**Table 1** Restoration of MUGS-degrading and MUG-degrading activities in cultured mouse Sandhoff Schwann cells and cultured human Sandhoff fibroblasts after addition of conditioned media from the transformed Chinese hamster ovary (CHO) cell lines

| | Conditioned medium | 5 mM M6P | MUGS-degrading activity | | MUG-degrading activity | |
|---|---|---|---|---|---|---|
| | | | (nmol/h/mg) | (%) | (nmol/h/mg) | (%) |
| Cultured mouse Schwann cells | | | | | | |
| Wild type (IMS32) | – | – | 992 | 100 | 2491 | 100 |
| Sandhoff (1113C1) | – | – | 188 | 19 | 177 | 5 |
| | + | – | 300 | 30 | 811 | 33 |
| | + | + | 203 | 20 | 316 | 13 |
| Cultured human fibroblasts | | | | | | |
| Normal | – | – | 981 | 100 | 4248 | 100 |
| Sandhoff | – | – | 52 | 5 | 63 | 1 |
| | + | – | 649 | 66 | 4201 | 99 |
| | + | + | 92 | 9 | 381 | 9 |

Cultured mouse Sandhoff Schwann cells (1113C1) and cultured human Sandhoff fibroblasts were cultured with conditioned media containing Hex isozymes from CHO transformants, as described in Materials and methods. Some experiments were performed in the presence of 5 mM M6P. After 3 days, the cells were harvested, and MUGS-degrading and MUG-degrading activities were measured. As a control, IMS32 cells from a wild-type mouse and human cultured fibroblasts from a normal subject were used. ($n=2$)

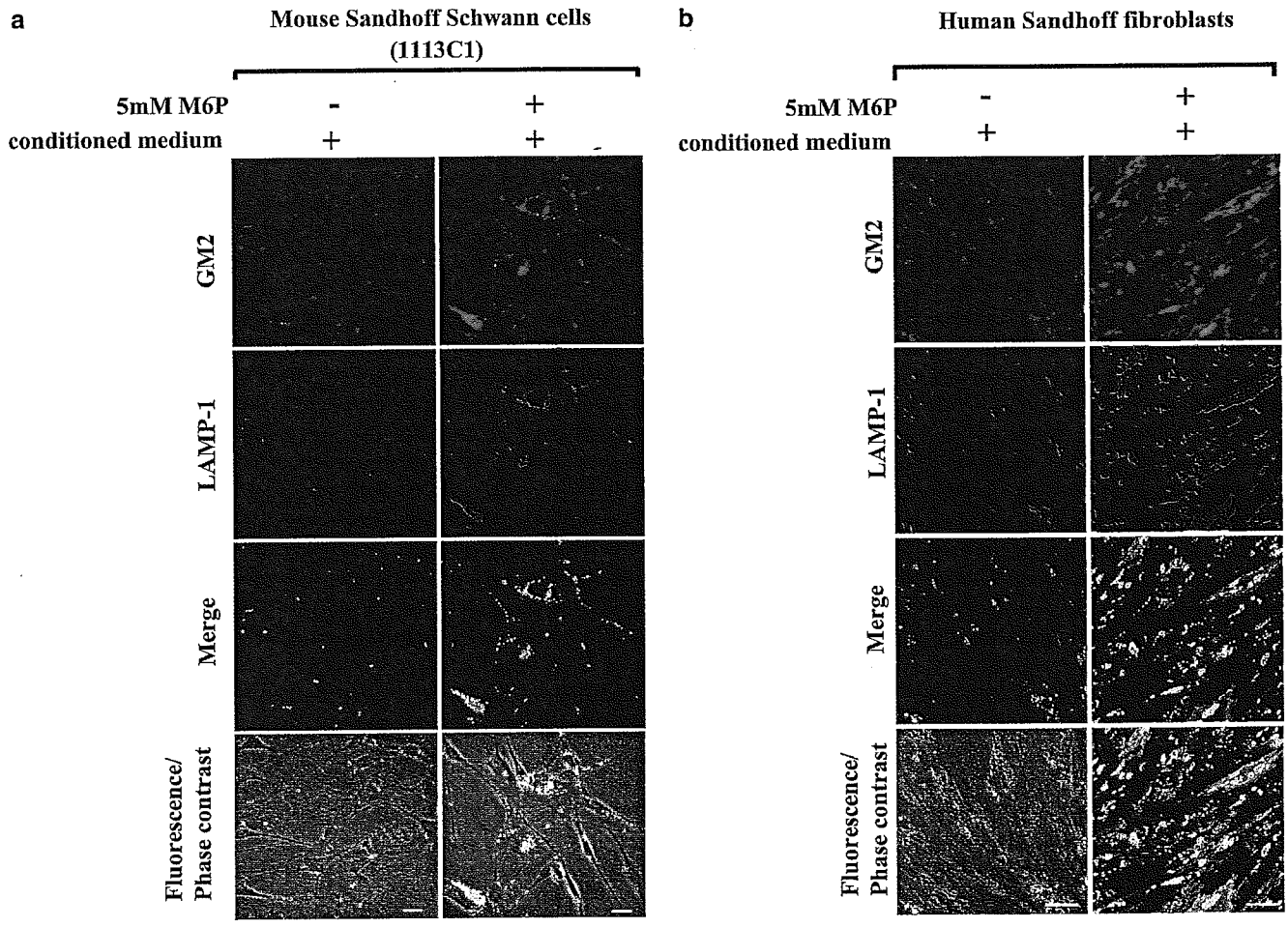


**Fig. 5** Administration of the recombinant human Hex isozymes to mouse Sandhoff Schwann cells and human Sandhoff fibroblasts. Cultured mouse Schwann cells (**a**) and human fibroblasts (**b**) with Sandhoff disease were stimulated with the conditioned media containing Hex isozymes from Chinese hamster ovary (CHO) transformants for 3 days. Double staining of these cells was carried out with a monoclonal antibody for GM2 ganglioside (*GM2, green*) and polyclonal antibodies for LAMP-1 (*LAMP-1, red*). *Fluorescence/Phase contrast images* with these two fluorescent probes are shown in *yellow*. Some experiments were performed in the presence of 5 mM M6P. *Bars*, 50 μm

human Sandhoff fibroblasts. Incorporation of the Hex isozymes was inhibited in the presence of 5 mM M6P in the culture medium, indicating that these Hex isozymes are taken up via cation-independent M6P receptors on the surface of cultured Schwann cells and cultured fibroblasts. Hex isozymes are known to be synthesized in the rough endoplasmic reticulum and are modified through the addition of high-mannose oligosaccharides. The Hex isozymes are transferred to the Golgi apparatus where further modification, including the addition of M6P residues to the nonreducing ends of the sugar chains and binding to the cation-independent M6P receptors, occurs. Then, the enzymes are transported to endosomes/lysosomes. In cultured fibroblasts, Hex isozymes are thought to be transported from the extracellular milieu to lysosomes through cation-independent M6P-receptor-mediated endocytosis (Kornfeld and Sly 2001). The recombinant Hex isozymes could also be incorporated into the cultured Schwann cells via cation-independent M6P receptors although the total number of cation-independent M6P receptors on the surface of cultured Schwann cells might be less than that on cultured fibroblasts.

The incorporated recombinant human Hex A degraded the accumulated GM2 ganglioside in the cultured mouse Sandhoff Schwann cells as well as in the cultured human Sandhoff fibroblasts. The enhanced immunofluorescence for LAMP-1 in these cells was normalized after the administration of Hex isozymes although an addition of M6P inhibited that. It suggests that the increased and enlarged lysosomes caused by the accumulation of GM2 ganglioside could be normalized by the uptake of Hex isozymes through cation-independent M6P receptors. This encourages us to develop ERT for Sandhoff disease although the problem of the blood–brain barrier and blood–nerve barrier must be solved in the near future.

In conclusion, we established immortalized Schwann cells from Sandhoff mice. The cells are useful for investigation and development of therapies for Sandhoff disease, i.e., we will be able to examine incorporation of a modified enzyme developed for therapy into neuronal cells using this cell line before performing an experiment with Sandhoff mice.

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Journal of Neuroscience Research 82:63–70 (2005)

# Motoneuron Degeneration After Facial Nerve Avulsion Is Exacerbated in Presymptomatic Transgenic Rats Expressing Human Mutant Cu/Zn Superoxide Dismutase

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We investigated motoneuron degeneration after proximal nerve injury in presymptomatic transgenic (tg) rats expressing human mutant Cu/Zn superoxide dismutase (SOD1). The right facial nerves of presymptomatic tg rats expressing human H46R or G93A SOD1 and their non-tg littermates were avulsed, and facial nuclei were examined at 2 weeks postoperation. Nissl-stained cell counts revealed that facial motoneuron loss after avulsion was exacerbated in H46R- and G93A-tg rats compared with their non-tg littermates. The loss of motoneurons in G93A-tg rats after avulsion was significantly greater than that in H46R-tg rats. Intense cytoplasmic immunolabeling for SOD1 in injured motoneurons after avulsion was demonstrated in H46R- and G93A-tg rats but not in their littermates. Facial axotomy did not induce significant motoneuron loss nor enhance SOD1 immunoreactivity in these tg rats and non-tg littermates at 2 weeks postoperation, although both axotomy and avulsion elicited intense immunolabeling for activating transcription factor-3, phosphorylated c-Jun, and phosphorylated heat shock protein 27 in injured motoneurons of all these animals. The present data indicate the increased vulnerability of injured motoneurons after avulsion in the presymptomatic mutant SOD1-tg rats. 

