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Overexpression of the Neural Growth-Associated Protein GAP-43 Induces Nerve Sprouting in the Adult Nervous System of Transgenic Mice

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Summary

Regulation of neurite outgrowth and structural plasticity may involve the expression of intrinsic determinants controlling growth competence. We have tested this concept by targeting constitutive expression of the growth-associated protein GAP-43 to the neurons of adult transgenic mice. Such mice showed striking spontaneous nerve sprouting at the neuromuscular junction and in the terminal field of hippocampal mossy fibers. In control mice, these nerve fibers did not express GAP-43, and did not sprout spontaneously. Lesion-induced nerve sprouting and terminal arborization during reinnervation were greatly potentiated in GAP-43-overexpressing mice. A mutant GAP-43 that cannot be phosphorylated by PKC had reduced sprout-promoting activity. The results establish GAP-43 as an intrinsic presynaptic determinant for neurite outgrowth and plasticity.

Introduction

Neurite outgrowth is a tightly regulated process both during development and in the adult. Precise regulation is essential to axonal navigation, synapse formation, plasticity, and regeneration. At the molecular level, nerve elongation coincides with the expression of a set of growth-associated proteins (GAPs) in the corresponding neuronal cell bodies (Skene, 1989; Bisby and Tetzlaff, 1992). Their discovery led to the proposal (the GAP hypothesis) that nerve growth is controlled in part by the expression of such proteins, which would play specific and important roles in neurite outgrowth (Skene and Willard, 1981; Skene, 1989). On the basis of their expression with respect to neurite outgrowth, GAPs can be subdivided into two classes. The expression of a first set of GAPs, including certain actin and tubulin isoforms, correlates with the actual process of long-distance axonal elongation (Bisby and Tetzlaff, 1992; McKerracher et al., 1993). These GAPs probably represent essential building components for axon formation. The expression pattern of the second set of GAPs, however, suggests a more general role in neurite outgrowth competence. Thus, their down-regulation during development coincides with regression of terminal arborization, and their reinduction after lesion correlates with regeneration competence in the adult (for references, see below). A key implication of the GAP hypothesis is that expression of intrinsic determinants of growth competence in neurons may provide a mechanism to regulate neurite outgrowth and structural plasticity in the nervous system. However, in the absence of supporting functional data, the role of general intrinsic mechanisms in the control of neurite outgrowth remains largely hypothetical.

A large body of correlative evidence is consistent with the possibility that the nervous system protein kinase C (PKC) substrate GAP-43 (Benowitz and Routtenberg, 1987; Skene, 1989) may be an intrinsic competence factor for nerve growth and plasticity. GAP-43 is a general early marker of postmitotic neurons, where it is expressed at high levels during axonal growth and synapse formation. It accumulates in axonal growth cones and presynaptic nerve terminals, where it associates with the cortical cytoskeleton (Skene, 1989; Meiri and Gordon-Weeks, 1990). Down-regulation of GAP-43 expression to essentially undetectable levels coincides with the regression of terminal arborization in several systems (Moya et al., 1988; Caroni and Becker, 1992). Interestingly, its expression is maintained in nervous system regions that have been associated with high plasticity (Benowitz and Routtenberg, 1987). Finally, upon target deafferentiation or nerve lesion, GAP-43 reinduction correlates with nerve sprouting and, in many cases, with competence for nerve regeneration (Schreyer and Skene, 1991; Doster et al., 1991; Tetzlaff et al., 1991).

GAP-43 appears to play a prominent competence role in neurite outgrowth. Its expession is not an absolute requirement for neurite outgrowth in vitro (Baetge and Hammang, 1991; Aigner and Caroni, 1993) and in vivo (Strittmatter et al., 1995). However, overexpression (Yankner et al., 1990) and depletion experiments in vitro (Aigner and Caroni, 1993; Shea et al., 1991) indicate that it greatly promotes neurite adhesion and persistent growth cone spreading and branching (Aigner and Caroni, 1995). In addition, mice lacking GAP-43 display defects in neuronal pathfinding during early development (Strittmatter et al., 1995). These findings suggest that the presence of GAP-43 in growth cones and nerve terminals may potentiate their responses to local growth and guidance signals.

To test the possibility that GAP-43 may be an intrinsic determinant of neurite outgrowth, we generated transgenic mouse lines that constitutively express GAP-43 in adult neurons. To define the role of PKC-mediated phosphorylation on GAP-43 function, we also generated transgenic mouse lines that expressed phosphorylation-site mutants of this protein. We show that the presence of GAP-43 leads to striking spontaneous sprouting of nerves

in their terminal fields. When neurite outgrowth was induced by established lesion protocols, a pronounced potentiation of the sprouting response was observed in the transgenic mice. In demonstrating that the expression of GAP-43 in adult neurons is sufficient to induce spontaneous nerve sprouting and to potentiate induced neurite outgrowth, these results strongly support the GAP hypothesis and indicate that GAP-43 is a crucial intrinsic determinant of neurite outgrowth and plasticity in the developing, adult, and regenerating nervous system.

Results

Generation of Transgenic Mouse Lines That Express GAP-43 in Adult Neurons

To achieve strong constitutive expression of GAP-43 selectively in the neurons of postnatal and adult mice, we used a mouse *Thy-1* expression cassette. This system provided comparatively late activation during nervous system development (postnatal day 4–10 [P4–10], depending on the type of neuron), approximately at a time when endogenous GAP-43 is down-regulated in most neurons. As a transgene, we used chick *GAP-43* cDNA sequences for most transgenic lines analyzed in this study. This allowed us to distinguish transgene and endogenous mouse GAP-43 at the nucleic acid and protein levels. In control experiments, the main findings of this study were confirmed with corresponding transgenic mice expressing mouse GAP-43. The transgenic lines were designated as follows: mice expressing wild-type GAP-43, GAP-43 (wt1–6); wild-type mouse GAP-43, GAP-43(wtm1,2); S42A mutant, GAP-43(A1–6); S42D mutant, GAP-43(D1–6).

Substantial levels of transgene mRNA and protein were expressed in the nervous system of 13 independent transgenic lines (Figure 1). In most lines, overall expression in the brain was comparable to peak levels during development (Figure 1B). Importantly, transgenic and endogenous GAP-43 could be detected independently (Figures 1B, 1C, and 1D). This allowed us to establish that by a variety of criteria, the regulation of endogenous GAP-43 expression was indistinguishable in control and transgenic mice (see below).

