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Review

Why do mature CNS neurons of mammals fail to re-establish connections following injury – functions of Bcl-2

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Abstract

Factors inside and outside neurons control the process of axonal growth and regeneration. Recently, it has become apparent that neurons are determined intrinsically for their ability to grow axons. In the mammalian CNS, the intrinsic machinery of neurons that triggers the growth of axons during early embryonic stages is shut down at a certain point in development; as a consequence, axon elongation and regeneration cannot occur in postnatal life. The proto-oncogene Bcl-2 has been recognized to act as a key regulator for the program of axon elongation inside neurons. However, expressing the gene Bcl-2 in CNS neurons is not sufficient to induce nerve regeneration in the adult CNS, eliminating the inhibitory mechanism in the mature CNS environment is still required. Recently, the formation of glia scar has been reported to be the major limiting factor in the CNS environment that blocks nerve regeneration. These new discoveries challenge the classical view of nerve regeneration in the mammalian CNS. It opens up a new dimension in the study of the cellular and molecular mechanisms underlying neurodevelopmental and neurodegenerative diseases.

Keywords: axon elongation; axon arborization; neuronal cell death; organotypic coculture; retinal ganglion cells; Bcl-2

Abbreviations: CNS, central nervous system; DRG, dorsal root ganglion; PNS, peripheral nervous system; RGC, retinal ganglion cell; E19 or P0, the day of mating is designated E0, day of birth is at E19 in mice and is designated P0

Introduction

Neurons contact and communicate with each other through long processes, named axons. In the adult CNS of mammals, which is composed of brain and spinal cord, axons regenerated poorly following injury. This leads to a catastrophic consequence to patients suffering from CNS damages, usually a result of trauma, stroke, or other neuronal diseases. Therefore, it presents challenging problems for neuroscientist. Why is it that the adult CNS of higher vertebrates, including mammals and birds, has little or no capacity for functional recovery? Why is it then, that axonal pathways can be formed and reformed once disrupted in the developing CNS as do peripheral nerves regenerate successfully throughout life with recovery of function after injury?

Factors influencing axon regeneration – extrinsic and intrinsic determinants

One conceptual approach towards understanding mechanisms that prevent axon regeneration in the adult CNS of mammals is to study factors influencing axonal growth during development. At the early embryonic stages, neurons actively extend nerve fibers and readily regenerate their axons following injury. Once matured, neurons of the mammalian CNS loss the ability to do so (Aubert *et al*, 1995). Thus, the loss in the growth and regenerative capacity of CNS axons can be induced by changes in the CNS environment or the intrinsic property of CNS neurons.

It is well-known that developing CNS environment is supportive for the growth and regeneration of nerve fibers; after maturation, the brain and spinal cord appear to be non-permissive, or even actively inhibitory for axonal regeneration following injury (Bray and Aguayo 1989; Bähr and Bonhoeffer 1994). The first evidence was provided by David and Aguayo in 1981, who observed CNS axons that normally do not regenerate in the CNS environment following injury could grow slowly into peripheral nerve grafts that were implanted into the CNS. Regenerating axons appeared to advance at a speed almost ten times slower than normal elongating axons. Such experiments led to the conclusion that (1) CNS neurons of adult mammals still possess intrinsic mechanisms for axonal regeneration if they are provided with an appropriate environment; and (2) adult CNS tissue of mammals is unfavorable for axonal regeneration.

For decades, strategies to promote CNS repair have been focused on the CNS environment. Two types of glial cells were found in the mature CNS – oligodendrocytes and astrocytes. These glial cells were thought to produce substances that are non-permissive for the growth of nerve fibers in adult (Keynes and Cook 1995). Oligodendrocytes represent the myelinating glia in the CNS. When these cells differentiate and begin to myelinate central axons, they synthesize glycoproteins that are known to actively repress axonal outgrowth *in vitro* and *in vivo*. In 1990, Schnell and Schwab characterized myelin-associated inhibitory proteins from the adult CNS, known as NI-35/250 (Schnell and Schwab, 1990). A monoclonal antibody IN-1 that neutralizes