**Key words:** axotomy; facial nerve; amyotrophic lateral sclerosis; ALS; mutant Cu/Zn superoxide dismutase; SOD1; transgenic rat

Since the discovery of the mutation of Cu/Zn superoxide dismutase (SOD1) in patients with familial amyotrophic lateral sclerosis (ALS) and the development of transgenic (tg) mice and rats expressing human mutant SOD1 that show clinicopathological characteristics comparable to human familial ALS, the mutant SOD1-tg animals have been the most widely used experimental models for elucidating the pathomechanism of and the therapeutic approach for familial ALS as well as sporadic ALS (Cleveland and Rothstein, 2001). Although the precise mechanism of motoneuron degeneration in mutant SOD1-tg animals is largely unknown, the mutant SOD1 is thought to have a gain of toxic function (Cleveland and Rothstein, 2001). In another animal model of motoneuron degeneration, peripheral nerve avulsion exhibits extensive loss of motoneurons in adult rats (Søreide, 1981; Wu, 1993; Koliatsos et al., 1994; Watabe et al., 2000; Sakamoto et al., 2000, 2003a,b; Ikeda et al., 2003; Moran and Graeber, 2004). The mechanism of motoneuron degeneration after avulsion also remains unclear, but peroxinitrite-mediated oxidative damage and perikaryal accumulation of phosphorylated neurofilaments have been demonstrated in injured motoneurons after avulsion (Martin et al., 1999). Both of these pathological features have also been shown in

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spinal motoneurons in mutant SOD1-tg animals as well as in patients with familial and sporadic ALS (Estévez et al., 1998; Cleveland, 1999; Cleveland and Rothstein, 2001). If motoneuron degeneration after peripheral nerve avulsion shares any underlying mechanisms of motoneuron death associated with SOD1 mutation, motoneurons in presymptomatic mutant SOD1-tg animals may be more susceptible to pathological insults following avulsion compared with their non-tg littermates. If this is so, we may be able to utilize facial nerve avulsion as an animal model for understanding the mechanisms of motoneuron degeneration in ALS. In the present study, we examined injured motoneurons after facial nerve avulsion in presymptomatic mutant human SOD1-tg rats and their littermates.

## MATERIALS AND METHODS

### Animals and Surgical Procedures

The experimental protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Metropolitan Institute for Neuroscience and Tohoku University Graduate School of Medicine. The tg rats expressing human mutant SOD1 (H46R, G93A) were generated as described previously (Nagai et al., 2001). Two types of rats with SOD1 mutations, H46R and G93A, were used for experiments. The H46R-tg rats develop motor deficits at about 140 days of age and die after 3 weeks, and G93A-tg rats show the clinical signs at around 120 days of age and die after 10 days (Nagai et al., 2001).

The presymptomatic female H46R (90 days old)- and G93A (80 days old)-tg rats were anesthetized with inhalation of halothane. Under a dissecting microscope, the right facial nerve was exposed at its exit from the stylomastoid foramen. With microhemostat forceps, the proximal facial nerve was avulsed by gentle traction and removed from the distal facial nerve as described elsewhere (Sakamoto et al., 2000, 2003a,b; Ikeda et al., 2003). As for axotomy, the right facial nerve was transected at its exit from the stylomastoid foramen, and a distal portion of the nerve, 5 mm in length, was cut and removed. The wound was covered with a small piece of gelatin sponge (Gelfoam; Pharmacia Upjohn, Bridgewater, NJ) and closed by fine suture.

### Motoneuron Cell Counting

At 2 weeks postoperation, rats were anesthetized with a lethal dose of pentobarbital sodium and transcardially perfused with 0.1 M phosphate buffer, pH 7.4 (PB), followed by 4% paraformaldehyde in 0.1 M PB. The brainstem tissue was excised, postfixed in the same fixative for 2 hr, dehydrated, and embedded in paraffin, and serial transverse sections (6-μm thickness) were made. Every fifth section (24-μm interval) was collected, deparaffinized, and stained with cresyl violet (Nissl staining), and facial motoneurons having nuclei containing distinct nucleoli on both sides of the facial nuclei were counted in 25 sections as described elsewhere (Sakamoto et al., 2000, 2003a,b; Ikeda et al., 2003). The data were expressed as the mean ± SEM, and statistical significance was assessed by Mann-Whitney U-test.

### Immunohistochemistry

Immunohistochemistry on paraffin sections was performed with the following primary antibodies: sheep anti-human SOD1 (1:1,000; Calbiochem, San Diego, CA), rabbit anti-human SOD1 (1:10,000; kindly provided by Dr. K. Asayama; Asayama and Burr, 1984), mouse monoclonal anti-phosphorylated neurofilament SMI-31 (1:1,000; Sternberger Monoclonals, Lutherville, MD), rabbit anti-ubiquitin (1:1,000; Dako, Glostrup, Denmark), rabbit anti-glial fibrillary acidic protein (GFAP; 1:1,000; Dako), rabbit anti-activating transcription factor-3 (ATF3; sc-188, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-c-Jun (sc-1694, 1:200; Santa Cruz Biotechnology), mouse monoclonal anti-phosphorylated c-Jun (sc-822, 1:200; Santa Cruz Biotechnology), rabbit anti-heat shock protein (Hsp) 25 that reacts with rat Hsp27 (SPA-801, 1:200; Stressgen, Victoria, British Columbia, Canada), and rabbit anti-phosphospecific ($Ser^{15}$)Hsp27 (1:200; Oncogene, San Diego, CA). For immunohistochemistry, deparaffinized sections were pretreated with 0.3% $H_2O_2$ in methanol and preincubated with 3% heat-inactivated goat or rabbit serum in 0.1% Triton X-100 in phosphate-buffered saline (T-PBS). In cases of immunostaining with mouse primary antibodies, MOM blocking kit (Vector, Burlingame, CA) was used according to the manufacturer's instructions to reduce nonspecific background staining. Sections were then incubated overnight at 4°C with the primary antibodies diluted in T-PBS, followed by the incubation with biotinylated rabbit anti-sheep, goat anti-rabbit, or goat anti-mouse IgG at a dilution of 1:200 and with ABC reagent (Vector), visualized by 3,3′-diaminobenzidine tetrahydrocholoride (DAB)-$H_2O_2$ solution and counterstained with hematoxylin. For negative controls, the primary antibodies were omitted or replaced by nonimmunized animal sera.

## RESULTS

Two weeks after avulsion of the right facial nerves in non-tg littermates, the number of surviving facial motoneurons declined to ~70% of that on the contralateral side, similar to that in normal rats, as described previously (Sakamoto et al., 2000). In SOD1-tg rats, only ~30–50% of motoneurons survived 2 weeks after avulsion, indicating that the loss of motoneurons was exacerbated in SOD1-tg rats compared with their non-tg littermates (Fig. 1, Table I). The numbers of surviving motoneurons in G93A-tg rats after avulsion (~35% of contralateral side) were significantly less than those in H46R-tg rats (~50% of contralateral side; Table I). The numbers of intact motoneurons at contralateral sides did not differ between tg rats and non-tg littermates, indicating that cell loss does not happen at this moment in the course of the disease with SOD1 mutations (Table I). Facial nerve axotomy did not induce significant loss of injured motoneurons in tg rats and non-tg littermates at 2 weeks postoperation (Fig. 1, Table I).