Spontaneous Motor Nerve Sprouting in *GAP-43*-Transgenic Mice

GAP-43 accumulates in axonal growth cones and presynaptic nerve terminals. Accordingly, in a first set of experiments, we determined whether the presence of GAP-43 in adult neurons is sufficient to induce spontaneous terminal nerve sprouting, a form of structural plasticity that is minimal in nontransgenic adult animals. We first searched for sprouting at the neuromuscular junction, an accessible and well-characterized system (Brown, 1984). Both in nontransgenic and transgenic mice, endogenous GAP-43 in motoneurons was down-regulated at P8–10 (Figure 1D), and no corresponding GAP-43 immunoreactivity was detected in intramuscular motoneuron processes after this stage (Figure 2C, top). In contrast, adult transgenic mice expressed significant levels of the transgene in intramuscular motor nerves and at the neuromuscular junction (see Figures 1D, 2C, and 4B). Such expression was detected from P8 onward throughout life, i.e., up to at least P180. In 13 out of 13 independent transgenic lines, expression of the transgene was followed by prominent sprouting of motor nerves at the neuromuscular junction (Figures 2A and 2B; see Figure 6). This sprouting was even more pronounced than the robust reaction induced in nontransgenic mice by paralysis of skeletal muscle with botulinum toxin A (BotA) (Figure 2B). Ultraterminal sprouting was first detected around P20, i.e., about 12 days following the onset of transgene expression, was maximal around P30, and then declined to 40%–70% of maximal values in older animals. As judged from immunocytochemical la-

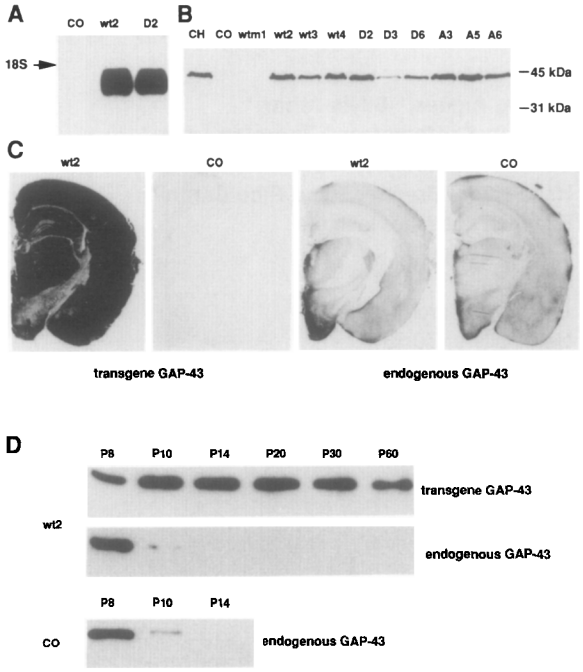

Figure 1. Independent Expression and Detection of Endogenous and Transgenic GAP-43 in 1-Month-Old Transgenic Mice Expressing Chick GAP-43

The transgenic lines are designated as follows: mice expressing wild-type GAP-43, GAP-43(wt1–6), or wt1–6; wild-type mouse GAP-43, GAP-43(wtm1,2), or wtm1,2; S42A mutant, GAP-43(A1–6), or A1–6; S42D mutant, GAP-43(D1–6), or D1–6.

(A) Specific detection of transgenic mRNA on Northern blots of mouse brains. CO, nontransgenic mouse.

(B) Expression of transgenic GAP-43. CH, E17 chick brain homogenate; CO, nontransgenic mouse. Note that in several lines, transgene levels in 1-month-old brains were comparable to maximal levels of endogenous GAP-43 in a chick brain (E17). Also note specificity of monoclonal antibody 5F10 for chick GAP-43 (no signal in line wtm1 expressing mouse GAP-43).

(C) Immunocytochemical detection of transgenic and endogenous GAP-43 on coronal sections of mouse brain (one sagittal half).

(D) Levels of transgenic and endogenous GAP-43 in the developing and adult gastrocnemius muscle. Immunoblots of muscle homogenates, ECL detection method. Endogenous GAP-43 was detected with monoclonal antibody 10E8, which does not cross-react with chick GAP-43. Note down-regulation of endogenous GAP-43 in control and wt2 mice between P8 and P14, and approximately constant levels of transgenic GAP-43 from P10 on.

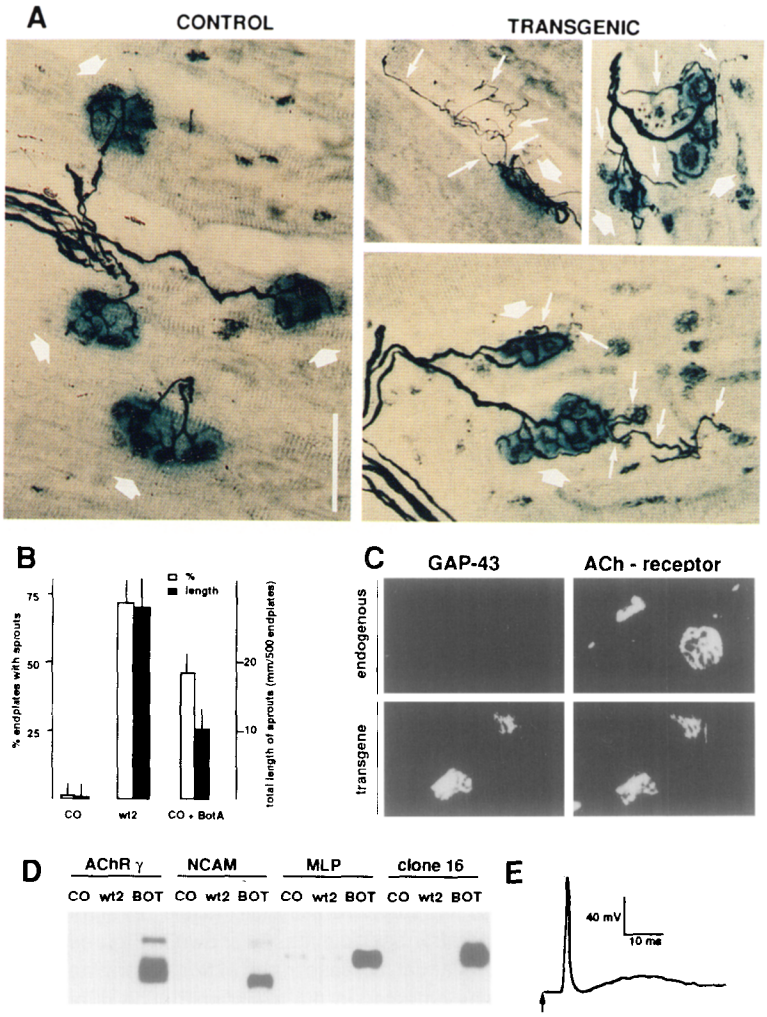

Figure 2. Spontaneous Ultraterminal Nerve Sprouting at the Neuromuscular Junction in the Gastrocnemius of 1-Month-Old *GAP-43*-Transgenic Mice (GAP-43(wt2) Line)

(A) Presence of ultraterminal nerve sprouts (small arrows) at neuromuscular junctions (large arrows) of transgenic (three panels on the right) mice. The combined silver-esterase reaction visualizes nerves (black) and acetylcholine esterase reaction product (blue; delimiting synaptic area).

(B) Quantitative analysis of ultraterminal sprouting in nontransgenic and transgenic mice; comparison with the sprouting reaction induced in the same type of muscle by local paralysis (8d) with BotA. n = 6.