the inhibitory effect of these proteins promoted, albeit to a limited extent, CNS axons to regenerate in adult (Schwab *et al*, 1993; Bregmen *et al*, 1995). Thereafter, other components of myelin extracts, in particular myelin-associated glycoprotein, were found to be inhibitory (McKerracher *et al*, 1994; Mukhopadhyay *et al*, 1994; Filbin 1995). Astroglial cells, on the other hand, respond to nerve injury with self-replication and form glial scars. The formation of glia scar was believed to present another physical or molecular barrier to the regenerating axons in addition to myelin-related inhibitors (Rudge and Silver, 1990; Schwartz *et al*, 1991; Silver 1994). Purified astrocyte populations obtained from glial scars were growth-inhibitory *in vitro* and *in vivo* (Smith *et al*, 1986; Bähr and Bunge, 1990; McKeon 1991; McMillian *et al*, 1994). Remarkably, Silver and his colleagues recently showed that in the absence of glia scar, the myelinated CNS white matter, indeed, supported the implanted DRG nerve fibers to extend for long distances (Davies *et al*, 1997). Abortive regeneration seemed to be associated with the levels of a product of reactive astroglia cells in the extracellular matrix, proteoglycans (McKeon *et al*, 1991). This finding strongly suggests that the formation of glia scar is likely to be the major limiting factor in the CNS environment that blocks nerve regeneration; while adult myelinated white matter itself can be highly permissive for nerve growth.

Besides the presence of inhibitory signals for axonal growth, the mature CNS appeared also to lack molecules that could facilitate axonal growth in development (Jessell, 1991). These include extracellular substances involved in axon adhesion and migration (Carbonetto and David, 1993; Chalmers *et al*, 1996), molecules for axon guidance and pathfinding, and soluble factors, such as neurotrophic factors for cell survival and nerve extension (Isachson 1995). Recently, the laboratory of Geoffrey Raisman reported that by transplanting ensheating cells from rat olfactory bulb to the lesion site of the spinal cord, a small number of corticospinal axons were induced to elongate following transection (Li *et al*, 1997). Mechanism underlying these ensheating cells to support nerve regeneration is not clear, it may involve multiple factors including secretory proteins and adhesion substrates. Nevertheless, this finding seems to reinforce the idea that adult CNS axons have the ability to reconnect themselves following injury if they are provided with permissive environment. It should be kept in mind that despite the numerous efforts to modify the CNS environment, the regeneration of CNS axons remained limited to only a small population of neurons.

Accumulating evidence indicate that not only the environment, but also the intrinsic property of CNS neurons changes during maturation. This change, unambiguously, contributes to the regeneraive failure of CNS axons. First, CNS neurons from an early embryonic stage did grow axons in culture, but the extent of axon elongation was highly variable depending on the region of neuron donors. Second, this growth capacity of neurons decreased with the age of neuron donors (Fawcett 1992; Davies 1989). Finally and most interestingly, immature neurons, such as neuroblast cells from human fetal brains, grew long axons when they were transplanted into the brains of adult rats (Wictorin *et al*, 1990; 1992). Axonal growth at this condition appeared not be inhibited at all by environmental factors in the brains of the adult hosts. These data, especially the fact that mature neurons were unable to establish connections as the embryonic neurons in a similar environment, suggest that the process of axonal regeneration may be controlled, at least in part, by factors intrinsic to neurons that are developmentally regulated.

Two major declines in the growth capacity of CNS axons during development

Significant progress has recently occurred in the understanding of the intrinsic mechanisms underlying the control of axonal growth during developmental and regenerative processes of neurons. Especially, the development of the model of neuronal organotypic cocultures enables independent variation of the developmental stage of neurons and their target tissues (Chen *et al*, 1995; Hafidi *et al*, 1995; Dusart *et al*, 1997). With this model, axotomized neurons were placed in the vicinity of appropriate target slices; contributions of source and target tissue in the process of axonal regrowth can be studied separately. It was discovered that developing neurons from the mammalian CNS underwent two declines of their capacity to regenerate axons following injury (Figure 1) (Chen *et al*, 1995). First, after an active stage of axonal elongation CNS neurons exhibit an abrupt tenfold reduction in the regenerative capacity of their axons in cocultures. It was evidenced that this decline in the regenerative capacity of severed axons was induced by changes in the property of CNS neurons rather than that of the environment. Second, neurons at a postnatal stage undergo a further decline of their regenerative capacity. This decline is induced by the non-permissive nature of the CNS environment and results in a complete prevention in axonal regeneration in adult (Chen *et al*, 1995).

