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MECHANISMS OF AXONAL PLASTICITY

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INTRODUCTION

The elongation of neuronal processes is important not only during development to shape the architecture of the mature brain, but also during reparative processes after injury and in several forms of physiological plasticity. In the adult peripheral nervous system both axonal elongation and terminal and collateral sprouting are well known phenomena. However, in the central nervous system, while collateral sprouting occurs in several brain regions (44, 52), axonal elongation is hampered by several factors that depend both on intrinsic properties of the neurons and on the environment (4, 24). Intrinsic properties vary among neurons, as shown by the fact that distinctive axonal populations show different growth capabilities when confronted with similar environmental conditions (5, 6, 11, 24, 43). The main environmental factors that impede axon growth are the glial scar at the lesion site (4, 33, 40), myelin associated growth-inhibitory molecules (47, 48) and lack of growth-permissive promoting substances that are instead present in the peripheral nervous system (4, 19).

Several proteins are expressed during development in association with axonal elongation and some of them are reexpressed during the reparative processes after injury in the mature brain (14). One of the most deeply investigated proteins of this group is GAP-43 also called B-50 (review: 26, 39). This protein is widely expressed in the brain during development and in most neurons it is downregulated at the time of synaptogenesis (49). However, in several neuronal populations both in the peripheral (34, 51, 56) and in the central nervous system (7, 8, 20, 38, 40) the expression is maintained in the adult. It has to be underlined that *in vivo* it appears to be localized mainly into the axon terminals (21, 25).

This protein is strongly associated with nerve regeneration (9, 49). While in the peripheral nervous system an overexpression is commonly associated with axotomy, this is not always true for the central nervous system. Peripheral nerve graft onto an optic nerve sectioned at about one millimeter from the eye results in regeneration of the retinal axons (57) and overexpression of GAP-43 (22). A similar overexpression is also detected in cells that do not regenerate into the graft, but all regenerating cells do overexpress GAP-43 (46). In contrast, when nerve section is more distally placed no regeneration (57) and no GAP-43 overexpression occur. This means that while GAP-43 is potentially active in promoting regeneration, this protein alone might be not sufficient without other factors to allow elongation of axonal processes.

More recent work has shown that a key role of this protein is to modulate

terminal arbor plasticity. Transgenic mice overexpressing GAP-43 show spontaneous sprouting (1, 30). In contrast, the suppression of GAP-43 expression hampers axonal outgrowth and pathfinding (1, 2, 53). This protein may therefore be considered an important molecule as an intrinsic factor in neuronal plasticity and particularly in guiding the growth of axons and their terminals.

Retrograde regulation of proteins associated with injury and regeneration in Purkinje cells

In order to better define the role of GAP-43 in axonal plasticity, we used a peculiar model offered in the cerebellar cortex. Mature Purkinje cells are characterized by their resistance to axotomy (23) and by the virtual absence of axonal regeneration even when provided with favorable environmental conditions, like embryonic neural tissue (43) or Schwann cells (11). These neurons lack a constitutive expression of immediate early genes that are associated with regenerative responses, like c-Jun and JunD (29), and have no nNOS (45) which is also associated with injury (28). At the same time these neurons never express GAP-43 (32) not even during development (17).

In a recent series of experiments (58) we have studied whether the above mentioned proteins can be expressed in Purkinje cells following axotomy as it occurs in many other neuronal populations (12, 28). The axons were cut in the cerebellar white matter. In these experiments we could detect two different responses. Those few neurons that were axotomised very close to cell body showed a strong upregulation of c-Jun and JunD early genes together with reactivity for NADPH diaphorase. In contrast, most cells that were axotomised more distally did not show any reaction (Tab. I). As far as GAP-43 is concerned, however, there was no expression even in the former population, although some of these cells became immunoreactive for the functionally similar protein CAP-23.

These findings suggest that in Purkinje cells some of the genes associated with injury and regeneration are normally inhibited by factors that are present along the axon and are transported retrogradely to the cell body. However, no GAP-43 expression is possible even when these factors are removed. To prove that retrogradely transported factors are indeed responsible for the repression, we have applied colchicine both *in vivo* and in organotypic cerebellar cultures. In both these conditions, colchicine promoted a similar gene expression as that found after axotomy close to cell body.