(C) Expression of transgenic (bottom) but not endogenous (top) GAP-43 at the neuromuscular junction of a transgenic mouse. Sections were counterstained with α-bungarotoxin to visualize postsynaptic acetylcholine receptors.

(D) Contents of denervation-sensitive mRNAs in skeletal muscle of nontransgenic and transgenic mice. As in nontransgenic (CO) animals, the gastrocnemius of GAP-43(wt2)-transgenic mice does not express genes induced by the absence of electrical activation (e.g., BotA-induced paralysis [BOT]).

(E) Action potential evoked by nerve stimulation (arrow) in a lateral gastrocnemius muscle fiber of a transgenic nerve–muscle explant preparation.

Scale bar in (A), 40 μm; in (C), 90 μm.

beling of motor nerves and immunoblots of muscle homogenates (see Figures 1D and 6C), there was no obvious change in transgene expression in motoneurons between P10 and P30. A slight decline was detected on immunoblots of older animals. However, since total muscle mass was significantly higher in these animals and immunocytochemical signals were not obviously reduced, it is not clear whether the decline reflected a specific decrease in motor nerve transgene contents. Therefore, while decreased sprouting after P30 may be due at least in part to decreased transgene levels, the first appearance of detectable ultraterminal sprouting around P20 did not correlate with transgene levels in the motoneurons. Additional factors affecting nerve sprouting may include changes in the local environment at the neuromuscular junction, and in further intrinsic properties of the motor nerves.

In nontransgenic mice, ultraterminal nerve sprouting is only detected upon suppression of nerve-induced electrical activation (Brown, 1984). In contrast, in all transgenic lines included in this analysis (10 out of 13; see Figure 6 for the three exceptions), sprouting occurred in the absence of obvious alterations in synaptic function. Thus, first of all, muscle genes known to be induced in inactivated muscle were not induced in these transgenic mice (Figures 2D and 6C); second, action potentials could be induced by nerve stimulation in all muscle fibers in nerve–muscle explants from corresponding muscle (Figure 2E); and third, no muscle atrophy was detectable in several muscle types displaying extensive sprouting, indicating that the extent of muscle activation in vivo was not impaired in any major way (data not shown).

Spontaneous sprouts in the transgenic mice did not appear to induce synaptic specializations. Thus, no acetylcholine esterase reaction product was detected along the sprouts (Figure 2A), and no ectopic acetylcholine receptor accumulations were detected (data not shown). Significantly, in spite of the presence of maximal levels of transgene during the final phase of synapse elimination (P9–P14; see Figure 1D), skeletal muscle in GAP-43 mice was not polyinnervated (data not shown). Therefore, our results indicate that the persistent presence of the presynaptic growth-associated protein GAP-43 in spinal motoneurons from P8 on leads to extensive nerve sprouting, but to no obvious alterations in neuromuscular connections.

Potentiation of Induced Nerve Sprouting in the Peripheral and Central Nervous System of *GAP-43*-Transgenic Mice

We next determined whether the presence of GAP-43 promotes lesion-induced nerve growth in adult animals. To define the range of induced sprouting reactions that may be affected by GAP-43, four distinct paradigms were investigated. As a first experimental system, we induced local paralysis of skeletal muscle with BotA. This toxin blocks calcium-induced transmitter release at the neuromuscular junction (Thesleff, 1989), leading to prominent ultraterminal nerve sprouting in the absence of GAP-43 mRNA induction in the corresponding spinal motoneurons (Bisby et al., 1993). To facilitate the detection of BotA-induced effects, these experiments were carried out in the gluteus muscle of 2-month-old mice, when spontaneous and BotA-induced sprouting were less pronounced. As shown in Figure 3A, when compared with nontransgenic mice, *GAP-43*-transgenic mice showed substantially potentiated BotA-induced sprouting. This was reflected in a strongly elevated proportion of endplates with sprouts, and in a significant increase in the total length of sprouts per endplate (Figure 3A).

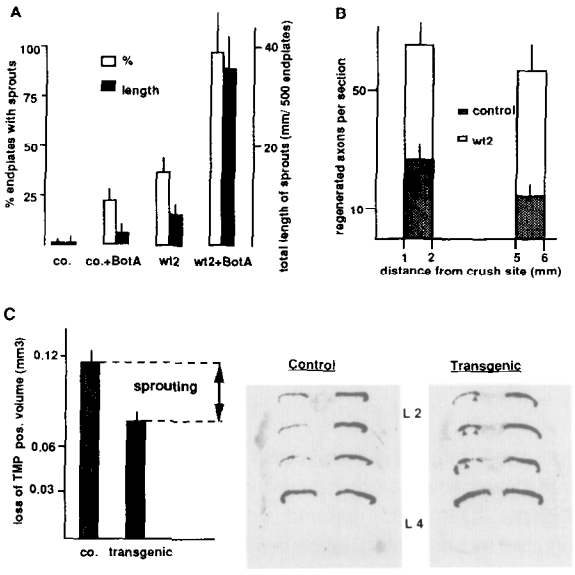

Figure 3. Potentiation of Induced Nerve Fiber Sprouting in *GAP-43*-Transgenic Mice (Line wt2)

(A) Sprouting in untreated and BotA-paralyzed (8d) gluteus muscles of 2-month-old mice. Note that to facilitate the detection of BotA-induced effects, these experiments were carried out under conditions slightly different from those of Figure 2 (gluteus instead of gastrocnemius; 2-month- instead of 1-month-old mice), where spontaneous and BotA-induced sprouting were less pronounced. Analysis as in Figure 2B; n = 3.

(B) Regenerative sprouting (at 64 hr) distal from sciatic nerve crush. Comparison for nontransgenic (control) and transgenic 2-month-old mice; n = 8.

(C) Sprouting of primary afferents in the upper dorsal horn of the spinal cord following rhizotomy. Deafferentation was assayed 3 weeks after unilateral dorsal root lesion (L1 to L3) by thiaminemonophosphatase (TMP) histochemistry. Left, quantitative analysis; right, representative example of the data. TMP label in lamina 2 of the spinal cord is strongly reduced on the lesioned side of control mice, but partially recovered (arrowheads) in transgenic mice, owing to the increased sprouting of remaining afferents from adjacent roots. Nontransgenic, n = 8; transgenic, n = 6. p = 0.0007 (Mann–Whitney test).

In a second approach, we analyzed regenerative neurite outgrowth after sciatic nerve crush. The distal nerve segment was examined at a time when the endogenous GAP-43 that is reinduced by the peripheral nerve lesion just begins to accumulate at the crush site (data not shown). As shown in Figure 3B, this early sprouting and regenerative reaction was potentiated in GAP-43 mice. Owing to the experimental design, the 4-fold increase in the number of neurite profiles 5–6 mm distal from the crush site is presumably due to the presence of transgenic GAP-43 in the growth cones of the crushed nerves.