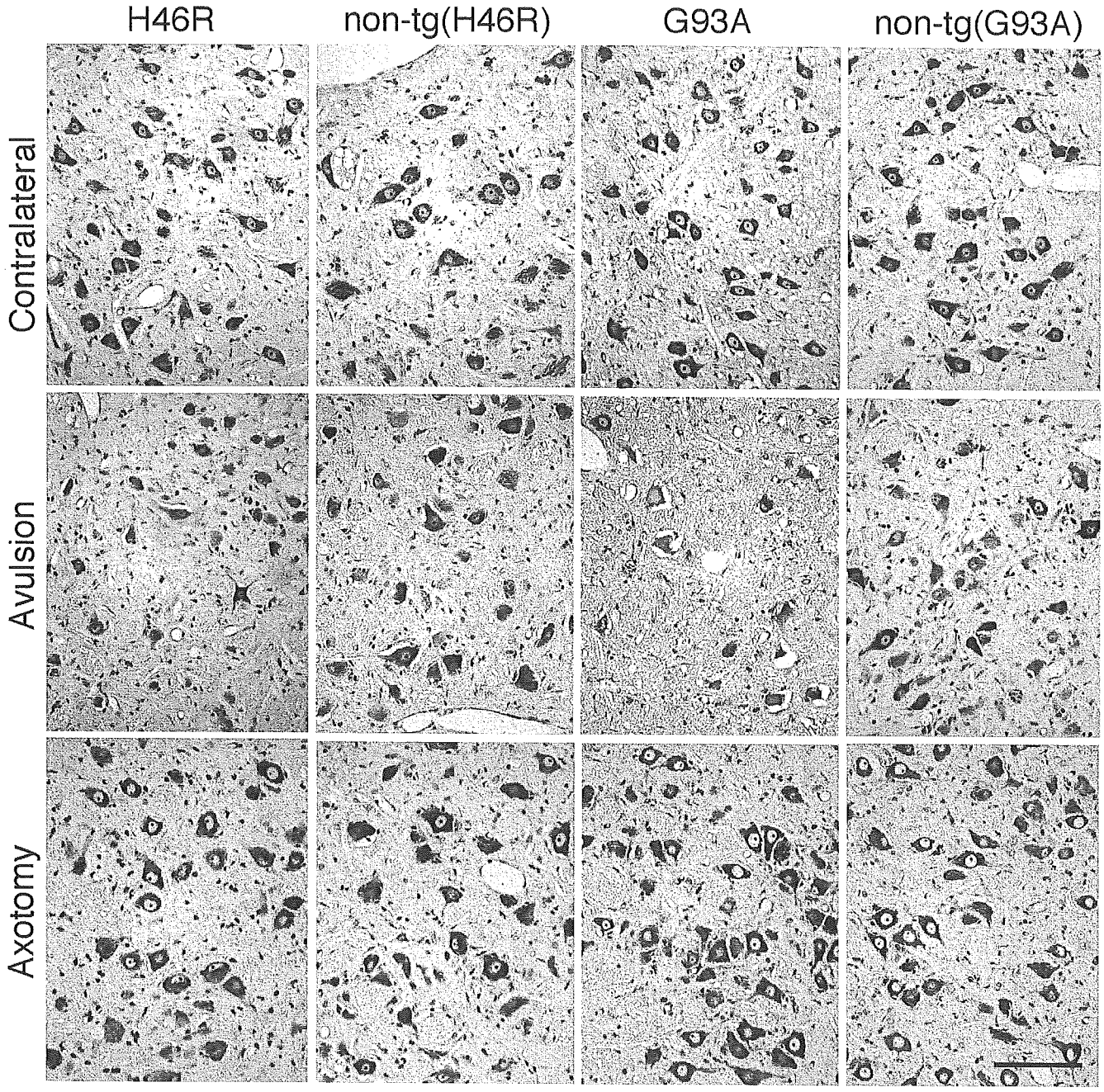


Fig. 1. Facial motoneurons of H46R- and G93A-transgenic (tg) rats and their non-tg littermates on the contralateral and ipsilateral (avulsion or axotomy) sides 2 weeks after facial nerve avulsion or axotomy. Nissl stain. Scale bar = 100 μm.

Examination of sections immunostained for SOD1 showed intense cytoplasmic immunolabeling for SOD1 in injured motoneurons after avulsion in H46R- and G93A-tg rats compared with uninjured motoneurons on the contralateral side that were not or were very faintly immunoreactive for SOD1 (Fig. 2). We used sheep and rabbit anti-SOD1 antibodies, both of which gave identical results. The cytoplasmic SOD1 immunolabeling patterns of injured motoneurons appeared diffuse in H46R-tg rats, whereas they were granular in G93A-tg rats. In G93A-tg rats, there were axons and vacuolar changes in the neuropil consistently immunoreactive for SOD1 at both uninjured and injured sides of facial nuclei (Fig. 2). There was no definite immunolabeling for SOD1 in either injured or uninjured motoneurons and their axons in non-tg littermates (Fig. 2). Facial nerve axotomy did not increase immunoreactivity for SOD1 in injured motoneurons of tg rats and non-tg littermates at 2 weeks

TABLE I. Survival of Motoneurons After Facial Nerve Avulsion and Axotomy†

| Rat (n) | Ipsilateral motoneuron number | Contralateral motoneuron number | Survival % |
|---|---|---|---|
| Avulsion | | | |
| NL (H46R) (n = 10) | 598 ± 18 | 813 ± 26 | 73.7 ± 1.2 |
| H46R (n = 8) | 402 ± 36* | 839 ± 27 | 47.5 ± 2.8* |
| NL (G93A) (n = 6) | 637 ± 56 | 822 ± 47 | 76.7 ± 3.0 |
| G93A (n = 7) | 306 ± 37* | 884 ± 44 | 34.7 ± 3.6*,** |
| Axotomy | | | |
| H46R (n = 5) | 751 ± 19 | 843 ± 23 | 89.2 ± 1.4 |
| NL (G93A) (n = 6) | 743 ± 15 | 835 ± 12 | 88.9 ± 0.6 |
| G93A (n = 5) | 741 ± 42 | 781 ± 45 | 94.9 ± 1.2 |

†Numbers of facial motoneurons and the percent survival at the ipsilateral (lesion) side relative to the contralateral (control) side 2 weeks after avulsion or axotomy. Results are presented as mean ± SEM. Statistical comparison was done by Mann-Whitney U-test. n = number of animals. NL. nontransgenic littermates.
*$P < 0.01$ vs. NL (H46R) and NL (G93A) rats after avulsion.
**$P < 0.05$ vs. H46R-transgenic rats after avulsion.

postoperation (Fig. 2). In contrast, immunohistochemical examination showed perikaryal accumulation of phosphorylated neurofilaments in injured motoneurons both after axotomy and after avulsion, as described previously (Koliatsos et al., 1989, 1994; Koliatsos and Price, 1996). There were no hyaline inclusions identifiable in HE-stained sections or ubiquitin-immunoreactive structures in both H46R- and G93A-tg rats and their non-tg littermates on either operated or contralateral sides (data not shown). Proliferation of astrocytes as evidenced by immunostaining for GFAP was observed at the injured sides in all the animals after avulsion and axotomy, and the degree of the astrocytic response appeared to correlate with the extent of motoneuron loss after avulsion; i.e., more intense GFAP immunostaining was demonstrated when less neuronal survival was observed (Fig. 3).

It has been shown that ATF3 is expressed, and c-Jun and Hsp27 are up-regulated and phosphorylated, in injured motoneurons after axotomy (Tsujino et al., 2000; Casanovas et al., 2001; Benn et al., 2002; Kalmár et al., 2002). Several reports have documented that ATF3, c-Jun, and Hsp27 cooperate to promote neuronal survival in vitro and in vivo, suggesting neuroprotective roles of these molecules (Pearson et al., 2003; Nakagomi et al., 2003). We then examined the expression of ATF3, c-Jun, and Hsp27 in injured motoneurons after facial nerve avulsion that causes extensive neuronal loss. In wild-type adult rats, intact facial motoneurons were constitutively immunoreactive for c-Jun and Hsp27 but not for ATF3, phosphorylated c-Jun, or phosphorylated Hsp27, whereas injured motoneurons become immunoreactive for ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 within 1 day after facial nerve avulsion and remain positive up to 4 weeks (Watabe et al., unpublished observations). In a similar manner, virtually all injured motoneurons were immunostained for ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 in H46R- and G93A-tg rats and their non-tg littermates 2 weeks after avulsion and axotomy as examined in this study (Fig. 3).

## DISCUSSION

We demonstrated that only 50% (H46R-tg rats) or 35% (G93A-tg rats) of motoneurons in mutant SOD1-tg rats survived 2 weeks after avulsion at their presymptomatic stage compared with 70% survival of motoneurons in their non-tg littermates, indicating that motoneuron degeneration after avulsion is significantly more severe in these presymptomatic mutant SOD1-tg rats. It is interesting to note that the loss of motoneurons in G93A-tg rats was significantly greater than that in H46R-tg rats after avulsion, insofar as the onset of paralysis is earlier and the disease progression is more rapid in G93A-tg rats compared with the H46R rats used in the present study (Nagai et al., 2001). The clinical courses of these tg rats are also likely to be relevant to those of human mutant SOD1-mediated familial ALS, in that the human H46R cases progress very slowly compared with the G93A cases (Nagai et al., 2001; Aoki et al., 1993, 1994). In contrast, we did not see significant motoneuron loss in the presymptomatic SOD1-tg rats and their non-tg littermates 2 weeks after facial nerve axotomy. Unlike avulsion, axotomy does not generally induce significant motoneuron death in adult rodents (Lowrie and Vrbová, 1992; Moran and Graeber, 2004), except that, in the case of adult Balb/C mice, the facial nerve axotomy leads to loss of >50% of the motoneurons at 30 days postoperation (Hottinger et al., 2000), and C57BL mice show late motoneuron loss (~60%) 8 weeks after facial nerve axotomy (Angelov et al., 2003). Mariotti et al. (2002) axotomized facial nerves of G93A-tg mice and their non-tg littermates at their presymptomatic stage and observed loss of facial motoneurons that was higher in G93A-tg mice than in non-tg littermates at 30 days postaxotomy; these data are relevant to our present data acquired from avulsion, but not axotomy, in rats, which probably is due to the use of different animal species. In contrast, Kong and Xu (1999) described axotomy of lumbar spinal or sciatic nerve in G93A-tg mice at the presymptomatic stage reducing the extent of axon degeneration at the end stage of the disease. They did