In order to identify possible factors located along the axons that repress these genes, we have focussed our attention on the myelin inhibitory molecules. It has been previously shown that in retinal axons grown under serum free conditions, glial cells are largely suppressed and GAP-43 expression is accompanied by axonal growth (37). In addition, an increase of GAP-43 expression has been shown in spinal cord when oligodendrocytes have been deleted (31). In our experiments we have applied *in vitro* hybridoma cells secreting IN-1 antibody and uninjured Purkinje cells showed a similar gene expression as that found after axotomy close to cell body. It should be underlined that GAP-43 was never expressed. Similar

results were obtained by applying *in vivo* the Fab fragment of this antibody and the expression was present also in distally axotomised Purkinje cells (Tab. I). This means that myelin inhibitory factors are responsible for the gene repression in Purkinje cells.

Table I. - *Expression of c-Jun, JunD, NADPH diaphorase and GAP-43 in Purkinje cells and inferior olive neurons in basal condition and following different manipulations.*

	PURKINJE CELLS	INFERIOR OLIVE
Axonal regeneration in permissive environment (embryonic material, Schwann cells)	NO	YES
Basal expression		
c-Jun	NO	NO
JunD	NO	NO
NADPH diaphorase	NO	NO
GAP-43	NO	YES
After axotomy (distal)		
c-Jun	NO	YES
JunD	NO	YES
NADPH diaphorase	NO	YES
GAP-43	NO	YES
After axotomy (close) or Colchicine or IN-1		
c-Jun	YES	
JunD	YES	
NADPH diaphorase	YES	
GAP-43	NO	

We have then grafted embryonic cerebellar tissue or Schwann cells to see whether some form of Purkinje cell regeneration could be detected at least in those neurons that showed early gene expression or NADPH diaphorase activity following axotomy. Since we never observed any Purkinje cell axon regenerating into the graft (unpublished observations), we have to assume that even our proximally axotomised reactive neurons remain unable to elongate neurites. This means that despite the upregulation of some genes that are usually associated with regenerative or injury responses, Purkinje cells are unable to regenerate.

GAP-43 overexpression promotes Purkinje cell axonal sprouting

Since Purkinje cells never showed GAP-43 expression it is possible that this protein plays a key role in regeneration by promoting the first step of axonal elongation. It is therefore interesting to study possible regenerative responses in transgenic mice overexpressing GAP-43 in this peculiar cell type (12). These mice had a normal architecture of the cerebellar cortex. However, following axotomy, Purkinje cells underwent morphological changes that were similar to those of wild

type mice (Fig. 1A-B). These changes consisted of hypertrophied recurrent collaterals and the formation of torpedoes (42, 23). In addition, numerous axonal sprouts, both at the level of the lesion (Fig. 1D) and of the torpedoes (Fig. 1C) were present in all Purkinje cells. In the former case, they appeared as short processes sometimes with branches, whereas at the level of the injury they appeared as a thin branched