To test for lesion-induced sprouting in the central nervous system (CNS), we carried out dorsal root lesions in nontransgenic and *GAP-43*-transgenic mice. A very small amount of sprouting of spinal cord afferents into the denervated zone has been demonstrated after such lesions in normal adult animals. This limited reaction correlates with the absence of an induction of GAP-43 expression in sensory neurons after dorsal root lesions (Chong et al., 1994). In *GAP-43*-transgenic mice, the size of the denervated zone after dorsal root lesions was much reduced when compared with that in nontransgenic mice, indicating an increased sprouting of primary spinal cord afferents in the transgenic mice (Figure 3C). These results are consistent with findings that sprouting in this system can be induced by peripheral nerve lesion or demyelination, procedures that lead to an induction of GAP-43 expression (Woolf et al., 1992; Schwegler et al., 1995).

Finally, we analyzed skeletal muscle reinnervation in control and transgenic mice. As shown in Figures 4A and 4C, a substantial increase in the extent of terminal arborization was detected in the transgenic mice during early reinnervation (e.g., 11 days after sciatic nerve crush). Subsequently, down-regulation of endogenous GAP-43 occurred at a comparable time in control and transgenic mice (Figure 4B). This process coincided with the down-regulation of denervation-sensitive genes in the reinnervated muscle (Figure 4D), and with the retraction of ultraterminal sprouts in nontransgenic mice (Figures 4A [bottom] and 4C). In contrast, the nerve retraction process was markedly delayed in the GAP-43 mice (Figures 4A and 4C). In summary, these results from distinct experimental approaches demonstrate that the expression of GAP-43 in adult neurons leads to a marked potentiation of their induced sprouting response, indicating that GAP-43 is an intrinsic determinant of neurite outgrowth in vivo.

Sprouting of Hippocampal Mossy Fibers in *GAP-43*-Transgenic Mice

To determine whether spontaneous nerve sprouting could also be detected in the CNS of *GAP-43*-transgenic mice, we visualized the terminals of hippocampal mossy fibers with a specific histological reaction. Under normal physiological conditions, spontaneous sprouting is absent in nontransgenic mice. A prominent sprouting reaction is sometimes, but not always, associated with kindling-induced epileptic seizures, or certain long-term potentiation–induc-

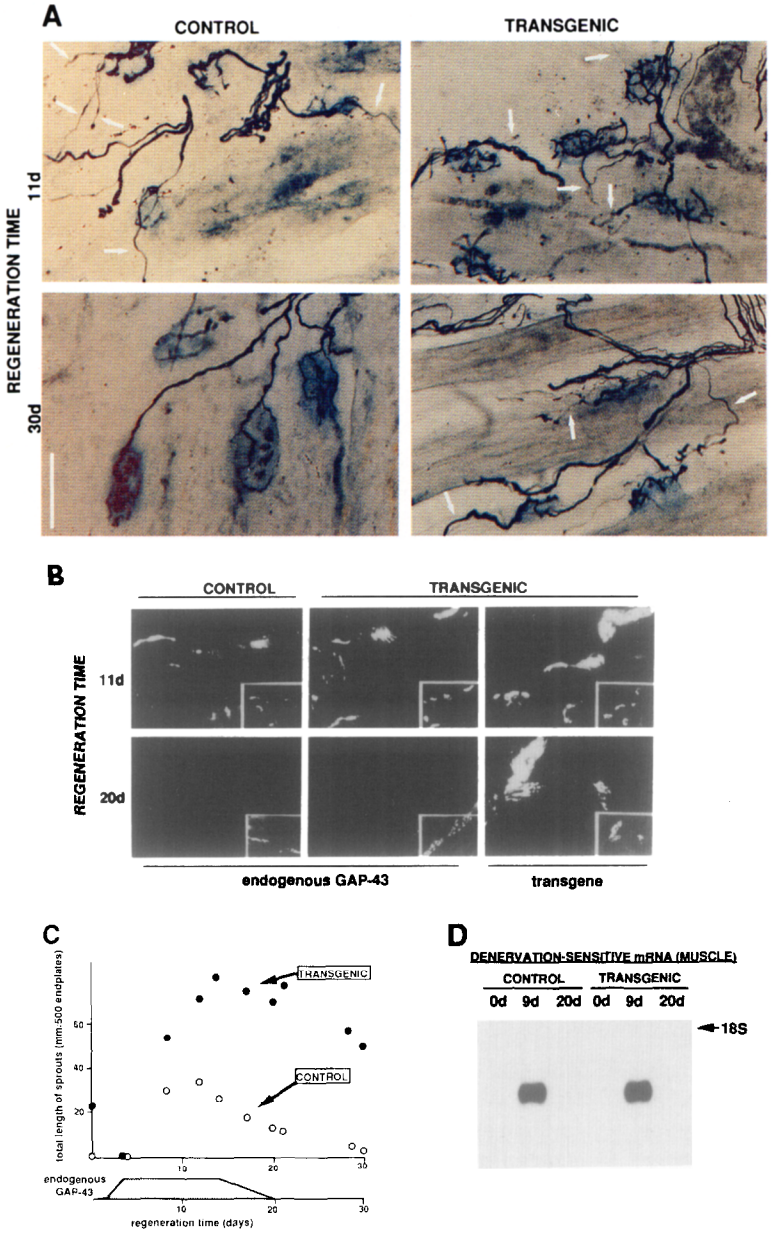

Figure 4. Expanded Terminal Arborization during and after Reinnervation of the Gastrocnemius Muscle in 1-Month-Old GAP-43(wt2)-Transgenic Mice

(A) Reinnervation pattern 11 and 30 days after mid-thigh sciatic nerve crush. Silver–esterase reaction; arrows, ultraterminal sprouts. Note elevated number of nerve profiles (bundles) and sprouts at 11 days in the transgenic mouse. At 30 days, most sprouts had retracted in the nontransgenic but not in the transgenic mice.

(B) Comparable down-regulation of endogenous GAP-43 immunoreactivity in regenerated intramuscular nerves of nontransgenic (control) and transgenic mice. To visualize nerves and neuromuscular junctions, the insets show the corresponding neurofilament-200 plus α-bungarotoxin counterstainings.

(C) Quantitative analysis of ultraterminal sprouts during reinnervation. Total length of sprouts as defined in Figure 2C. Each point represents one experimental animal. Values at 0 days are corresponding spontaneous sprouting data. At 4 days, all endplates were denervated. The lower trace is a qualitative representation of endogenous GAP-43 immunoreactivity levels in the corresponding motor nerves (see also [B]).

(D) Comparable induction and down-regulation of denervation-sensitive muscle mRNA (clone 16; see Figure 2D) during the de- and reinnervation experiment in nontransgenic and transgenic mice. The numbers are days after sciatic nerve crush.