In coculture experiments, the growth of axons and the appearance of glia and myelins in this *in vitro* model replicated their developmental sequences *in vivo*. It was shown that CNS axons of neonatal mice readily projected into brain slices obtained from older mice. However, most neurons ($\sim$90%) of older animals did not project axons to target tissues derived from early neonatal mice (Figure 2) (Chen *et al*, 1995; Li *et al*, 1995; Dusart *et al*, 1997). These data demonstrate that developmental alterations of neurons, rather than of the target environment, determine this early regenerative failure of the efferent CNS axons.

Later, regeneration of CNS axons is further blocked by the subsequent maturational change of the CNS environment. The observations were inconsistent with the previous finding that during maturation, CNS environment switched from permissive to inhibitory for the growth and regeneration of CNS axons (Smith *et al*, 1986). The number of regenerating axons of the postnatal CNS, albeit limited to a small population of neurons ($<$10%), declined further in cocultures prepared from postnatal tissues (Figure 1); finally, no axonal regeneration was observed when cocultures were prepared from adult tissues (Chen *et al*, 1995). Altering the CNS environment by the supplement of embryonic tectum to adult neurons could partially overcome the second regenerative failure observed in the adult CNS

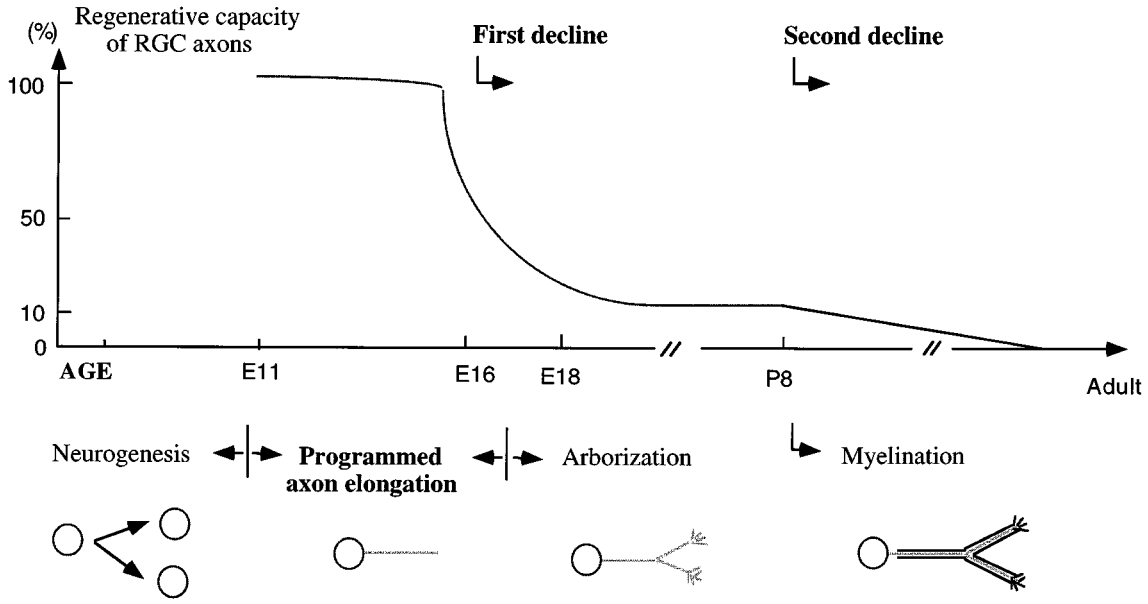

Figure 1 Schematic diagram summarizing the connective stages of axonal development of mouse RGCs. X-axis represents the age of animals that used to prepare the coculture while Y-axis represents the regenerative capacity of RGC axons. The growth capacity of embryonic RGCs is standardized as 100%. During development, most RGCs are born and migrate to the top layer of retina prior to E11 in mice. From E11 through E16, retinal axons grow in a mode of axon elongation that was shown *in vivo* and *in vitro*. This 'programmed axon elongation' ends at E18 (first decline in regenerative capacity of RGC axons) and follows by a period of axon arborization. During the second and third week of postnatal life axon myelination starts, and CNS environment becomes non-permissive for axon elongation and arborization. This transition of the CNS environment causes a second decline in the regenerative capacity of RGC axons

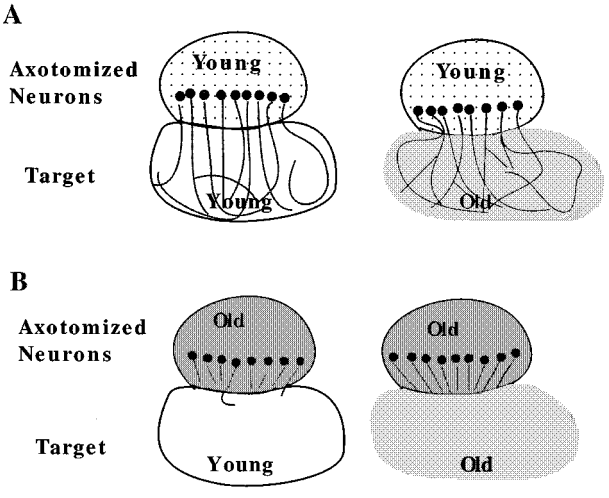