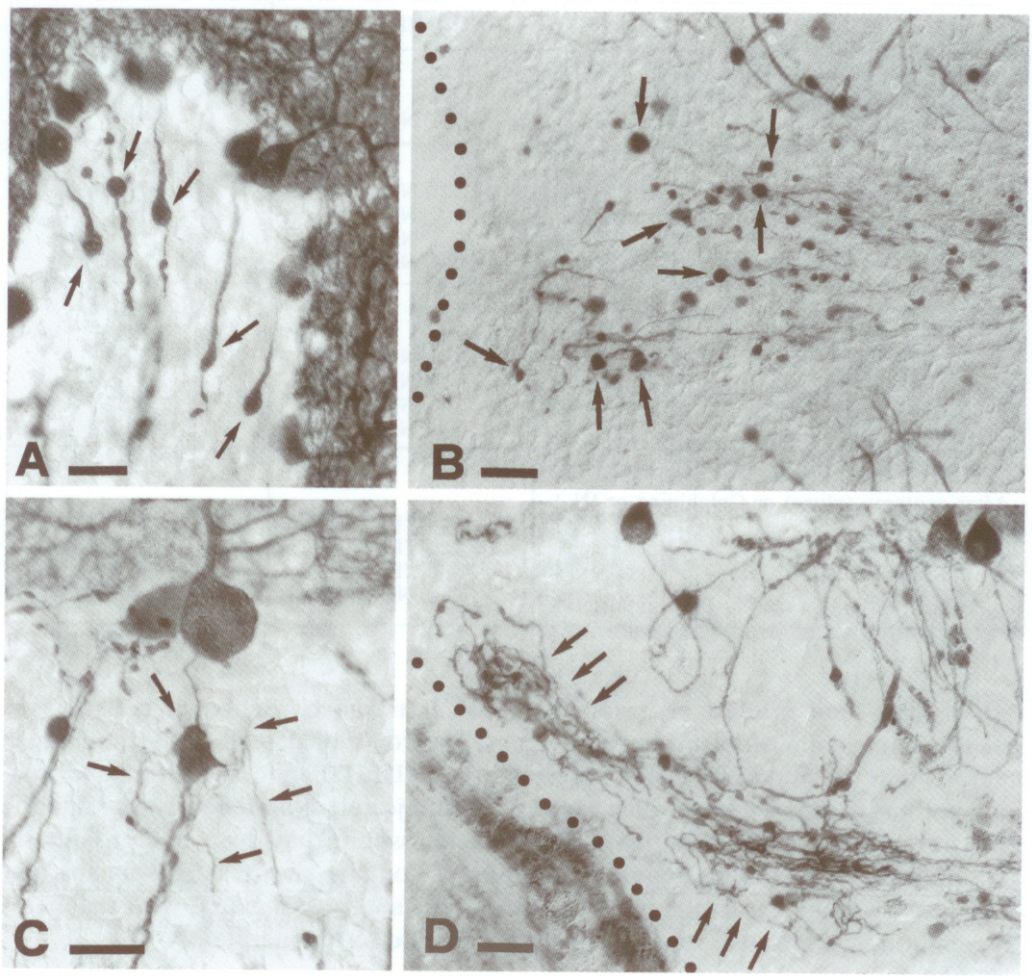

Fig. 1. - *Response to axotomy in wild-type and GAP-43 overexpressing Purkinje cells.*

Micrograph A and B show the axonal modifications of injured wild-type Purkinje cells. Prominent torpedoes (arrows in A) characterize the initial portion of the axon in the granular layer. By contrast, the severed axons (B) remain apposed to the lesion site (dotted line) terminating with large terminal clubs (some are pointed by arrows). Similar modifications occur in GAP-43 overexpressing Purkinje cells (C and D). However, numerous thin processes (arrows in C) bud from the torpedoes, whereas a profuse sprouting (arrows in D) is present at the severed axon tips abutting the injury site (dotted line in D). Anti-calbindin immunostaining in A-C, anti GAP-43 immunolabeling in D. Scale bars, 50 μm in A, B, D, 30 μm in C.

plexus typical of sprouting phenomena. In fact, their length was limited and they never appeared as fully developed axons. In conclusion, axotomised Purkinje cells, in the absence of the most common proteins associated with injury and regeneration were able to show regenerative responses in the presence of GAP-43.

We have then attempted to see whether these transgenic Purkinje cells were able to regenerate in front of embryonic cerebellar tissue or Schwann cell grafts. The axotomised Purkinje cells showed similar regenerative responses, but no regeneration was observed inside the grafts. Therefore, we conclude that although Purkinje cells overexpressing GAP-43 were able to grow new processes, they were unable to regenerate their axons inside the transplants. It should be underlined that axotomised inferior olive cells were instead able to grow their axons into the Schwann cell environment and, when they could face the cerebellar cortex across the graft, they were able to reach and reinnervate Purkinje cells (11). The different behavior between these two cell populations indicates that the failure of a full axonal elongation of the Purkinje cells is not due to the lack of suitable environmental cues, but rather to their intrinsic inability to express growth-associated genes. On the other hand, the role of GAP-43 in regeneration is limited and multiple parallel pathways are necessary for the regulation of axonal elongation.