Scale bar in (A), 40 μm; in (B), 100 μm.

ing protocols (Ben-Ari and Represa, 1990). As shown in Figure 5A, the distribution of endogenous GAP-43 immunoreactivity in the hippocampus of control and transgenic mice was undistinguishable. Furthermore, the mossy fibers of dentate gyrus granule cells (arrowheads in Figure 5A) did not express endogenous GAP-43 but did express transgenic GAP-43. Figure 5B demonstrates that in such mice (7 out of 13 transgenic lines), prominent mossy fiber sprouting could be detected. Some transgenic lines with strong mossy fiber sprouting (wt2, D2) exhibited a striking tendency for spontaneous epileptic states, whereas lines with less pronounced sprouting (e.g., A3) showed no signs of epileptic behavior. In wt2 and D2 mice, mossy fiber sprouting was detectable from the fourth postnatal week (P21–23) on, and seizure episodes appeared later. Significantly, treatment of wt2 or D2 mice with the broad-range seizure-suppressing agent phenobarbital from P18 to P34 did not detectably affect the extent of mossy fiber sprouting at P35 (Figure 5B), supporting the notion that sprouting in these mice was not a consequence of seizure-like episodes. Therefore, these findings indicate that expression of GAP-43 in hippocampal mossy fibers can promote massive sprouting in their terminal region.

The Nonphosphorylatable Mutant GAP-43(S42A) Has Reduced Sprout-Promoting Activity

The unique PKC site Ser-41 (Ser-42 in the chick) of GAP-43 is a major physiological target for phosphorylation in the nervous system (Benowitz and Routtenberg, 1987). To define the role of this phosphorylation reaction for the activity of GAP-43, we analyzed spontaneous and induced nerve sprouting in transgenic mice expressing either GAP-

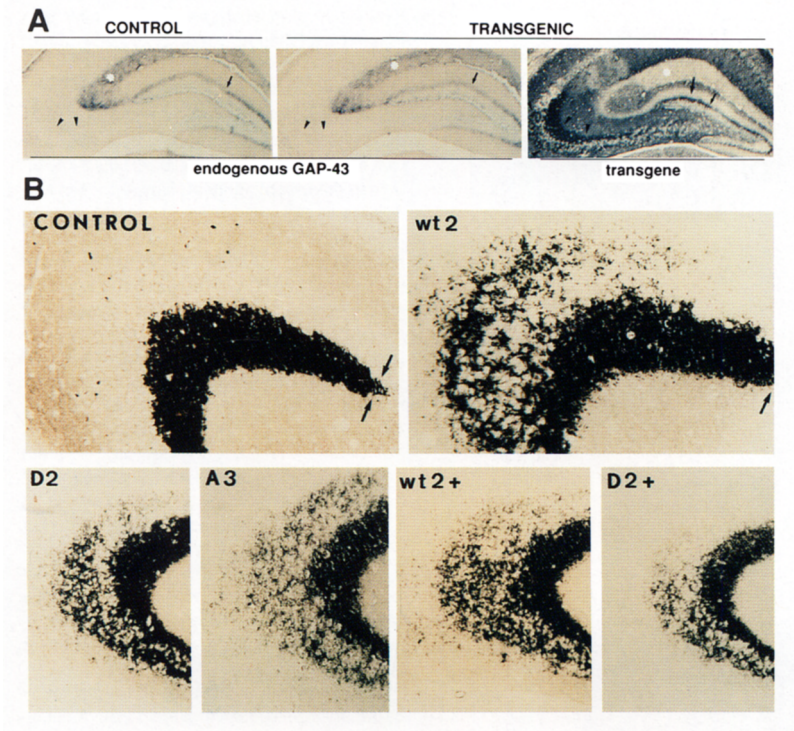

Figure 5. Sprouting of Mossy Fibers in the Hippocampus of GAP-43-Overexpressing Mice

(A) Expression of endogenous and transgenic GAP-43 in the hippocampal formation. Immunocytochemistry, visualization with alkaline phosphatase reaction. Note undistinguishable expression of endogenous GAP-43 in 2-month-old nontransgenic and transgenic mice (wt2 line). Signal is detected in the C/A region of the dentate gyrus cortex (arrow), and in stratum lacunosum-moleculare (asterisk). No endogenous GAP-43 is expressed in mossy fibers (arrowheads). The transgene is expressed in most hippocampal axonal pathways, with the exception of the middle perforant pathway (larger arrow) and stratum lacunosum-moleculare. Prominent expression is detected in the mossy fibers, which display sprouting in CA3 (arrowheads).

(B) Visualization of mossy fiber terminals in CA3 with the selective TIMM reaction. In the upper two panels, the outer reach of the mossy fiber pathway is marked by the arrows. Control, nontransgenic mouse; wt2+ and D2+, mice from the corresponding transgenic lines were treated with the antiepilecticum phenobarbital from P18 to P34 and analyzed at P35. All untreated mice were 2 months old. Note prominent mossy fiber sprouting into the pyramidal cell layer of CA3 in the transgenic mice. Also note prominent sprouting in the phenobarbital-treated mice, and in A3 mice, which showed no signs of seizures.

43(S42A) or GAP-43(S42D). The S42A mutant cannot be phosphorylated by PKC, whereas the S42D mutant mimicks phopshorylated GAP-43 and does not bind calmodulin (Liu and Storm, 1990; Chapman et al., 1991). As shown in Figure 6, the S42A nonphosphorylatable mutant (A3 and A5 mice) also did promote spontaneous (Figure 6A) and BotA-induced sprouting at the neuromuscular junction (Figure 6B), but to a much lesser extent than wild type. In contrast, the S42D mutant (D2 and D6 mice) had an activity comparable to that of wild-type GAP-43 (Figures 6A and 6B). The diminished activity of the nonphosphorylatable mutant was not due to reduced expression levels of the transgene at the neuromuscular junction or in the whole brain (Figures 6C and 1B). These results indicate that the status of Ser-41(42) affects the neurite-promoting activity of GAP-43. Phosphorylation of GAP-43 by PKC may potentiate its sprout-promoting activity. However, because the S42D mutant did not induce more sprouting than the wild-type protein, the results are also consistent with the possibility that both phosphorylation and calmodulin binding may be involved in the neurite-promoting activity of this protein.

A Motor Behavioral and Cerebellar Phenotype in *GAP-43*-Transgenic Mice Is Abolished by the S42D Mutation, Which Suppresses Calmodulin Binding

Does the expression of GAP-43 in the transgenic mice lead to a behavioral phenotype? As mentioned above, we noticed a striking predisposition for spontaneous seizure-like episodes in some lines of mice with strong mossy fiber sprouting in the hippocampus. However, the most consistent phenotype in *GAP-43*-transgenic mice consisted in a characteristic adduction of the hindlimbs when the animals were lifted by the tail. Significantly, when only lines with robust expression of the transgene were considered, it was detected in 3 out of 3 GAP-43(A), 7 out of 7 GAP-43(wt, or wtm), but in none of 3 GAP-43(D) lines (Figure 6D), in which calmodulin binding by GAP-43 is abolished. We carried out a detailed analysis of gross nervous system histology. No differences between transgenic and control mice could be detected in immunostainings of the brain and spinal cord for myelin basic protein, glial fibrillary acidic protein, the synaptic vesicle protein synaptophysin, the calcium-binding protein parvalbumin, or nonphosphorylated neurofilaments as detected by monoclonal antibody SMI 31. Cresyl violet stainings also did not reveal obvious differences, except for a striking reduction of cells, mainly stellate interneurons in the cerebellar molecular layer of certain lines (Figure 6D). In most affected lines, this loss was less than 20% (indicated by a check mark in Figure 6D). However, in the three exceptional lines wt4, wt5, and A6, it exceeded 70%. Detailed analysis revealed that it was due to elevated apoptotic cell death, which was restricted to development (P7–15) and involved cerebellar interneurons and granule cells. In the same three exceptional lines, but in none of the other transgenic lines, progressive loss of spinal motoneurons was detected from the fourth postnatal week on. This loss was restricted to the lumbar portion of the spinal cord, where it exceeded 50% in certain older animals (range of 20%–35% for 3-month-old mice) and was accompanied by induction of