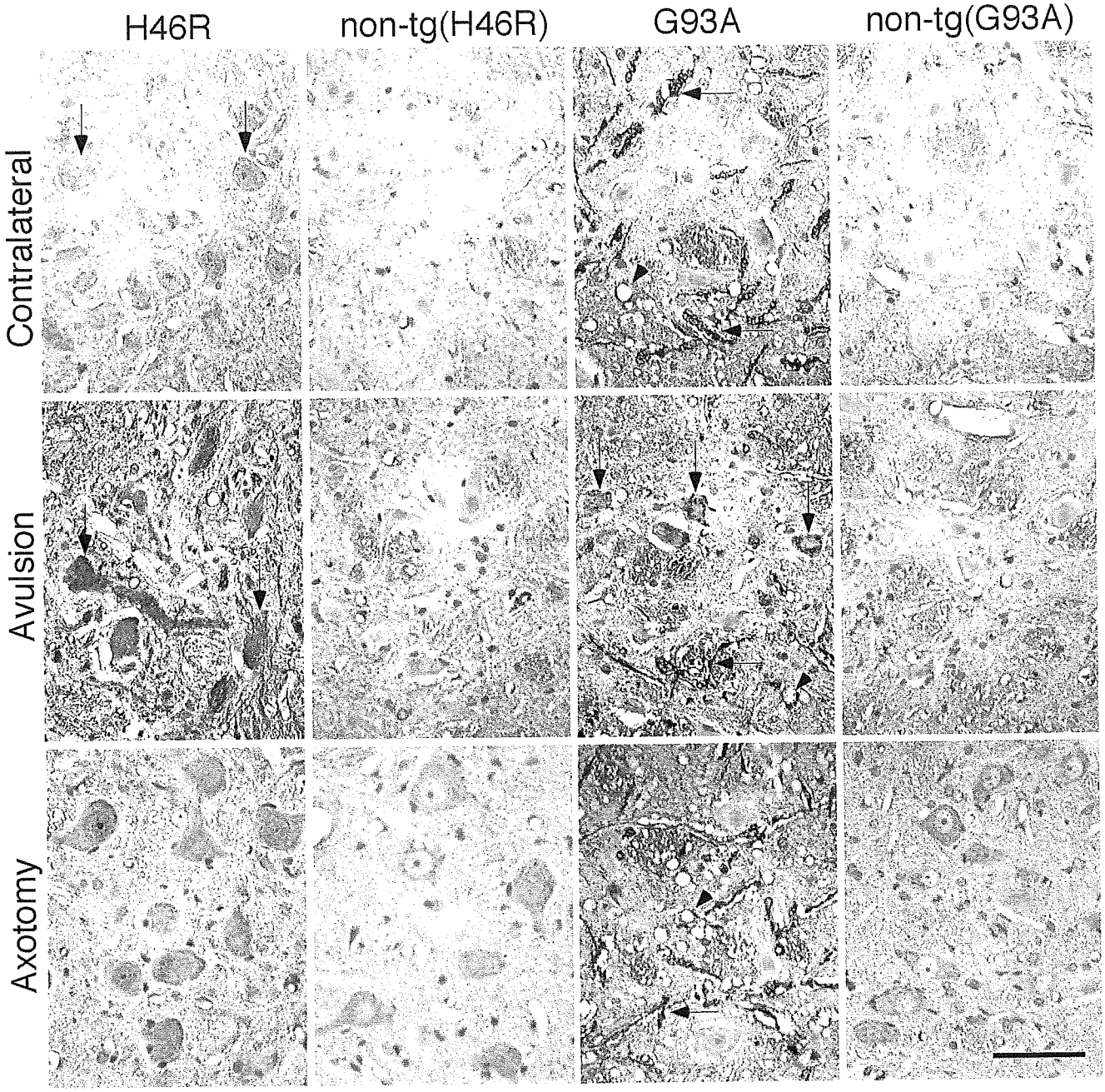


Fig. 2. SOD1 immunohistochemistry of facial motoneurons of H46R- and G93A-tg rats and their non-tg littermates on the contralateral and ipsilateral (avulsion or axotomy) sides 2 weeks after facial nerve avulsion or axotomy. Counterstained with hematoxylin. Note immunostained motoneurons (vertical arrows), axons (horizontal arrows), and vacuoles in neuropil (arrowheads) in H46R- and G93A-tg rats. Scale bar = 50 μm.

not evaluate the response of the cell bodies of spinal motoneurons, so it remains unknown whether SOD1 mutation affects the viability of spinal motoneurons after axotomy. In the present study, we demonstrated that motoneuron degeneration after facial nerve avulsion, but not after axotomy, is exacerbated in presymptomatic mutant SOD1-tg rats at 2 weeks postoperation. These data clearly indicate the increased vulnerability of facial motoneurons to proximal nerve injury in the presymptomatic SOD1-tg rats.

It has been shown that SOD1 is abundantly expressed in cell bodies, dendrites, and axons of wild-type mouse and rat motoneurons in vivo (Pardo et al., 1995; Moreno et al., 1997; Yu, 2002). In the present

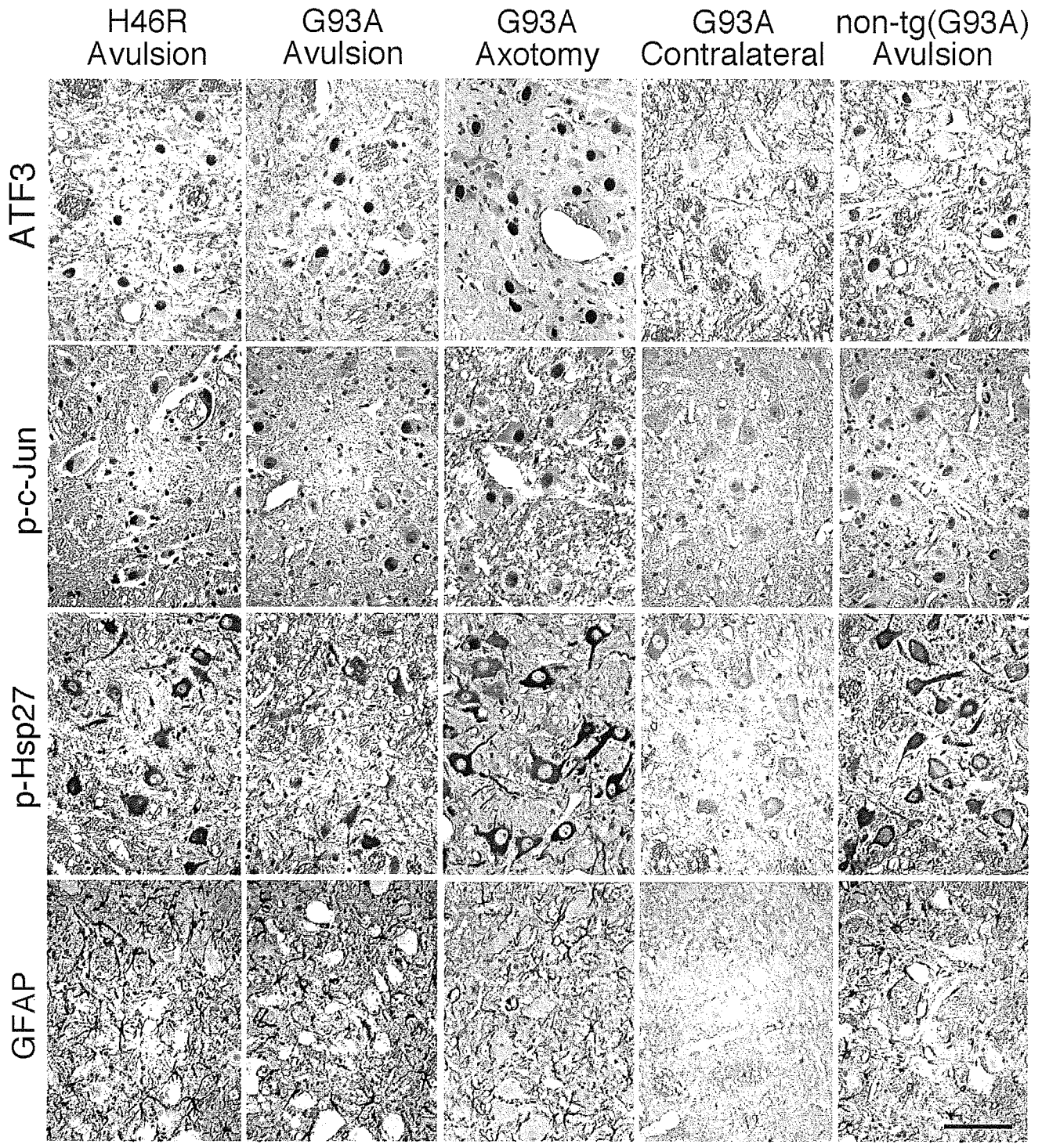


Fig. 3. Immunohistochemistry for ATF3, phosphorylated c-Jun (p-c-Jun), phosphorylated Hsp27 (p-Hsp27), and GFAP of facial nuclei in H46R-tg rat, G93A-tg rat, and non-tg littermate on the ipsilateral (avulsion or axotomy) and contralateral sides 2 weeks after facial nerve avulsion or axotomy. All injured motoneurons are immunostained for ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 in these rats after avulsion or axotomy. The intensity of GFAP immunoreactivity appears parallel to the extent of motoneuron loss (see also Fig. 1). Counterstained with hematoxylin. Scale bar = 50 μm.

study, we did not observe immunoreactivity for SOD1 in facial motoneurons of nontransgenic littermates with sheep and rabbit anti-human SOD1 antibodies; it is postulated that the antibody concentrations (i.e., 1:1,000–10,000) used in this study are below the detection levels for immunostaining rat SOD1 antigen on paraffin sections. Instead, we demonstrated some facial motoneurons showing very faint immunoreactivity for SOD1 in H46R-tg rats on paraffin sections. In G93A-tg rats, axons and vacuoles in neuropil were intensely immunoreactive for SOD1 at both uninjured and injured sides. The increased immunostaining for SOD1 in injured motoneurons of SOD1 (H46R and G93A)-tg rats may therefore indicate that human mutant SOD1 protein is accumulated in the cytoplasm of facial motoneurons after avulsion. When several mutant SOD1 genes that include G93A were transfected to COS7 cells, the mutant SOD1s, but not wild-type SOD1, aggregated in association with the endoplasmic reticulum (ER) and induced ER stress (Tobisawa et al., 2003). Accumulation of mutant SOD1 in injured motoneurons after avulsion may therefore potentiate ER stress and exacerbate motoneuron death in the presymptomatic mutant SOD1-tg rats, although the mechanism of accumulation of SOD1 remains unknown. Whether up-regulation of cytoplasmic mutant SOD1 expression or retrograde accumulation of mutant SOD1 from injured axons was induced in these neurons awaits further investigations. In addition, facial nerve axotomy, as opposed to avulsion, did not increase immunoreactivity for SOD1 in injured motoneurons of SOD1-tg rats and their non-tg littermates, which seems consistent with the absence of significant motoneuron loss in these rats as described above. As for wild-type SOD1, previous reports documented no change in SOD1 mRNA levels or SOD1 immunoreactivity in injured motoneurons after facial or sciatic nerve axotomy in wild-type rats (Yoneda et al., 1992; Rosefeld et al., 1997).