Another issue related to GAP-43 expression concerns possible local regulatory mechanisms. We have seen above that in many neurons of the peripheral and central nervous system GAP-43 expression is switched off at the time of synaptogenesis because target molecules act as a repressive signal. In these neurons, axotomy is usually able to induce or enhance the expression. On the other hand, it is currently assumed that those central neurons that maintain a basal protein expression are endowed with the capacity of continuous synaptic remodeling. Protein Kinase C (PKC) phosphorylates GAP-43 (3) and transgenic mice overexpressing this protein showed enhanced sprouting (1). A persistent change in phosphorylation occurs during long-term potentiation (35). In this case a protein activation may be important to modulate the remodeling. Recent experiments have shown that NMDA receptors and even retrograde signals are important to modulate GAP-43 activity. The question then arises whether in those neurons that express a poor basal level of GAP-43, an induced remodeling of the terminal arbor, in the absence of axotomy, is sufficient to induce a GAP-43 upregulation or overexpression (9).

Role of GAP-43 in remodelling intact axons

It has been recently shown that the developmental down-regulation of the GAP-43 mRNA is prevented by muscle paralysis and intramuscular injection of IGF-1 (15). Furthermore, GAP-43 expression in the axon terminals of motoneurons has been obtained following intramuscular injection of insulin-like growth factors (16). GAP-43 immunoreactivity increases following partial muscle denervation (36) while GAP-43 mRNA does not change during sprouting of motoneurons induced by partial denervation and by application of botulinum toxin to the muscle (10). In GAP-43 overexpressing mice, spontaneous sprouting was not accompanied by induction of endogenous GAP-43. From these experiments it has been concluded that nerve sprouting in the adult can be an exclusive local reaction (14).

We have looked for possible changes in GAP-43 immunoreactivity during remodeling of uninjured motoneuron terminals (57). This occurs in Duchenne Muscular Dystrophy (DMD) an X-linked recessive disorder characterized by lack of dystrophin expression (41). In this disease, muscle degeneration is accompanied by insufficient muscle regeneration. A similar lack of dystrophin occurs in the *mdx* mutant mouse. Here muscle regeneration leads to a complete set of new fibers which become resistant to degeneration (13, 18, 54, 55). This pattern of degeneration-regeneration implies a continuous remodeling of the motoneuron terminal arbors. Torres and Duchen (55) have showed a lack of damage to motoneurons in *mdx* mice.

Gastrocnemius and quadriceps muscles of *mdx* mice have been immunolabeled with an anti-GAP-43 polyclonal antibody. In wildtype mice, we observed scattered immunopositive nerve fibers. Some of them were running in bundles through the adipose tissue along vascular peduncles while others were thin terminal plexuses across the wall of the vessels. No GAP-43 immunopositive neurites were detected in contact with acetylcholinesterase-stained endplates. These data confirm previous observations that in normal muscles only autonomic nerve fibers innervating arteries display GAP-43 immunoreactivity (27, 34, 51). In our *mdx* mice GAP-43 immunopositivity was strikingly more abundant in nerve bundles and strongly positive along the course of the blood vessels. In addition, they were also present in axons running towards (Fig. 2A) and terminating inside endplates identified by acetylcholinesterase staining (Fig. 2 B-C). Occasionally, two positive fibers approached the endplate from two opposite directions, suggesting a polyinnervation of the muscle fibers (Fig. 2D). A quantitative evaluation of the number of positive axons revealed that they were abundant in muscle regions characterized by a degeneration-regeneration pattern, while in regions of fully regenerated muscle fibers, identified by the presence of central nuclei, these fibers were significantly lower in number. This finding suggests that GAP-43 immunoreactivity is a transitory event related to the ongoing process of terminal remodeling in the absence of direct motoneuron injury. The nature of the signals that induce the increase of GAP-43 immunoreactivity is unknown.