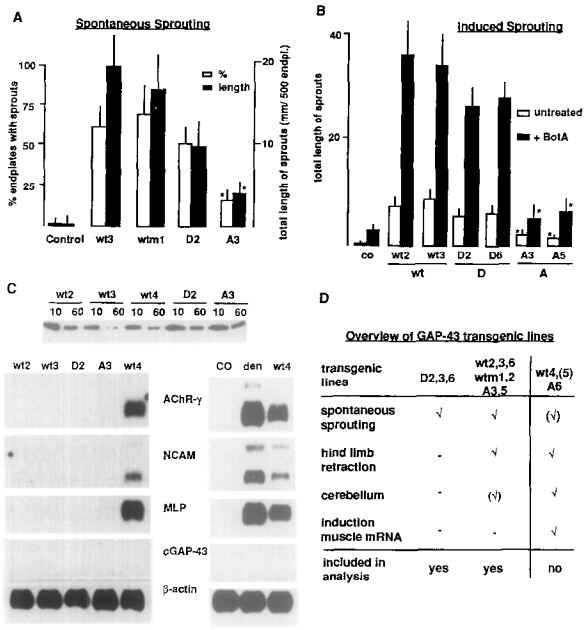

Figure 6. GAP-43(S42A), a Mutant That Cannot Be Phosphorylated by PKC, Has Reduced Sprout-Promoting Activity

(A) Quantitation of spontaneous ultraterminal sprouting in 1-month-old gastrocnemius in nontransgenic (control) and transgenic (wt3, wtm1, D2, A3) lines. Data comparable to those of Figure 2; n = 6. The differences between wt2 (Figure 2C), wt3, wtm1, and D2 mice were not significant, whereas A3 mice displayed significantly reduced sprouting (asterisk; $p < 0.0005$, Mann–Whitney test).

(B) Comparison of spontaneous (untreated) and BotA-induced (d8) sprouting in gluteus muscle from 2-month-old nontransgenic (co) and various transgenic lines. Data comparable to those of Figure 3A; n = 3. Values for wt and D lines were not significantly different, whereas values for A lines were significantly lower (asterisks; $p < 0.001$, Mann–Whitney test).

(C) Contents of transgenic GAP-43 and expression of denervation-sensitive genes in the gastrocnemius muscle of *GAP-43*-trangenic lines. Top panel, immunoblot of muscle homogenates from P10 and P60 mice, as indicated. Detection with monoclonal antibody 5F10, as described in Figure 1D. Bottom panels, Northern blot analysis of RNA from 1-month-old mice. CO, nontransgenic mouse; den, 8d denervated muscle from a nontransgenic mouse; MLP, muscle LIM protein; cGAP-43, transgene mRNA. Note that the exceptional line wt4 showed signs of denervation, whereas the other lines did not. Also note that consistent with its neuron-specific pattern of expression, no transgene mRNA was detected in skeletal muscle.

(D) Summary of characteristic phenotypic properties of *GAP-43*-transgenic lines. Note that the three exceptional lines (wt4, wt5, A6) with marked losses of cells in the molecular layer of the cerebellar cortex and in the lumbar spinal cord, and with signs of denervation at the gastrocnemius muscle, were not included in the analysis of GAP-43-induced sprouting. Check, positive; minus, negative; check in parentheses, intermediate phenotype (cerebellum), or presence of partial denervation (sprouting).

activity-sensitive genes in the corresponding muscles (Figure 6C). Electrophysiological analysis revealed the presence of functionally denervated muscle fibers from P21 on. This was preceded by progressive loss of evoked transmitter release, suggesting a possible involvement of calmodulin binding by GAP-43 in this process. The cerebellar and motoneuron loss was not due to obviously elevated overall expression of the transgene in these lines (see Figures 1B and 1D), and its causes are presently not clear. Owing to the fact that partial denervation by itself induces local nerve sprouting, these lines were excluded from the analysis.

Discussion

The expression of the nervous system protein GAP-43 correlates with neurite outgrowth, an expanded terminal field, and predisposition for nerve sprouting during development and in the adult. By expressing GAP-43 in adult neurons of transgenic mice, we now directly demonstrate that the presence of this protein greatly potentiates spontaneous and induced nerve sprouting and plasticity. Taken together, the results support and extend the GAP hypothesis by demonstrating that GAP-43 is a presynaptic intrinsic determinant of neurite outgrowth and plasticity.

The Expression of GAP-43 in Adult Neurons Leads to Spontaneous Sprouting

Nerve sprouting has been studied most extensively at the neuromuscular junction, where induction of ultra- and preterminal sprouting requires massive impairment of muscle activation (Brown, 1984). Sprouting is invariably preceded by characteristic reactions in the inactive muscle, such as the induction of activity-sensitive genes in skeletal muscle fibers and muscle interstitial cells (Laufer and Changeux, 1989; Sanes, 1989). These reactions, which lead to the expression of diffusible growth factors and of certain cell adhesion and extracellular matrix proteins, are thought to cause local stimulation of a nerve-sprouting reaction aimed at restoring functional neuromuscular activity. GAP-43 expression is not detectable in adult motoneurons, and its reinduction apparently requires nerve lesion. Therefore, sprouting induced by partial denervation or BotA paralysis does not require the expression of GAP-43. We now demonstrate that in *GAP-43*-transgenic mice, robust sprouting can occur in the absence of any detectable induction of activity-sensitive genes in the muscle, in spite of an electrophysiologically functional neuromuscular transmission apparatus, and in the absence of any detectable sign of muscle atrophy (lines wt2, wt3, wt6, wtm1, wtm2, D2, D6, D3, A3, and A5).

Recent studies indicate that the terminal Schwann cell can respond to muscle denervation by process extension (Reynolds and Woolf, 1992), and that nerve sprouts preferentially grow along terminal Schwann cell processes (Son and Thompson, 1995). However, no transgene mRNA could be detected in skeletal muscle (Figure 6C), and denervation led to rapid disappearance of transgenic GAP-43 at the neuromuscular junction (data not shown), indicating that, like other glial cells, terminal Schwann cells did not express transgene. Therefore, by these criteria, expression of GAP-43 in presynaptic adult spinal motoneurons directly led to spontaneous intramuscular nerve sprouting in the presence of a functional neuromuscular system.