Figure 2 Schematic diagram illustrating neuron-target interactions in cocultures. Coculture models were established using tissues from three different regions of the CNS, (1) tissues of retinal explants containing RGCs and tectal slices as target tissue; (2) tissues from the entorhinal cortex and hippocampal slices,or (3) cerebeller slices containing Purkinje cells or deeper nuclei of the cerebellum. (**A**) Explants obtained from a young animal that contained neurons of origin were placed next to slices of target tissue derived from animals at two different ages – young or old; (**B**) Explants from old animals that contained neurons of origin were cocultured next to slices of target tissues derived from animals at two different ages. Axonal regeneration was observed mainly with explants that contained neurons from young animals, regardless of the age of target tissues. These data indicated that factors intrinsic to neurons play a determining role in the capacity for axotomized neurons to regenerate their axons

cocultures. However, the first major decline in the regenerative capacity of CNS neurons, which was controlled by a developmental program of neurons, was unaffected (Chen *et al*, 1995).

It ought to be mentioned that the behavior of postanatal CNS axons appeared very different from that of the younger neurons. When cocultures were prepared from embryos, regenerating CNS axons that innervated target tissue were largely unbranched, grew rapidly and formed fascicles (Chen *et al*, 1995). They appeared as elongating axons (Jhaveri *et al*, 1991). But as animal is older, most CNS neurons lose the ability for axonal elongation, and the small number of axons that regenerated advanced slowly as arborizing axons. Modification of the CNS environment, such as confronting adult CNS neurons with embryonic target tissue, induced these postnatal axons to arborize but not to elongate. These data are inconsistent with previous reports that up to adulthood, CNS neurons retain some ability to slowly regenerate their axons when provided with an appropriate environment (Bray *et al*, 1989; Schnell and Schwab, 1990). However, axonal arborization merely resulted in functional restoration following CNS damage. For regeneration to occur, the program that triggers axon elongation during the early embryonic stage has to be turned on following injury.

Bcl-2 and the first decline of regenerative capacity

CNS neurons actively grow their axons during a certain period of development, namely a stage of 'programmed axon

elongation'. Recently, the proto-oncogene Bcl-2 has been identified as a key regulator that controls this genetic program for axonal elongation (Chen *et al*, 1997).