CONCLUSIONS

Altogether the three groups of experiments described above support the view that GAP-43 plays a key role in axonal plasticity. Recent work suggests that GAP-43 may relate to growth cone guidance rather than axonal elongation (1, 53). In a cell population, like the Purkinje cells, that does not show any attempt to regenerative phenomena, the simple addition of this protein is able to induce sprouting along their axons and at their sectioned end (12). In addition, when the Purkinje cells are induced to express several growth associated genes, but not GAP-43, no regenerative events are possible (58). This failure occurs even when the sectioned fibers are in the same environmental conditions that allow other cell populations, like the inferior olivary neurons, to regenerate and to reinnervate their target

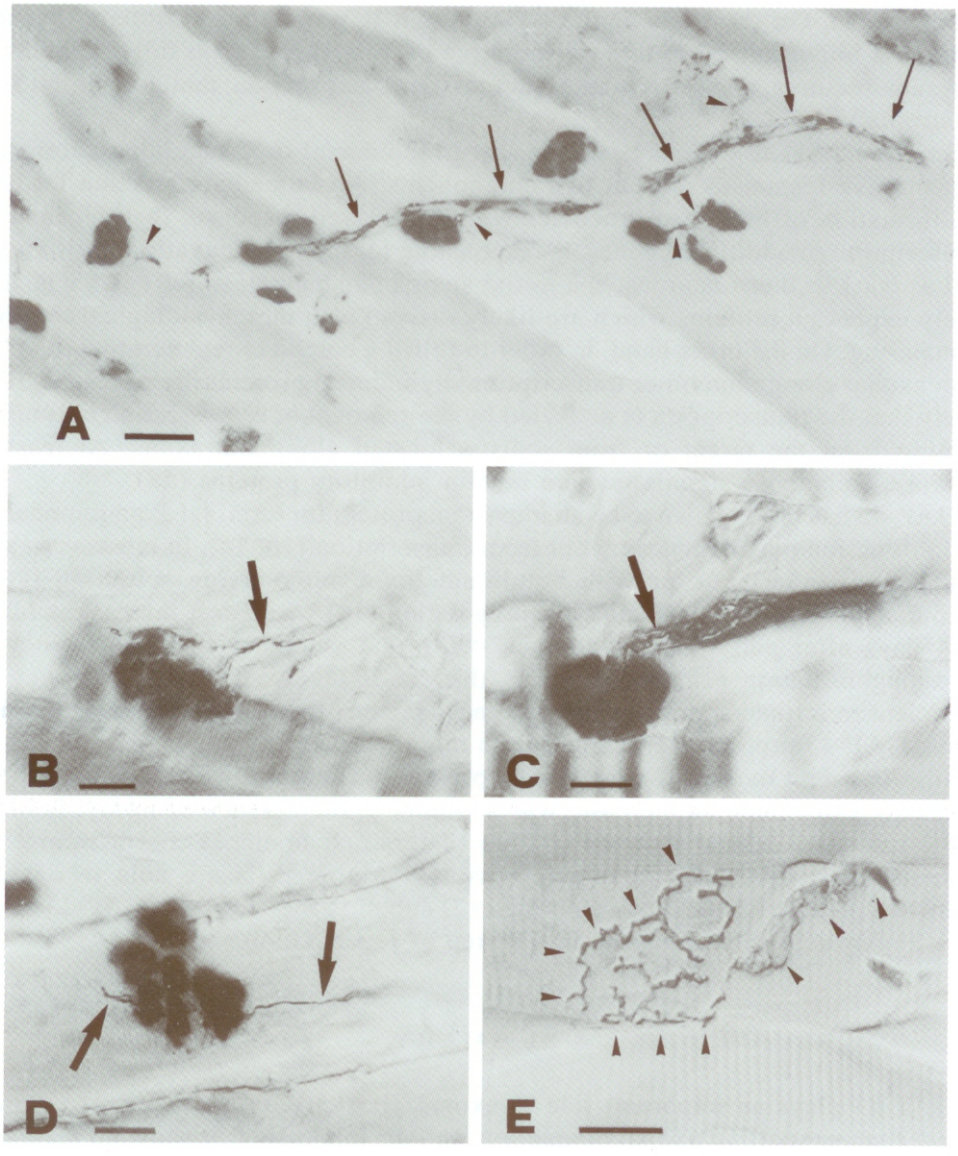

Fig. 2. - *GAP-43 immunoreactivity in terminal axonal branches at motor endplates of the* mdx *mouse (A-D) and S-100 immunostaining of Schwann cell processes (E) at a motor endplate of a wild-type mouse.*