How does GAP-43 induce spontaneous nerve sprouting? Analysis of GAP-43-depleted sensory neurons in vitro revealed that the presence of this GAP greatly promoted adhesion and sustained spreading or branching of the growth cone (Aigner and Caroni, 1995). Accordingly, the

presence of GAP-43 in the adult transgenic mice may stabilize spontaneous lamellar and filopodial structures at the nerve terminal (Robbins and Polak, 1988), thus allowing the formation of persistent nerve sprouts in the presence of an otherwise subthreshold environment for nerve sprouting (Figure 7). According to this interpretation, nerve sprouting and terminal arborization would be affected by the combined actions of extrinsic factors such as diffusible and surface-bound molecules that may promote or inhibit neurite outgrowth, and by intrinsic factors such as GAP-43 that would favor neurite outgrowth.

This study demonstrates that overexpression of GAP-43 induces and potentiates nerve sprouting in an adult context. A recent study demonstrated that in GAP-43-deficient mice, growth cone guidance during development is impaired (Strittmatter et al., 1995). The results of both in vivo studies and those from our in vitro depletion studies (Aigner and Caroni, 1995) complement each other and are all consistent with the model presented in Figure 7, which proposes that GAP-43 potentiates growth cone responsiveness to signals from the local environment. This would promote growth cone guidance and the formation and growth of sprouts.

What are the functional consequences of GAP-43-induced nerve sprouting? We found no evidence that intramuscular sprouts would induce ectopic synaptic structures. This may not be surprising, since, in mammals, in an active neuromuscular system the synaptic apparatus tends to be stable, and the formation of ectopic synapses is prevented. Perhaps less expected was the observation that elimination of polyinnervation at the neuromuscular junction of GAP-43 mice proceeded to completion, and essentially on schedule (data not shown). Endogenous GAP-43 is down-regulated between P8 and P12, i.e., during synapse elimination (Caroni and Becker, 1992), and maximal levels of transgene were detected between P9 and P14 (Figure 1D). It would, therefore, have been conceivable that the excess growth potential in the GAP-43 mice may have interfered with the elimination process. Our results are consistent with the proposal by Lichtman and his coworkers (Balice-Gordon and Lichtman, 1994) that synapse elimination is an activity-sensitive process initiated and controlled by the postsynaptic muscle cell. Accordingly, presynaptic growth favored by intrinsic components such as GAP-43 may provide potential presynaptic elements, but activity-sensitive processes on the postsynaptic side would determine whether and how synapses would form.

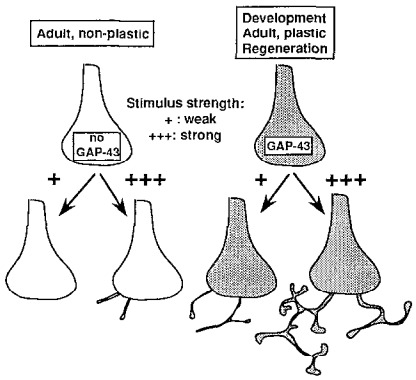

Figure 7. Proposed Role of GAP-43 as an Intrinsic Presynaptic Potentiator of Local Growth Reactions in Response to Extrinsic Signals

Most nerve terminals in the adult express little or no GAP-43 (left). Weak stimuli elicit no growth reactions (e.g., no spontaneous sprouting at the neuromuscular junction), whereas stronger stimuli (BotA, rhizotomy) elicit a limited sprouting reaction. In contrast, growth cones and nerve terminals contain substantial levels of GAP-43 during development and regeneration, but also in certain systems (e.g., pyramidal neurons in the associative cortex) in the adult (right). This leads to potentiated reactiveness to local growth stimuli.

GAP-43 Potentiates Induced Nerve Sprouting

The induced sprouting experiments demonstrate that the presence of GAP-43 in the sprouting nerves leads to a marked gain-of-function phenotype, indicating that GAP-43 is an intrinsic potentiator of neurite outgrowth. These findings are in good agreement with a large body of evidence suggesting the existence of a strong correlation between the presence of GAP-43 and nerve sprouting. The BotA experiments shown in Figure 3A clearly support the model of GAP-43 as an intrinsic amplifier of growth and plasticity stimuli, as proposed in Figure 7. Thus, BotA blocks calcium-induced transmitter release at the nerve terminal, leading to a sprout-promoting environment, but it does not induce GAP-43 expression in spinal motoneurons. In the presence of GAP-43 in the motoneurons, the sprouting reaction was greatly potentiated. In the spared roots paradigm, sprouting in the CNS is induced by signals from a vacated terminal field. Very limited invasion by neighboring intact sensory neurons in nontransgenic mice correlates with an absence of GAP-43 induction in these neurons, whereas the presence of GAP-43 in the transgenic mice potentiated this type of sprouting. Importantly, potentiation of sprouting in the transgenic mice was apparently not due to general induction of a growth state in presynaptic neurons, since endogenous GAP-43 and the related growth-associated protein CAP-23 (Widmer and Caroni, 1990; Caroni et al., unpublished data) were regulated normally and were not induced in these experiments.

Spontaneous sprouting and potentiation of induced sprouting were substantially reduced in mice expressing GAP-43(S42A), a mutant that cannot be phosphorylated by PKC. These results suggest that phosphorylation of GAP-43 by PKC potentiates its effects on nerve sprouting. However, the fact that the S42D mutant (GAP-43(D)-transgenic lines) did not promote sprouting to a larger extent than the wild-type protein suggests that the full activity of GAP-43 may involve reversible phosphorylation by PKC and calmodulin binding. Comparative analysis of the transgenic mice revealed a further aspect of the regulation of GAP-43 at its PKC phosphorylation site. Thus, the hindlimb adduction and cerebellar phenotypes were detected in GAP-43(wt)- and GAP-43(A)- but not in GAP-43(D)-transgenic lines (Figure 6D). Since GAP-43(S42D) does not bind calmodulin, these findings support the notion that binding of calmodulin is involved in the activities of this growth-associated protein.

Conclusions

This study indicates that GAP-43 is an intrinsic determinant of presynaptic plasticity modulated by PKC. One implication is that, given its potency, mechanisms controlling the levels of GAP-43 are likely to play an important role in defining the potential for presynaptic plasticity. Perhaps reflecting its importance, such regulation appears to operate at multiple levels, including transcription, mRNA stability, and posttranslational modifications. A further major question is whether the presence of GAP-43 in adult neurons may promote nerve regeneration. Efficient long-distance regeneration of lesioned nerves may require full induction of a growth program, a reaction that seems to be prevented in the CNS of higher vertebrates. However, it will be of interest to detemine whether the presence of GAP-43 may improve the limited regeneration that can be achieved by presently known interventions.