Bcl-2 is a proto-oncogene which was isolated from the breakpoint of the t(14;18) chromosomal translocation in a B-cell lymphoma (Tsujimoto *et al*, 1985; Bakhshi *et al*, 1985). It has been shown to block the programmed death of many cell types and protect neurons from diverse death-inducing stimuli, such as axotomy, growth factor deprivation, glucocorticoids, ischemia, or oxidant stressors (Merry and Korsmeyer, 1997). The expression patterns of Bcl-2 in mice were reported by Korsmeyer and colleagues (Table 1) (Merry *et al*, 1994; 1997). The expression of Bcl-2 was widely detected in developing neurons of the PNS and CNS. It was most prominent in differentiating neurons that began to grow nerve fibers duing embryonic stages, and levels of its expression declined thereafter. Though Bcl-2 was also detected in the CNS areas containing neural progenitor cells (areas with active neurogenesis), differentiating neurons or neurons undergoing neurite outgrowth seemed to express substantially higher levels of Bcl-2 than proliferating cells or stem cells. For example, the developing cortical plate, the mantle layer of the spinal cord, and the differentiating neurons of the olfactory epithelium, all had higher levels of Bcl-2 expression than regions that contain the corresponding stem cells. Especially, in neurons that retained the ability to grow or regenerate axons throughout life, such as peripheral sympathetic and sensory ganglia neurons, Bcl-2 was expressed up to adulthood (Merry *et al*, 1994). The fact that the expression pattern of Bcl-2 coincides with the period of neurite outgrowth implicates a role of Bcl-2 in the regulation of axonal elongation in neural development.

Using the approach of immunofluorescence staining, it was shown again that the pattern of expression of Bcl-2 in RGCs correlated with the regenerative capacity of retinal axons (Table 1). Bcl-2 expressed high in the RGC layer when retinae extended axons. Correlating with the onset of the first regenerative failure of retinal axons, the expression of Bcl-2 in the RGC layer decreased to an undetectable level at the same day. Following this observation, it was then demonstrated by using cocultures prepared from mouse tissues carrying a Bcl-2 transgene or a null mutation that Bcl-2 is critical for the growth and regeneration of RGC axons. First, using cocultures prepared from early embryos that resulted from Bcl-2 heterozygous matings (Veis *et al*, 1993), a quantitative correlation between the level of the expression of Bcl-2 and the number of retinal axons that regenerated into tectal slices was discovered. As compared to cultures prepared from wild-type mice, the number of axons was reduced by about 50% in cultures prepared from Bcl-2 (+/−) mice and by about 80% in cultures prepared from Bcl-2 (−/−) mice. Although Bcl-2 is known to have a strong anti-apoptotic activity, no significant loss of RGCs in the retinae of Bcl-2 heterozygous and null mutants was observed at this age. It suggests that the different capacity of axonal growth presented by RGCs of wild-type and Bcl-2-deficient mice cannot be accounted for by differences in the number of RGCs in their retinae. Second, using transgenic mice that constitutively expressed Bcl-2 in neurons (Martinou *et al*, 1994), it was found that Bcl-2 is not only essential but also sufficient for inducing axonal growth machinery of the adult CNS neurons. In mice expressing the Bcl-2 transgene, adult RGCs regenerated axons in cocultures when they were confronted with embryonic tectal slices, a permissive environment for axonal growth and regeneration. Retinal axons of Bcl-2 transgenic mice advanced at a fast speed and extended as elongating axons. The regeneration of axons required the expression of Bcl-2 in retinae but not in tecta. Embryonic tecta attracted the same number of axons regardless of whether they originated from wild-type mice, mice deficient in Bcl-2, or mice constitutively expressing Bcl-2.

Axon regeneration was also achieved *in vivo* in neonatal mice in whose neurons Bcl-2 was constitutively expressed. After transection at the optic tract, retinal axons of wild-type mice were restricted to tissues proximal to the lesion site and did not regenerate. In contrast, retinal axons of Bcl-2 transgenic mice grew in large numbers across the lesion site and reinnervated the entire superior colliculus (tectum). Bcl-2 appears to play an important role in the regulation of retinal axon elongation *in vitro* and *in vivo*. Although the molecular mechanisms underlying the activity of Bcl-2 is not clear, it is possible that Bcl-2 is primarily involved in the regulation of neural differentiation and, subsequently, influences the machinery of axonal growth inside neurons.

