON crush (Fig. 5B), this value dropping to 116 and 52 cells at 1 and 2 months post-ON crush respectively. The sprouts wander randomly in the various retinal laminae since the PN in this case was inaccessible for innervation. When ON crush was performed without intravitreal PN implantation, no RGCs were observed to sprout (Fig. 5A). These results suggest that diffusible factors emanating from the intravitreal PN alone is sufficient to induce sprouting of ALPs and that innervation of the PN graft is not necessary at least for the short term development of ALPs.

The ALPs arising from the axotomized RGCs could originate from three sites of the cell: the dendritic tree, soma and intraretinal axon (Fig. 6). However, a distinct preference for certain sprout initiation loci was observed: ALPs tend to arise most commonly from the dendrites while only 6% to 16% of the cells sampled from 2 weeks to 2 months post-ON crush possessed ALPs coming directly from the soma. The frequency of sprouting from the intraretinal axon laid in between with 26% to 40% of the sampled cells bearing axonal sprouts. Moreover, the
The highest number of sprouts come from the dendritic compartment (maximum 20), with the intraretinal axon in between (maximum 9), and the soma having only 1 to 2. If a PN is apposed to the cut end of the ON to induce axonal regeneration into the graft, only 0.34% of the regenerating cells exhibited dendritic or intraretinal axonal sprouts (Cho and So 1992). These observations suggest the existence of an hierarchical order of sprouting within an axotomized RGC, with the injured axonal stump being the most favoured sprouting site. Only when the damaged axon cannot regrow will ALPs be sprouted from other parts of the cell, and in this case dendrites are more only attractive than the intraretinal axon and soma. Determining the mechanisms which govern the intracellular distribution of sprouting loci could provide insights on how a developing neuron achieves its highly specific morphology.

In addition to ALP formation, the somata of the sprouting RGCs also exhibited changes in parallel with the sprouting response. For example, a drastic increase in the cross-sectional area of the soma occurred which at the peak could be more than 4 times the area of normal RGCs. Sprouting RGCs also displayed irregular somatic profiles such as surface foldings and filamentous protrusions (Fig. 5C, D). The temporal pattern of variation of these somatic changes can be correlated with the intensity of sprouting of ALPs from the dendritic tree, suggesting that the morphological changes of the soma is another manifestation of the growth behaviour exhibited by the sprouting RGCs. Interestingly, these somatic changes are more vigorous when compared to that of RGCs undergoing axonal regeneration in a PN graft, an indication that RGCs sprouting ALPs are in a more active growth state than RGCs regenerating their axons.

Conclusions

The above results highlight the exceptional ability of a mature mammalian CNS neuron to reorganize its neuronal geometry after injury. Sprouting of ALPs can be viewed as an attempt of the injured neuron to replace its damaged axon under the circumstances when axonal regeneration becomes impossible. In this regard, the feasibility of utilizing ALPs as a means of reconstructing interrupted neural pathways should be examined. However, the structural and functional properties of ALPs must first be characterized and compared with that of axons. In addition, the factors governing the initiation, growth,
stabilization and elimination of ALPs have to be elucidated in order to avoid the formation of aberrant and misdirected target connections. Studies of the ultrastructure and biochemical features of ALPs can also shed light on how a neuron maintains the identity of its processes.

References


Cho EYP, So K-F (1993) Sprouting of axon-like processes from axotomized retinal ganglion cells is influenced by the distance of axotomy from the cell body and the mode of transplantation of the peripheral nerve. Restor Neurol Neurosci 6:29-34


Kiernan JA (1985) Axonal and vascular changes following injury to the rat's optic nerve. J Anatomy 141:139-154


Ramon y Cajal S (1928) Degeneration and regeneration in the nervous system, Oxford University Press.