It has been demonstrated that ATF3 is expressed, and c-Jun and Hsp27 are up-regulated and phosphorylated, in injured adult motoneurons after axotomy (Tsujino et al., 2000; Casanovas et al., 2001; Benn et al., 2002; Kalmár et al., 2002). As for the neuroprotective nature of these molecules, it has been reported that ATF3 enhances c-Jun-mediated neurite sprouting in PC12 and Neuro-2a cells (Pearson et al., 2003), and ATF3 and Hsp27 cooperate with c-Jun to prevent death of PC12 cells and superior cervical ganglion neurons (Nakagomi et al., 2003). Hsp27 is induced and phosphorylated in adult, but not in neonatal, motoneurons after axotomy, and axotomized neonatal motoneurons that lack Hsp27 die by apoptosis, suggesting that phosphorylated Hsp27 is necessary for motoneuron survival after peripheral nerve injury (Benn et al., 2002). However, there have been no reports concerning the expression of ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 in injured motoneurons after avulsion. In the present study, we have demonstrated that, even after avulsion that causes extensive motoneuron death, ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 were fully up-regulated in both SOD1-tg and non-tg rats. These results suggest that neuroprotective effects of Hsp27 cannot overcome yet unidentified stress(es) induced by facial nerve avulsion. On the other hand, a recent report demonstrated that facial motoneurons of c-Jun-deficient mice are resistant to axotomy-induced cell death, suggesting that c-Jun promotes posttraumatic motoneuron death (Raivich et al., 2004). In addition, it has been shown that mutant SOD1 binds to Hsp27 and forms aggregates, suggesting that this binding of Hsp27 to mutant SOD1 blocks antiapoptotic function of Hsp27 and leads to motoneuron death (Okado-Matsumoto and Fridovich, 2002). The effects of phosphorylated c-Jun and Hsp27 and their association with mutant SOD1 accumulation should be further investigated to elucidate the mechanism of exacerbated motoneuron death in SOD1-tg rats after avulsion.

In this study, we have demonstrated that motoneuron degeneration after facial nerve avulsion is exacerbated in presymptomatic mutant SOD1-tg rats compared with their non-tg littermates. Mutant SOD1 accumulation and its association with c-Jun and Hsp27 may have a key role leading to enhanced motoneuron death. In this context, motoneuron death after avulsion may share, at least in part, a common mechanism with the motoneuron degeneration associated with SOD1 mutation.

## ACKNOWLEDGMENT

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Workshop: Recent Advances in Motor Neuron Disease

# Peripheral nerve avulsion injuries as experimental models for adult motoneuron degeneration

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**We have used adult rat peripheral nerve avulsion models to evaluate the effects of neuroprotective molecules on motoneuron degeneration. The right facial nerves of adult Fischer 344 male rats were avulsed and adenoviral vectors encoding glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), transforming growth factor-β2 (TGFβ2), and growth inhibitory factor (GIF) were injected into the facial canal. The treatment with the vectors significantly prevented the loss of lesioned facial motoneurons, improved choline acetyltransferase (ChAT) immunoreactivity and suppressed the induction of nitric oxide synthase activity in these neurons. In separate experiments, animals were orally administered a solution of a neuroprotective compound T-588 after avulsion. Both free oral administration and oral tube administration of T-588 improved the survival of injured motoneurons and ameliorated their ChAT immunoreactivity. These results indicate that the gene transfer of GDNF, BDNF, TGFβ2, and GIF and oral administration of T-588 may prevent the degeneration of motoneurons in adult humans with motoneuron injury and motor neuron diseases.**

**Key words:** avulsion, adenovirus, facial nerve, motoneuron, neurotrophic factor.

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by selective loss of motoneurons leading to progressive paralysis and death by respiratory failure. Approximately 10% of ALS cases are familial and include mutations in the Cu/Zn superoxide dismutase (*SOD1*) gene and in the *ALS2/alsin* gene putatively encoding a ras GTPase. Since the development of transgenic (tg) mice expressing human mutant *SOD1* that show clinicopathological characteristics similar to human familial ALS, the mutant *SOD1*-tg animals have been the most widely used experimental models to elucidate the pathomechanism of and the therapeutic approach against familial ALS as well as sporadic ALS.[1] Although the precise mechanism of motoneuron degeneration in mutant *SOD1*-tg animals is largely unknown, the mutant *SOD1* is thought to have a gain of toxic function. Most cases of ALS, however, are considered to be sporadic and of unknown etiology. With regard to another animal model of adult motoneuron degeneration, avulsion of peripheral nerves exhibits extensive loss of motoneurons in adult rats.[2–12] The mechanism of motoneuron degeneration after avulsion also remains unclear, but peroxinitrite-mediated oxidative damage and perikaryal accumulation of phosphorylated neurofilaments have been demonstrated in injured motoneurons after avulsion.[13] Both of these pathological features have also been shown in spinal motoneurons in mutant *SOD1*-tg animals as well as in patients with familial and sporadic ALS.[1,14,15] To explore therapeutic strategies against motoneuron injury and motoneuron degeneration such as ALS, we have used adult rat peripheral nerve avulsion models and examined the effects of neuroprotective molecules on injured motoneurons.[5–12]

## ADULT RAT PERIPHERAL NERVE AVULSION

In neonatal rats, peripheral nerve axotomy (transection) causes extensive motoneuron death through an apoptotic process associated with Bax and caspase-3 pathways.[16–19] In adult rats, however, axotomy does not induce significant motoneuron death.[20,21] Adult motoneuron degeneration

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and death can clearly be demonstrated after avulsion of peripheral (facial, hypoglossal, vagal and spinal) nerves.[2–12] In contrast to axotomy, avulsion causes complete disappearance of peripheral nerve components including Schwann cells that produce several molecules neuroprotective for motoneurons. The precise mechanism of adult motoneuron death after peripheral nerve avulsion remains unclear. Some investigators reported that the degeneration of motoneurons following avulsion is due to apoptosis,[13,22] whereas others described that it more closely resembles necrosis.[23] In our facial nerve and spinal root avulsion models as described below, no typical morphological features of apoptosis were identified in injured motoneurons, as described by other investigators.[21,24] Herpes simplex virus vector-mediated expression of Bcl-2 prevented degeneration of spinal motoneurons after root avulsion,[25] whereas caspase inhibitors failed to rescue avulsed spinal motoneurons in adult rats,[26] suggesting that adult motoneuron death after avulsion is not simply due to an apoptotic mechanism.

We have performed avulsion of facial nerve and cervical spinal root in adult rats. Adult Fischer 344 male rats (12–14 weeks old, 200–250 g) were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg). As for facial nerve avulsion, the right facial nerve was exposed at its exit from the stylomastoid foramen under a dissecting microscope.[6–11] Using microhemostat forceps, the proximal facial nerve was avulsed by gentle traction and removed from the distal facial nerve (Fig. 1). As for cervical root avulsion, the right seventh cervical segment (C7) nerve was exposed by separating the surrounding cervical muscles and connective tissue until the point where the vertebral foramen was identified.[5] Using microhemostat forceps, the C7 ventral and dorsal roots and dorsal root ganglia (DRG) were avulsed and removed from the peripheral nerve (Fig. 2). After 2–8 weeks, rats were anesthetized with a lethal dose of pentobarbital sodium and transcardially perfused with 0.1 M phosphate buffer, pH 7.4 (PB) followed by 4% paraformaldehyde in 0.1 M PB. The brain stem tissue containing facial nuclei and their intramedullary nerve tracts or the cervical spinal cord tissue was dissected and immersion fixed in the same fixative for 2 h. The tissues were either cryoprotected, or dehydrated and embedded in paraffin, and serial transverse sections were made. For motoneuron cell counting, every fifth section was picked up and stained with cresyl violet (Nissl staining). In 25 sections, motoneurons having nuclei containing distinct nucleoli on both sides of the facial nuclei or the Rexed's lamina IX of C7 spinal cord were counted. The data were expressed as the mean ± SD and statistical significance was assessed by Mann–Whitney *U*-test. Two to eight weeks after facial nerve and C7 root avulsion, there was marked atrophy of facial nucleus and C7 spinal ventral horn, respectively, and the loss of motoneurons with prominent glios (Figs 1–3). The number of motoneurons gradually decreased and reached 20–30% of contralateral side by 4–8 weeks after avulsion (Fig. 4).

## ADENOVIRAL GENE TRANSFER OF NEUROPROTECTIVE FACTORS

Several neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), NT4/5, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT1), insulin-like growth factor-1 (IGF1), and glial cell line-derived neurotrophic factor (GDNF), have been shown to prevent the death of facial and spinal motoneurons, suggesting them as potential therapeutic agents for motoneuron injury and motor neuron diseases such as ALS.[27,28] Recombinant adenoviral vectors encoding BDNF, NT3, CNTF, CT1, and GDNF cDNAs have been used to protect neonatal rat facial and spinal motoneurons from axotomy-induced death[29–32] as well as spinal motoneurons in the mouse mutant progressive motor neuronopathy.[33,34] In adult rats, as described above, axotomy does not generally induce significant motoneuron death.[20,21] Injured motoneuron death in adult rats can be demonstrated 2–4 weeks after the avulsion of facial and spinal nerves.[2–12] Previously, local administration of BDNF, GDNF, or transforming growth factor-β2 (TGFβ2) protein has been reported to prevent the death of adult rat motoneurons after avulsion of spinal root and hypoglossal nerve.[35–40] We investigated whether adenoviral gene transfer of these neurotrophic factors can prevent the death of facial and spinal motoneurons after avulsion in adult rats.[5–10]