Experimental Procedures

Generation of *GAP-43*-Transgenic Mice

A 8.2 kb EcoRI genomic DNA fragment encompassing the murine *Thy-1.2* gene was used to construct the expression cassette (a gift from G. A. Evans and S. Chen, Salk Institute, San Diego, CA). This drove constitutive transgene expression in postnatal and adult neurons, with no expression in the thymus, and weak expression in the lung (see Vidal et al., 1990). The phosphorylation-site mutants of chick GAP-43 were as described (Widmer and Caroni, 1993). To avoid possible regulation of *GAP-43* mRNA stability or translation due to regulatory sequences in the untranslated regions, the cDNA sequences only contained 4 nt (chick) and 6 nt (mouse) of 5′-untranslated region, and 23 nt (chick) and 10 nt (mouse) of 3′-untranslated region. Founders were analyzed by genomic Southern blots to verify that no rearrangements had occurred. A survey of mouse tissues revealed very weak expression in the lung and no further expression outside the nervous system. In the nervous system, we detected prominent expression in a diverse range of neuronal types in the PNS and CNS, but no expression in glial fibrillary acidic protein–positive astrocytes, peripheral nerve Schwann cells, terminal Schwann cells at the neuromuscular junction, or GalC-positive oligodendrocytes (data not shown).

Detection of mRNAs and Proteins

Total RNA was isolated and analyzed on Northern blots with digoxigenin-labeled riboprobes, as described (Arber et al., 1994). Clone 16 corresponds to a 0.9 kb mRNA expressed in skeletal and heart muscle (Arber et al., unpublished data). In mouse skeletal muscle, it is downregulated between P8 and P12, and it is rapidly and dramatically induced by denervation or paralysis. A rat cDNA coding for the γ subunit of the acetylcholine receptor was a kind gift from A. Buonanno (the National Institutes of Health, Bethesda, MD). A rat *N-CAM* cDNA fragment (nucleotides 638–1058 of the sequence published by Small et al. [1987]; this probe hybridizes to all major splice forms of *N-CAM*) was cloned from a rat brain cDNA library. For immunoblots, brains or gastrocnemius muscles were homogenized in SDS–PAGE sample buffer, and 40 μg of protein was loaded per slot (14% polyacrylamide gels). GAP-43 was visualized with either monoclonal antibody 5F10 (specific for chick GAP-43; Widmer and Caroni, 1990), monoclonal antibody 10E8 (detects mouse GAP-43 but not chick GAP-43; gift of K. Meiri, State University of New York at Syracuse, Syracuse, NY), monoclonal antibody 7B10 (detects chick GAP-43 and mouse GAP-43; gift of K. Meiri), an antiserum to the last 12 carboxy-terminal residues of chick GAP-43 (Aigner and Caroni, 1993), or an antiserum to the carboxy-terminal residues KEDPEADQEHA of rat and mouse GAP-43 (this antiserum is selective for rodent GAP-43; Aigner et al., unpublished data). Immunocytochemistry was performed on 12 μm cryostat sections of 4% formaldehyde-fixed tissues, as described (Widmer et al., 1990). Neurofilament-160 and neurofilament-200 were detected with specific monoclonal and polyclonal antibodies, respectively (Sigma). Rhodamine-α-bungarotoxin was from Molecular Probes (Eugene, OR). Bound antibodies were visualized with biotin-conjugated second antibodies, followed by lucifer yellow–conjugated streptavidin, and with rhodamine-conjugated second antibodies (all secondary antibody reagents were from Molecular Probes, Eugene, OR).

Electrophysiology on Nerve-Muscle Explants

For electrophysiological experiments, muscles were pinned out in a Sylgard-covered Petri dish filled with a solution containing 40% Leibovitz L15 medium and the following: 140 mM NaCl, 4 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 5 mM HEPES. The solution was bubbled with 95% O_2 and 5% CO_2 and adjusted to pH 7.3 with $NaHCO_3$. Gassing was continued for the entire period of the experiment. Intracellular recordings and nerve stimulation were conventional.

Lesion Protocols

Local paralysis was induced by a single subcutaneous injection of 0.8 pg of purified BotA (gift of C. Montecucco, Pavia, Italy, and S. Catsicas, Geneva, Switzerland) over the right gluteus maximus of 1- or 2-month-old mice. Paralysis was obvious after 24 hr and was verified as described (Caroni et al., 1994). Paralyzed muscle was analyzed 8 days after the BotA injection. For crush experiments, mice were anesthetized, and the right sciatic nerve was exposed at mid-thigh level. The nerve was crushed over 1 mm, with appropriate watchmaker tweezers, by applying pressure for 15 s. For the short-term regeneration experiments, 4 out of 18 animals displayed absence of regeneration over more than 10% of the crush, presumably owing to mechanical barriers, and were not included in the analysis. The rhizotomy experiments were carried out as described. In brief, dorsal roots L1–L3 were sectioned and corresponding ganglia removed unilaterally in 1-month-old normal and transgenic mice.

Detection and Analysis of Sprouting

Intramuscular nerves and neuromuscular junctions were visualized on 50 μm cryostat sections of gastrocnemius and gluteus maximus muscle, as described (Pestronk and Drachman, 1978; Caroni et al., 1994). For the unambiguous identification of sprouts, only nerve profiles longer than 5 μm that clearly emerged from the endplate area (ultraterminal sprouts) were included in the analysis. At least 500 endplates per animal were analyzed. Microscope images were fed to a computer, and quantitative analysis of sprout length was performed with Image 1.4 software, as described (Caroni et al., 1994). BotA-induced and spontaneous sprouts had a similar appearance (in most cases shorter than 100 μm; thin, with frequent apparent changes of growth direction) and were therefore analyzed in the same manner. To provide a measure of the total length (mass) of the sprouts in a given muscle, the lengths of all ultraterminal sprouts in randomly selected fields were summed and normalized to 500 neuromuscular junctions (these included all neuromuscular junctions in a given field, i.e., those with and without sprouts). Regenerative nerve growth 64 hr after sciatic nerve crush was analyzed by counting the number of neurofilament-160-positive profiles 1–2 mm and 5–6 mm distal from the crush site. To this end, the entire width of the sciatic nerves was analyzed on 12 μm longitudinal cryostat sections. At the time of analysis, neurofilament immunoreactivity had essentially disappeared in the degenerating nerves, and regenerating profiles, which mainly occurred in loose bundles, could be clearly identified as thin, brightly stained continuous structures. In the rhizotomy experiments, sprouting from adjacent intact afferents was detected by the reduction of the thiaminemonophosphatase-devoid volume, as described (Schwegler et al., 1995). Mossy fiber terminals in the hippocampus were visualized with a modified TIMM staining procedure (Sloviter, 1982) on coronal sections that included the septal part of the hippocampal formation. For some experiments, to prevent the appearance of seizures, mice (six per experimental group) received one intraperitoneal injection of 20 μg/g of phenobarbital every 24 hr, from P18 to P34. Phenobarbital is a standard broad-range antiepilecticum, and this dosage effectively prevents seizures in mice (M. Schmutz, personal communication). The animals were sacrificed at P35 and analyzed for sprouting in the hippocampus.

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