The arrows in A indicate a bundle of GAP-43 immunolabeled axons in the gastrocnemius muscle. Single fibers (arrowheads) emanate from this bundle and end on acetylcholinesterase labeled endplates. The high magnification micrographs in B and C show two examples of single GAP-43 immunoreactive axons (arrows) ending in endplates with typical morphology. By contrast, the endplate shown in D appears to be broken up in several small islands. Note that two distinct immunoreactive axons (arrows) terminate in two different islands of this endplate. The comparison between the micrograph E, showing the morphology of Schwann cell processes, and B and C, revealing GAP-43 immunoreactive fibers, suggests that in *mdx* mice GAP-43 is detected in terminal axon branches of motoneurons. Scale bars: 50 μm in A, 25 μm in B-D, 20 μm in E. Reprinted with permission from (56).

neurons (11). On the basis of these findings it is possible to postulate that this protein is important to initiate regenerative events. However, a full regenerative program requires a more complex set of gene expression that has still to be determined.

Smith and Skene (50) have shown that in the dorsal root ganglion neurons the regenerative responses present two components. An immediate reaction leads to the formation of branched processes while a delayed response, that depends on transcription of additional genes, is responsible for axonal elongation. This means that at least in these neurons, the initial steps of regeneration relies on constitutively expressed proteins which are likely driving also the physiological terminal remodeling. On the other hand, in order to fulfill a complete regenerating program with axonal elongation other transcriptionally induced proteins are necessary. It is likely that the transcription is activated by the removal of repressive signals either coming from the target or from sources located along the axons. At least in Purkinje cells these signals involve myelin inhibitory proteins (58).

GAP-43 has been shown to be an important protein for terminal axon remodeling and this action may be independent from transcription (10, 14). In muscles treated with a local anesthetic or with botulinum toxin, motoneuron sprouting is not accompanied by a GAP-43 mRNA upregulation (10). A modest increase is found at 4 days after partial denervation, a delay that is higher than the latency of sprouting initiation (10). This means that basal levels of the protein may be sufficient to initiate sprouting by local mechanisms. However, in the latter condition a GAP-43 protein overexpression in motoneuron terminals is present (36). A similar transient overexpression is also present during terminal remodeling in the *mdx* mice (56). Such an increase, which occurs in the absence of injury, is likely to be important to improve the growth of terminal branches. Furthermore, the demonstration that GAP-43 overexpressing Purkinje cells are able to develop branched plexuses typical of sprouting (12) supports the view that GAP-43 might be a key protein promoting the initial step of regeneration.

SUMMARY

GAP-43 plays an important role in axonal plasticity by guiding growth cones rather than supporting axonal elongation. In Purkinje cells that show no regenerative responses and no GAP-43 expression after axotomy, the simple addition of GAP-43 gene induces the formation of branched plexuses typical of sprouting growth. Purkinje cells can express some growth-associated proteins, but never GAP-43, when axotomy is made very close to cell body or when an antibody for the myelin-associated inhibitory molecules is applied to intact cells both *in vivo* and *in vitro*. Also in these conditions they are unable to show new axonal profiles even in a permissive environment that allows inferior olive cells to regenerate and reinnervate their target cells. We suggest that GAP-43 is a key molecule to initiate axon growth while other genes are necessary to develop a full regenerative program.

In *mdx* mice, which are characterized by a pattern of muscle degeneration-regeneration accompanied by a remodeling of intact motoneuron terminal arbor, GAP-43 is transiently overexpressed during the remodeling period. Although there is evidence that sprouting can occur in the absence of GAP-43 overexpression, our results support the view that GAP-43 is important for the terminal arbor plasticity.

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