### Adenovirus preparation

Replication-defective recombinant adenoviral vectors encoding human GDNF (AxCAhGDNF), mouse BDNF fused with Myc epitope-His/IRES-EGFP at the 3′ end (AxCAmBDNFME), rat CNTF and CT1 fused with the mouse nerve growth factor (NGF) signal sequence at the 5′ ends (AxCANrCNTF and AxCANrCT1, respectively), human IGF1 (AxCAhIGF1), mouse TGFβ2 (AxCAmTGFβ2), and rat growth inhibitory factor (GIF), also called metallothionein-III (MT-III), fused with Myc epitope at the 3′ end (AxCArGIFM) were generated using a cassette cosmid pAxCAwt (TaKaRa, Osaka, Japan) carrying an adenovirus type-5 genome lacking the E3, E1A, and E1B regions to prevent virus replication (Fig. 5).[5,9,10] The cosmid pAxCAwt contains the CAG (cytomegalovirus-enhancer-chicken β-actin hybrid) promoter on the 5′ end and a rabbit globin poly (A) sequence on the 3′ end. The cosmids were cotransfected to 293 cells

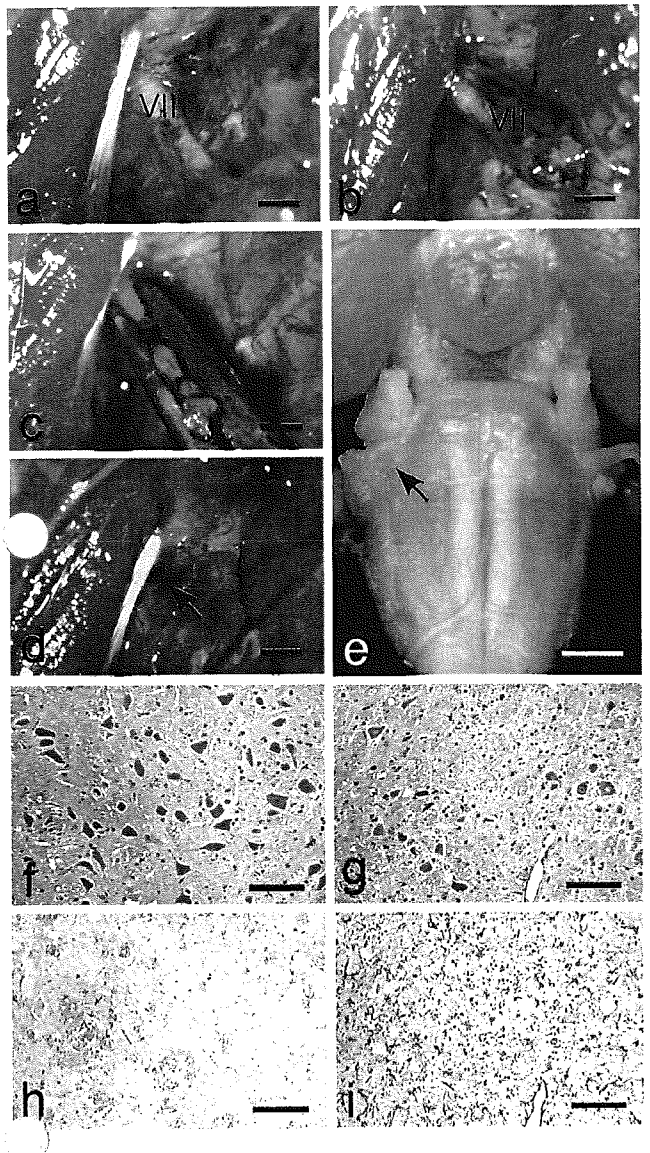


**Fig. 1** Facial nerve avulsion of adult rat. (a–d) Avulsion surgery: (a) the right facial nerve (VII) is exposed at its exit from the stylomastoid foramen; (b) the distal portion of the facial nerve is cut, and (c) the proximal facial nerve is avulsed by gentle traction using microhemostat forceps; (d) an arrow indicates the stylomastoid foramen after avulsion. Bars (a–d) 1 mm. (e) Perfusion-fixed brain tissue 4 weeks after avulsion showing the absence of the extra-axial portion of the facial nerve on the lesioned side (arrow). Bar, 2 mm. (f–g) Photomicrographs of facial motoneurons at the contralateral (f,h) and ipsilateral (g,i) side 4 weeks after the right facial nerve avulsion stained with HE (f,g) and immunolabeled with GFAP (h,i). Bars (f–i) 100 μm. Note the loss of motoneurons with gliosis on the lesioned side.

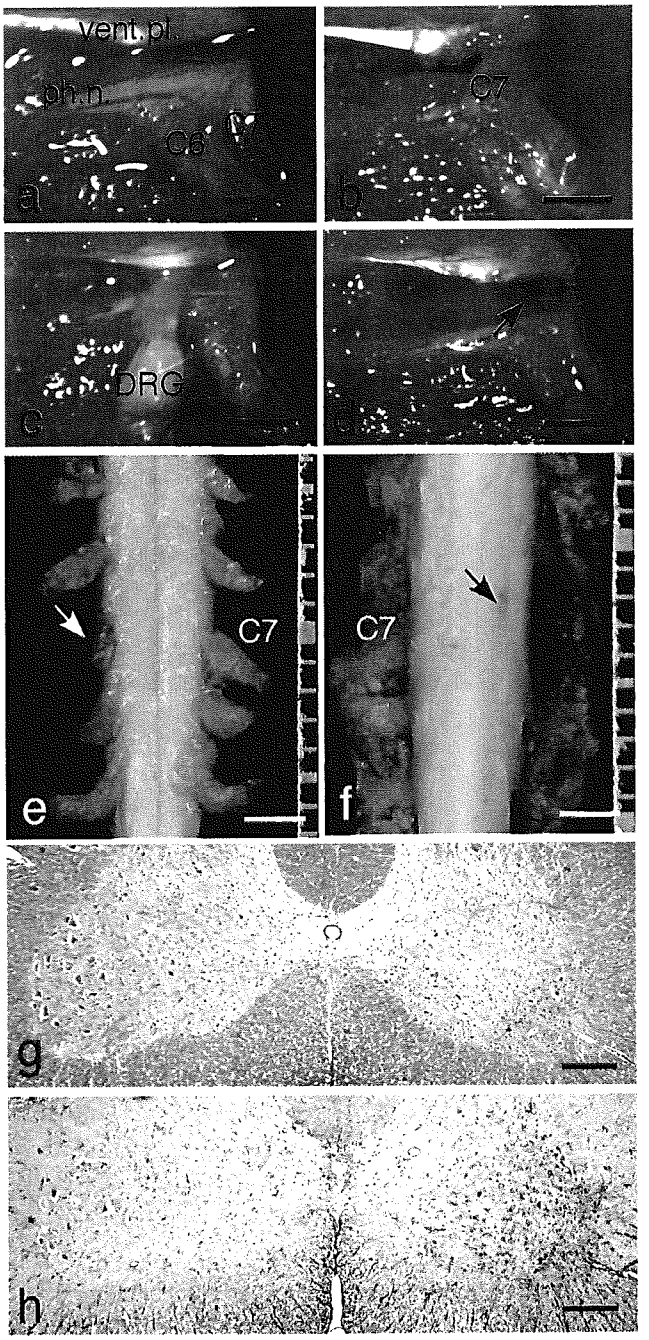


**Fig. 2** Spinal root avulsion of adult rat. (a–d) Avulsion surgery: (a) the right sixth (C6) and seventh (C7) cervical segment nerves are identified underneath the ventral plate (vent. pl.); (b) the C7 distal portion is cut, lifted upon the phrenic nerve (ph. n), and the C7 nerve is exposed until the point where the vertebral foramen is identified; (c) using microhemostat forceps, the C7 ventral and dorsal roots and dorsal root ganglia (DRG) are avulsed and removed; (d) an arrow indicates the C7 intervertebral foramen after avulsion. Bars (a–d) 1 mm. (e,f) Perfusion-fixed spinal cord tissue 6 weeks after avulsion showing the absence of C7 ventral (e) and dorsal roots (f) and DRG on the lesioned side (arrows). Bars (e,f) 2 mm. (g,h) Photomicrographs of spinal cord 6 weeks after C7 root avulsion stained with KB (g) and immunolabeled with GFAP (h). Bars (g,h) 200 μm. Note the atrophy of C7 ventral horn with loss of motoneurons and gliosis on the lesioned (right) side.

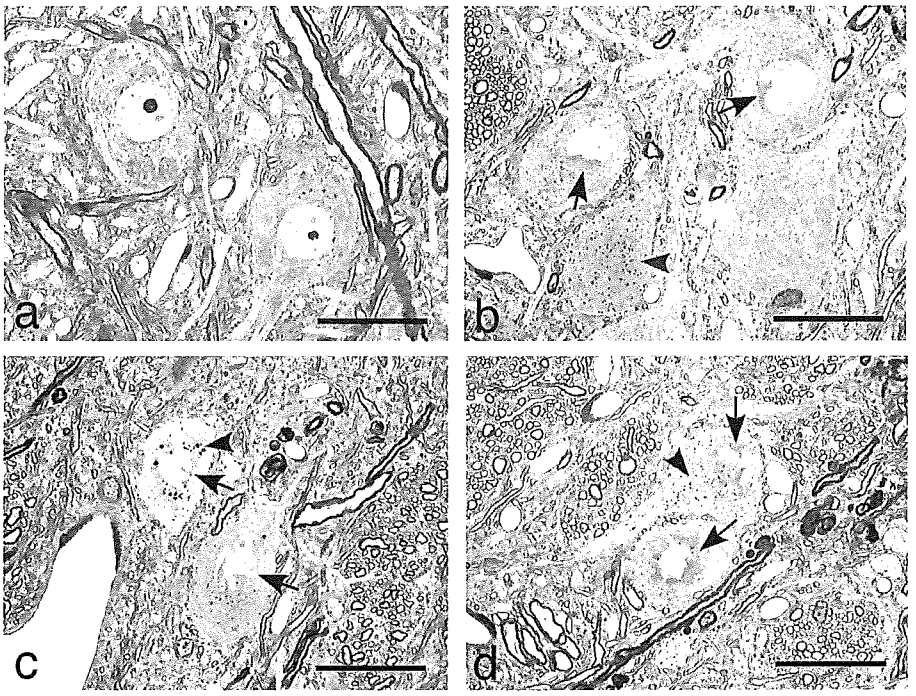


**Fig. 3** Photomicrographs of Epon-embedded semithin sections of facial nucleus at the (a) contralateral and (b–d) ipsilateral side 1 (b), 2 (c), and 4 (d) weeks after avulsion. Shrunken motoneurons show dispersed Nissl substance, nuclear caps (arrows) and intracytoplasmic granules (arrowheads) but no morphological features of apoptosis after avulsion. Decreased numbers of neurites in neuropil and degradation of myelin are also noted. (Toluidine Blue). Bars (a–d) 30 μm.