Table 1 The expression of Bcl-2 and the capacity of axotomized neurons to regenerate their axons

		Bcl-2	Axon regeneration
Bcl-2 (+/+)	Emb PNS	++	++
	Ad PNS	++	++
	Emb CNS	++	++
	Ad CNS	−	−
	Ad olfactory bulb	++	++
	Ad dentate gyrus	++	++
	<E16 RGCs	++	++
	>E18 RGCs	−	−
Bcl-2 (+/−)	<E16 RGCs	+	+
Bcl-2 (−/−)	<E16 RGCs	−	−
Bcl-2 tg	>E18 RGCs	++	++

Emb: embryonic; Ad; adult; Bcl-2 (+/+): wild-type mice; Bcl-2 (+/−): Bcl-2 heterozygous mice; Bcl-2 (−/−): Bcl-2 knockout mice; Bcl-2-tg: Bcl-2 transgenic (overexpressing) mice; <E16: animals younger than E16; >E18; animals older than E18

Bcl-2: a role in axon regeneration or neuronal survival?

Since the anti-apoptotic function of Bcl-2 is well established, it thus raised the possibility that the growth-promoting activity of Bcl-2 may simply be a consequence of its effect on neuronal survival after axotomy (Bonfanti *et al*, 1996; Dubois-Dauphin *et al*, 1994). Many neurons undergo apoptosis following injury, especially if the lesion is close to their cell bodies. Presumably, this was because axotomized neurons are deprived of the trophic support which is normally provided

by their distant targets and by the non-neuronal cells that surround their axons.

Accumulating evidence now indicates that neuronal survival is necessary but not sufficient for axon regeneration after axotomy. First, most neurons in the adult brain survive axotomy if the lesion occurs far from their cell bodies, but fiber regeneration does not occur (Schwab and Bartholdi, 1996). For instance, more than 50% of adult RGCs survived over 3 months after distal optic nerve transection (about 10 mm from the eyeball), but their severed axons did not regenerate to form new connections (Bray *et al*, 1991). Another example is mature Purkinje cells. These cells were among the most resistant neurons to axotomy-induced cell death, but were refractory to axonal regeneration (Dusart and Sotelo, 1994; Rossi *et al*, 1995). Second, deprivation of neurotrophic factors was considered as one of the most critical factors for axotomized neurons to die after injury, and supplying exogenous neurotrophic factors to axotomized RGCs did increase the survival of retinal neurons and the arborization of retinal axons. However, it did not affect axon elongation or regeneration (Mansour-Robaey *et al*, 1994). Third, an anti-apoptotic drug, the cysteine protease inhibitor Z-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk), was found to support neuronal survival but had no effect on axonal regeneration in culture. Combining dissociated cell culture system and the technique of retrograde labeling of RGCs, it was recently reported that the addition of ZVAD-fmk prevented the death of RGCs after axotomy, but these RGCs did not grow axons in the presence or absence of neurotrophic factors (Chen *et al*, 1997). However, in cultures prepared from retinae of Bcl-2 overexpressing mice, most survived RGCs sent out long axons. These findings suggest that the induction of axonal regrowth requires more than the mere survival of neurons. While the caspase inhibitor, ZVAD-fmk, and many neurotrophic factors only supports neuronal survival or axon arborization, Bcl-2 promotes both neuronal survival as well as axonal regeneration (Figure 3).