Legend

Fig. 1 Schematic diagram illustrating the experimental protocols employed to study the responses of RGCs after axotomy. 
(A) Transplantation of an autologous peripheral nerve (PN) segment to the retina of the eye. 
(B) Grafting of a PN to the retina together with intraorbital crushing of the optic nerve (ON). 
(C) Part of a retinal whole mount from (A) showing the distribution of RGCs which had regenerated into the graft after application of HRP to the graft at 1 month post-grafting. HRP-labelled RGCs were located in a sector peripheral to the grafting site (G). x=optic disc. 
(D) Part of retinal whole mount from (B) showing the location of HRP-labelled regenerating RGCs at not only peripheral but also central (towards the optic disc) to the grafting site. The RGCs located close to and central to the graft have elicited axon-like processes which undergo long distance growth in the PN when their damaged axons reside in the ON and cannot regenerate (see Fig. 2).

Fig. 2 Camera lucida tracings of axotomized RGCs from silver-stained retinal whole-mounts of animals in which a peripheral nerve was grafted to the retina and concurrently the optic nerve was crushed intraorbitally. 
(A) Three silver-stained ganglion cells located central to the graft exhibited sprouting of an axon-like process from the cell body to grow towards the graft. The processes have the characteristics of axons in that
they are long and sparsely branched, and they tend to maintain a constant thickness without tapering. In their course of elongation towards the graft, they can exhibit either a relatively undeviated trajectory (e.g. cell 1) or formed loops and turns (cell 2 and 3). In each cell, the original axon (which had the damaged stump residing in the optic nerve) could still be traced towards the optic disc. G: grafting site; OD: optic disc; p: axon-like process; a: axon. Scale bar= 100 μm.

(B) An axotomized ganglion cell with an axon-like process (p) emerging from the dendrite. The process heads towards the graft (located beyond the upper right corner) while the axon (a) runs in the direction of the optic disc (OD). Scale bar= 100 μm.

Fig. 3 (A), (B) Schematic diagram illustrating the determination of the orientation of the initial trajectory of the axon-like process (p) with respect to the graft. The average initial trajectory of the process is defined as a line drawn from the initiation site of the process (from the soma) to a point on the process which 100 μm distant from it (the distance between the 2 arrowheads). The angle β is the orientation of the average initial trajectory of the process with respect to the graft (G). The orientation can be smaller than 90° (as in A) or greater than 90° (in B). A smaller angle of orientation would suggest that the initial course of the process might be under the influence of the graft.

(C) Histogram illustrating the initial trajectory of the axon-like process with respect to the graft. It was found that 59% of the cells had an initial course which was oriented at 30° or less with respect to the grafting site, suggesting that at least in the early stage of initiation and growth of the axon-like process, it was under the active influence of the graft, presumably via diffusible trophic factors.

Fig. 4 Implantation of a short PN into the vitreous body (V) of an eye through a small opening at the superior aspect of the limbus. The ON was also crushed intraorbitally at the same time. The PN was found either to be located close to the limbal insertion site or attached to the lens (L) in the vitreous. In either case there was no direct physical contact between the PN and retina.

Fig. 5 Silver staining of retinas after intraorbital ON crush alone (A) or concurrent intravitreal PN implantation (B-D). In the presence of an intravitreal PN, some axotomized RGCs exhibited sprouting of axon-like processes and they were deeply stained (B). No such sprouting was seen in (A) without an intravitreal PN. (C) and (D) illustrated the
different soma morphologies of the sprouting RGCs. Scale = 50µm (A,B); 10µm (C,D)

Fig. 6 Photomicrographs of silver stained RGCs which exhibit sprouting of axon-like processes from various cellular compartments after axotomy.
(A) Sprouting from the dendritic tree. A sprout (labelled by arrows) could be seen originating from a dendrite. Sprout elongation occurred in various retinal laminae and was extremely random as suggested by the complex looping behaviour.
(B) Sprouting from the intraretinal axon. Two sprouts (initiation site denoted by arrows) could be identified arising from the parent axon (a). The sprouts were not directed towards the optic disc (OD) as was the parent axon but instead exhibited random growth.
(C) Sprouting from the soma. The axon-like sprout (*) arose at a point (arrowhead) on the cell almost directly opposite to the axon initiation site (arrow). The 2 processes could be distinguished by the trajectory and location: the original axon (a) resided in the nerve fibre layer and projected towards the optic disc (OD) whereas the sprout displayed loops and was not confined to a specific lamina. Scale bar = 25µm (A-C)
Fig. 2
Fig. 3