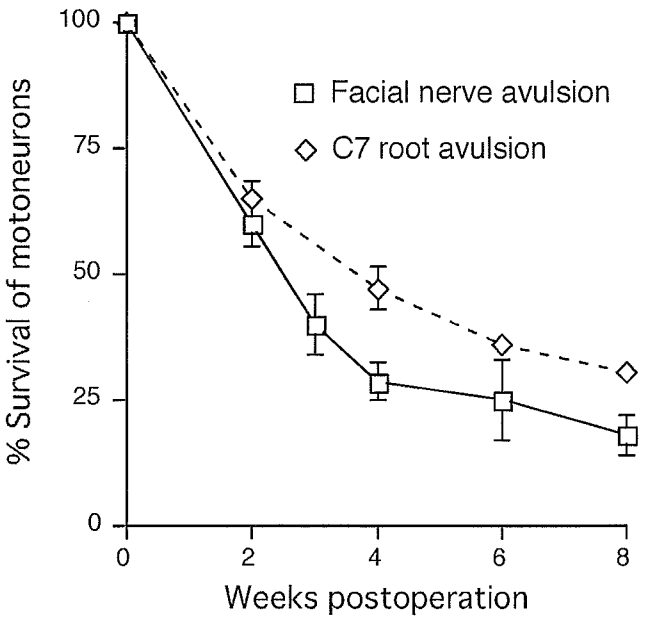


**Fig. 4** The percentages of surviving facial motoneurons at the ipsilateral (lesion) side relative to the contralateral (control) side after facial nerve and seventh cervical (C7) root avulsion.

with the adenovirus genome lacking the E3 region.[41] Recombinant adenoviral vectors were propagated and isolated from 293 cells, and purified by two rounds of CsCl centrifugation. A recombinant adenoviral vector encoding bacterial β-galactosidase gene (AxCALacZ) were used as a reporter adenovirus.[42] The ability of recombinant adenoviral vectors to induce expression of neurotrophic factors *in vitro* was confirmed by Western blot analysis of conditioned media or total cell lysates derived from COS1 cells infected with the vectors.[5,9,10] *In vitro* neurotrophic activity of conditioned media from COS1 cells infected with the vectors were checked by the survival assays using E14 rat mesencephalic and E15 rat spinal motoneuron cultures, and the proliferation assay using an immortalized Schwann cell line IMS32.[5,9,43–45]

## Transduction of injured adult motoneurons by adenoviral vectors

Immediately following the avulsion of facial nerve, a microsyringe was inserted into the stylomastoid foramen and 20 μL solution of either adenoviral vectors ($1 \times 10^8$ pfu each for single and combined injection) or PBS was injected through the facial canal. The wounds were covered with a small piece of gelatin sponge (Gelfoam; Pharmacia Upjohn, Bridgewater, NJ, USA) and suture closed.[6] As for spinal root avulsion, a small piece of Gelfoam presoaked with 10 μL solution of either adenoviral vectors ($1 \times 10^8$ pfu) or PBS was placed in contact with the lesioned C7 intervertebral foramen.[5]

One week after the avulsion and the treatment of AxCALacZ, injured facial and C7 spinal ventral motoneurons and their axons were stained with X-gal histochemistry.[5,6] This indicates the diffusion of the virus through the facial canal or intervertebral foramen, its adsorption to

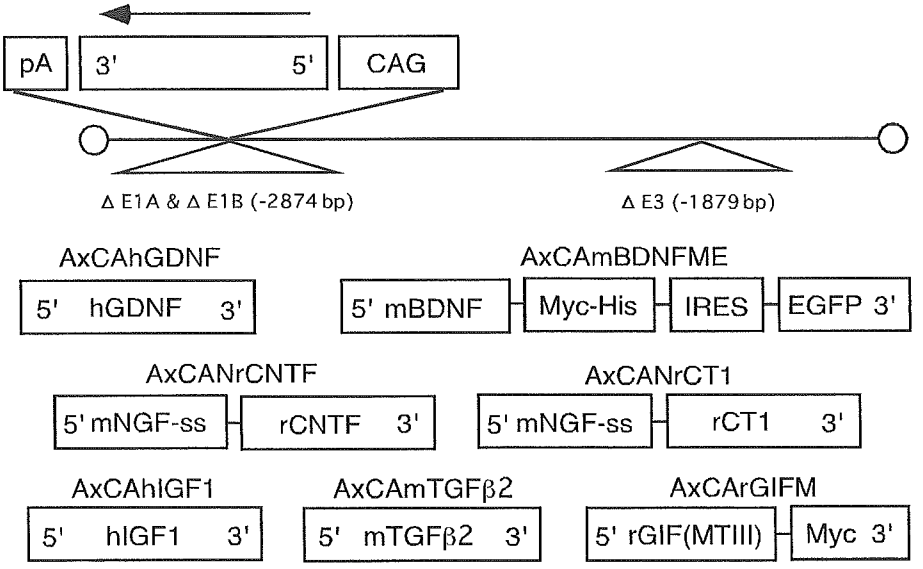


**Fig. 5** Adenoviral vectors encoding human glial cell line-derived neurotrophic factor (AxCAhGDNF), mouse brain-derived neurotrophic factor (AxCAmBDNFME), rat ciliary neurotrophic factor (AxCANrCNTF), rat cardiotrophin-1 (AxCANrCT1), human insulin-like growth factor-1 (AxCAhIGF1), mouse transforming growth factor-β2 (AxCAmTGFβ2), and rat growth inhibitory factor (AxCArGIFM). mNGF-ss, mouse nerve growth factor (NGF) signal sequence.

injured axons, retrograde transport of the virus to soma of the motoneurons, and successful induction of the virus-induced foreign gene in these neurons. Retrograde transport of adenovirus encoding β-galactosidase gene from the proximal stump of axotomized peripheral nerves or from the innervated skeletal muscles to soma of motoneurons and successful β-galactosidase expression in these neurons has been demonstrated in neonatal and adult rodents.[29–32,46,47] Because the avulsion of peripheral nerves can cause significant injured motoneuron death in adult rodents, we consider that facial nerve and spinal root avulsions are excellent model systems to examine the effects of adenoviral vectors expressing various neurotrophic factors and neuroprotective molecules on the survival of adult motoneurons.

In a similar manner, 1 week after avulsion and the treatment with AxCAhGDNF, AxCAmBDNFME, AxCANrCNTF, AxCANrCT1, AxCAhIGF1, AxCAmTGFβ2, and AxCArGIFM, intense cytoplasmic immunolabeling for GDNF, BDNF/Myc, CNTF, CT1, IGF1, TGFβ2, and GIF/Myc, respectively, was demonstrated in 10–20% of facial motoneurons exclusively on the ipsilateral side of the facial nucleus.[6–10] There was no definite immunostaining for these factors in facial motoneurons on the side contralateral to the adenovirus treatment as well as those in unoperated control animals or operated animals without adenovirus treatment. These results led us to expect the autocrine and paracrine neurotrophic effects of these vectors on injured motoneurons after avulsion.[6–10]

## Neuroprotective effects of adenoviral vectors

Four weeks after facial nerve avulsion, the treatment with AxCAhGDNF, AxCAmBDNFME, AxCAmTGFβ2, and AxCArGIFM significantly prevented the loss of facial motoneurons after avulsion as compared to the treatment with PBS or AxCALacZ (Fig. 6). In addition, it has been known that peripheral nerve avulsion as well as axotomy induces rapid decrease of ChAT immunoreactivity in injured adult motoneurons.[38,48] We demonstrated that the treatment with AxCAhGDNF, AxCAmBDNFME, AxCAmTGFβ2, and AxCArGIFM after avulsion attenuated the decrease of ChAT immunoreactivity in lesioned facial motoneurons.[5–10] Similar immunohistochemical results have been demonstrated by local or s.c. administration of BDNF and GDNF protein after facial nerve axotomy[48,49] or by continuous intrathecal infusion of BDNF protein after sciatic nerve avulsion[38] in adult rats, indicating neuroprotective effects of these factors on adult motoneurons. Furthermore, AxCAhGDNF, AxCAmBDNFME, AxCAmTGFβ2, and AxCArGIFM suppressed nitric oxide synthase (NOS) activity in lesioned facial motoneurons after avulsion.[5–10] It has been reported that NOS activity is induced in lesioned adult facial and spinal motoneurons after avulsion, suggesting that the induction of NOS activity plays a significant role in the initiation of adult motoneuron death.[3,50] The NOS inhibitors, such as nitroarginine or Nω-nitro-L-arginine methyl ester, have been shown to prevent the induction of NOS activity and the subsequent motoneuron death after avulsion.[50,51] Taken together, these results indicate the neuroprotective effects of AxCAhGDNF, AxCAmBDNFME, AxCAmTGFβ2, and AxCArGIFM on the injury and death of adult facial motoneurons. In contrast, the treatment with AxCANrCNTF, AxCANrCT1 and AxCAhIGF1 failed to prevent the loss of facial motoneurons after avulsion; neither improvement of ChAT immunoreactivity nor suppression of NOS activity was observed. Additionally, we also exam-