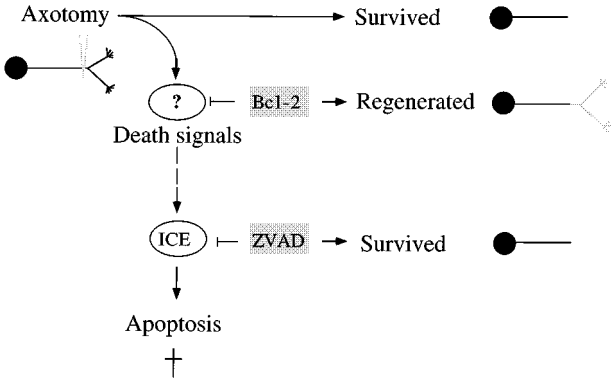

Figure 3 The induction of axonal regrowth requires more than the mere survival of neurons. Diagram represents a general view of events occurring after axotomy. CNS neurons that survived axotomy do not regenerate their axons. Under most circumstances, injured neurons go through a process of apoptotic cell death, which can be blocked by the overexpression of Bcl-2 or supplement of the ICE inhibitor, ZVAD, to neurons. However, while ZVAD only supports neuronal survival, Bcl-2 promotes both neuronal survival as well as axonal regeneration

Several other studies also supported the avenue that Bcl-2 may play a direct role to regulate neurodifferentiation and axonal growth in addition to its anti-apoptotic activity. Davies and colleague using dissociated cultures prepared from trigeminal ganglia of Bcl-2 deficient mice observed that when neurons were maintained under a similar culture condition, cells from Bcl-2 homozygous knockout ($-/-$) mice showed a marked decrease in their axonal growth rate than those from wild-type (+/+) mice (Hilton *et al*, 1997). This reduction in the rate of axon extension was irrespective of whether the neurons were grown with neurotrophic factors, such as NGF, BDNF, or NT-3. In contrast, overexpression of Bcl-2, showed by two other groups using a neural crest-derived or a midbrain-derived dopaminergic cell lines, enhanced neurite outgrowth (Zhang *et al*, 1996; Oh *et al*, 1996). These results are consistent with the observation that Bcl-2 regulates not only cell survival but also the growth or regenerative process of CNS axons.

Bcl-2 and the second decline of regenerative capacity

As we know that expressing the Bcl-2 transgene in neurons overcomes the first regenerative failure induced by changes of the intrinsic property of neurons, it remains interesting to see whether neurons containing Bcl-2 regnerate their axons in adult brain which presents a non-permissive environment for axonal growth. Evidence has been provided that while neurons derived from Bcl-2 transgenic mice regenerated axons to embryonic tectal slices that contained a permissive environment for axon elongation, they did not send any axon to tectal slices prepared from adult brain (Chen *et al*, 1997). Axonal regeneration was completely blocked by environmental substrate provided by adult tectal slices even if neurons (expressing the Bcl-2 transgene) had the intrinsic machinery for nerve extension. Supplement of neurotrophic factors and IN-1 antibody to the coculture did not seem to have any effect on nerve elongation under such conditions. Thus, it appears that adult neurons overexpressing the Bcl-2 transgene differ from fetal neurons (which also express Bcl-2) in that axons of adult neurons are able to recognize the inhibitory signals in the adult CNS but not fetal axons. In conclusion, expressing Bcl-2 transgene in RGCs activates the intrinsic machinery of neurons for axonal regeneration, but it cannot overcome the second regenerative failure that is induced by maturational changes in the CNS environment.

Concluding remarks and future directions

The recent breakthrough in the field of nerve regeneration came from the identification of a program intrinsic to neurons which controls axonal elongation and regeneration and from the finding that Bcl-2 is critically involved in the regulation of this program. In the future, molecular mechanisms through which Bcl-2 regulates the growth and regeneration of CNS axons remain to be elucidated. Investigation of the role of other Bcl-2 family members in the process of axonal growth

would provide important information for the identification of molecular pathways of Bcl-2. Especially, further insights into these issues should help to clarify whether Bcl-2 triggers an independent downstream pathway of its anti-apoptotic activity that is upstream of the caspases to promote axonal regeneration. However, it should be kept in mind that to achieve the functional restoration following CNS damage in adult mammals, a better understanding of the inhibitory mechanism of the CNS environment is required. We suggested that introducing Bcl-2 exogenously or finding other means to achieve Bcl-2 expression in mature neurons can be a promising, new approach to treat CNS nerve injuries and perhaps neurodegenerative diseases, but a combinatorial strategy for co-treatment with other agents that modify the surroundings of severed axons is essential.

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