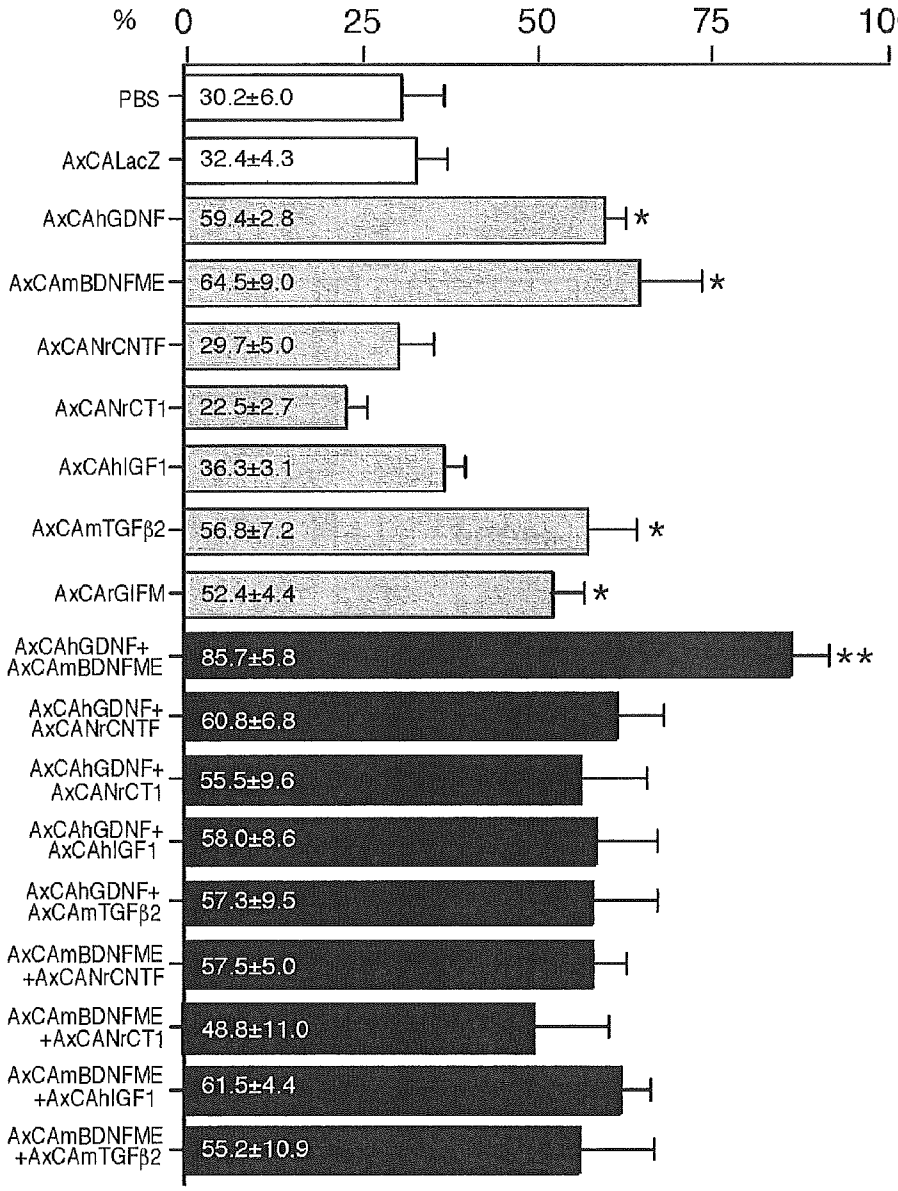


**Fig. 6** The percentages of surviv... facial motoneurons at the ipsilateral (lesion) side relative to the contralateral (control) side after avulsion and treatment with PBS, AxCALacZ (white bars), AxCAhGDNF, AxCAmBDNFME, AxCANrCNTF, AxCANrCT1, AxCAhIGF1, AxCAmTGFβ2, or AxCArGIFM (shaded bars) as well as pairwise combinations of these vectors (black bars). Results are presented as mean ± SD (PBS: $n = 8$, others: $n = 4$). Statistical comparison was done by Mann–Whitney $U$-test. $^{*}P < 0.05$ versus PBS-, AxCALacZ-, AxCANrCNTF-, AxCANrCT1- and AxCAhIGF1-treated groups; $^{**}P < 0.05$ versus AxCAhGDNF- and AxCAmBDNFME-treated groups.

ined pairwise combinations of these adenoviral vectors and demonstrated additive neurotrophic effects of AxCAhGDNF and AxCAmBDNFME on injured motoneurons after avulsion (Fig. 6). Such combined effects of GDNF and BDNF have been demonstrated *in vitro* using fetal rat motoneurons.[52]

## Neuroprotective activity of GIF (MT-III)

Among these neuroprotective factors that rescued injured motoneurons in our avulsion experiments, GIF (MT-III) is a unique molecule whose expression is reduced in Alzheimer's disease brains. GIF, a CNS-specific member of the MT family, is a 68 amino acid small, cysteine-rich protein that binds zinc and copper with high affinity.[53] GIF exhibits protective effects against glutamate-, nitric oxide-, and β-amyloid-induced neurotoxicity.[54,55] GIF acts as a hydroxy radical scavenger and inhibits tyrosine nitration by peroxynitrite.[56] The formation of hydroxy radical-modified DNA and RNA as well as peroxynitrite-modified proteins has been demonstrated in injured motoneurons after avulsion.[13] Our results therefore indicate that GIF may protect injured motoneurons from oxidative stress by hydroxy radical and peroxynitrite.[10]

GIF mRNA is down-regulated in post-mortem spinal cord tissues of human sporadic ALS,[57] whereas up-regulated in the spinal cord of human mutant *SOD1*-tg mice (G93A mice) as the animals age and develop weakness.[58,59] G93A mice deficient of GIF exhibit reduced survival and accelerated motoneuron death compared with

G93A mice with normal GIF expression.[60] These reports suggest that GIF may have protective roles against motoneuron degeneration in ALS. In addition, zinc is important in maintaining the *SOD1* structure, and some variants of mutant *SOD1 in vitro* exhibit markedly reduced affinity for zinc and enhanced nitration activity by peroxynitrite.[61] The induction of either wild-type or mutant *SOD1*, depleted of zinc, into cultured motoneurons is found to provoke nitric oxide-dependent neuronal death that is accompanied by elevated level of nitrotyrosine.[1,62] As GIF is an important regulator of zinc in CNS and has a scavenging effect for peroxynitrite,[56] GIF may protect motoneurons in patients with ALS by modulating zinc and/or preventing tyrosine nitration by peroxynitrite. It is therefore conceivable that GIF may prevent the degeneration of motoneurons in patients with motoneuron injury and motor neuron diseases such as ALS.[10]

## ORAL ADMINISTRATION OF T-588

R(-)-1-(benzo[b]thiophen-5-yl)-2-[2-(N,N-diethylamino) ethoxy]ethanol hydrochloride (T-588) has been developed as a candidate for a neuroprotective agent against neurodegenerative diseases. This low molecular weight (330 Da) compound is a synthetic derivative of acetylcholine.[63] Orally administered T-588 is efficiently transported into the CNS.[64] It has been demonstrated that T-588 promotes neurite outgrowth of cultured spinal ventral horn cells[65] and delays the progression of motor deficits in the wobbler mouse.[64] We investigated whether oral administration of T-588 can protect injured motoneurons after facial nerve avulsion in adult rats.[11]

After avulsion of the right facial nerve, the animals were freely administered a solution of 0.05% (w/v) T-588 or received T-588 (3–30 mg/kg per day) through an oral tube for 1–4 weeks. The loss of injured motoneurons was significantly prevented in rats freely administered 0.05% T-588 solution ($62.7 \pm 5.3\%$, $n = 8$ and $50.1 \pm 4.8\%$, $n = 11$ at 3 and 4 weeks postoperation, respectively) in comparison with vehicle-treated animals ($42.4 \pm 6.4\%$, $n = 8$ and $31.2 \pm 6.4\%$, $n = 10$ at 3 and 4 weeks postoperation, respectively). In separate experiments, the loss of injured motoneurons was also significantly prevented by oral tube administration of 30 mg/kg per day T-588 ($52.4 \pm 8.0\%$, $n = 10$) as compared to vehicle ($34.8 \pm 13.8\%$, $n = 8$) at 4 weeks after avulsion. T-588 treatments also ameliorated ChAT immunoreactivity in injured motoneurons and the tissue ChAT enzyme activities at 1 week postoperation examined. These results indicate that oral administration of T-588 ameliorates the survival of injured motoneurons and supports their neuronal function after facial nerve avulsion in adult rats. It has been shown that T-588 activates mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) pathway in cultured rat newborn astrocytes and inhibits astrocyte apoptosis induced by $Ca^{2+}$ stress.[66] T-588 may therefore modify the MAP/ERK pathway in injured motoneurons and surrounding glial cells after facial nerve avulsion. Our results indicate that T-588 may be a promising therapeutic agent for motoneuron injury and motor neuron diseases in humans.

## CONCLUSION

Using peripheral nerve avulsion models, we have identified neuroprotective activities of GDNF, BDNF, TGFβ2, GIF, and T-588 against degeneration of adult motoneurons. These factors may prevent the degeneration of motoneurons in adult humans with motoneuron injury and motor neuron diseases. Further investigations are required to elucidate pathomechanisms of motoneuron degeneration after peripheral nerve avulsion that may help understand the pathogenesis of ALS in humans.

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