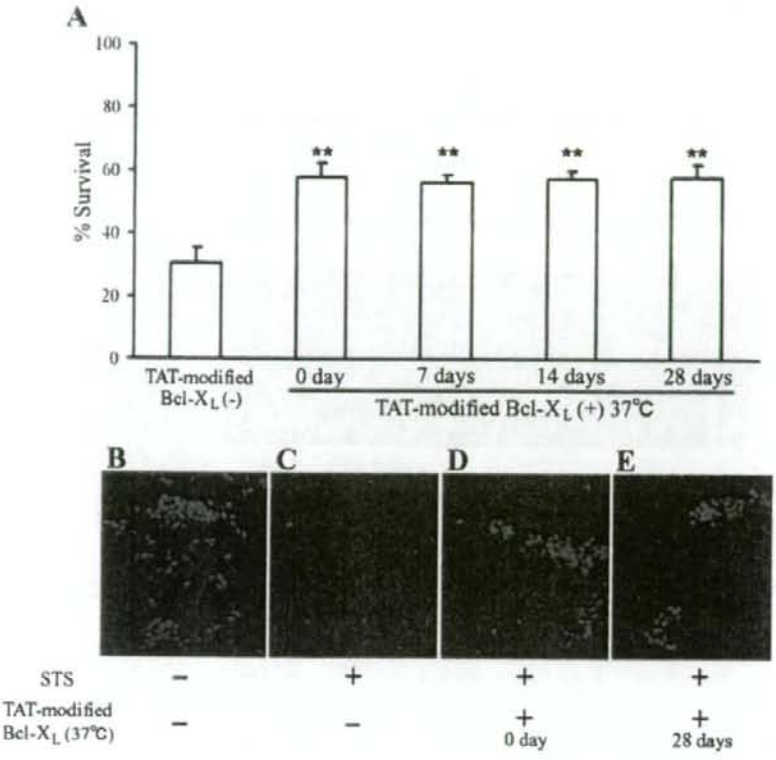

Fig. 1. The antiapoptotic effects of TAT-modified Bcl-X_L are retained at 37°C for 7, 14, and 28 days and prevent STS-induced apoptosis in neuroblastoma SH-SY5Y cells. **A:** TAT-modified Bcl-X_L incubated at 37°C for 7, 14, or 28 days markedly inhibits STS-induced cell death, as efficiently as TAT-modified Bcl-X_L incubated for 0 days. SH-SY5Y cells were pretreated with 3 nM TAT-modified Bcl-X_L for 1 hr and incubated with 50 nM STS for 1 day. Living cells were counted by staining with propidium iodide (red) and Hoechst 33342 (blue). The means of five independent experiments are presented with the SD. $^{**}P < 0.01$ compared with non-TAT-modified Bcl-X_L (one-factor ANOVA followed by Tukey-Kramer post hoc comparison). **B–E:** Representative pictures of living (blue round nuclei) and apoptotic (blue fragmented nuclei) cells incubated without (B) or with (C–E) 50 nM STS (×100). Representative pictures of cells pretreated with 3 nM TAT-modified Bcl-X_L incubated at 37°C for 0 (D) or 28 (E) days.

RESULTS

Antiapoptotic Effects of TAT-Modified Bcl-X_L Remain Active at 37°C for 28 Days In Vitro

To confirm that the antiapoptotic effects of TAT-modified Bcl-X_L could remain active in osmotic mini-pump-implanted mice for 28 days, we used STS to induce apoptosis in SH-SY5Y cells treated with TAT-modified Bcl-X_L. TAT-modified Bcl-X_L that had been incubated at 37°C for 7, 14, or 28 days markedly inhibited cell death as efficiently as fresh TAT-modified Bcl-X_L (Fig. 1A–E), suggesting that the antiapoptotic effects of TAT-modified Bcl-X_L could be retained at 37°C for 28 days.

An Intrathecally Infused TAT-Fused Protein Is Transferred Into Neurons of the Lumbar Cord

To examine the distribution of TAT-fused protein in the lumbar cord when delivered intrathecally, aCSF, TAT-GFP (3.5×10^{-2} nmol or 3.5×10^{-1} nmol, administered over 7 days), or TAT-modified Bcl-X_L (3.5×10^{-1} nmol, administered over 7 days) was intrathecally infused into a lumbar site of non-Tg wild-type B6SJL male littermates (WT) at 91 days of age, for 7 days, using a 200-μl-volume osmotic mini-pump, which was designed for a nominal infusion rate of 1 μl/hr for 7 days. Immunohistochemical staining of the lumbar cord using an anti-GFP antibody revealed immunoreactivity in large cells of the ventral horn with a diameter greater than 20 μm (Fig. 2Ab, arrows) and small cells of the dorsal horn with a diameter smaller than 20 μm following infusion of 3.5×10^{-2} nmol of TAT-GFP; stronger immunoreactivity was observed following infusion of 3.5×10^{-1} nmol of TAT-GFP (large cells of the ventral horn; Fig. 2Ac). Double-immunofluorescence analysis using an anti-GFP antibody and SMI32, a monoclonal antibody that reacts with a nonphosphorylated neurofilament protein, revealed an overlap between the distributions of these two antigens (Fig. 2Ba–c). Immunohistochemical staining of the lumbar cord using an anti-Bcl-X_L antibody, a monoclonal antibody that reacts with both endogenous mouse Bcl-X_L and a modified Bcl-X_L, revealed stronger immunoreactivity in large cells of the ventral horn with a diameter greater than 20 μm (Fig. 2Cb) and small cells of the dorsal horn with a diameter smaller than 20 μm following infusion of 3.5×10^{-1} nmol of TAT-modified Bcl-X_L compared with the ventral horn following infusion of aCSF (Fig. 2Ca). These results indicate that intrathecally administered TAT-fused proteins are dose-dependently transferred into neurons, including motor neurons, in the lumbar cord.

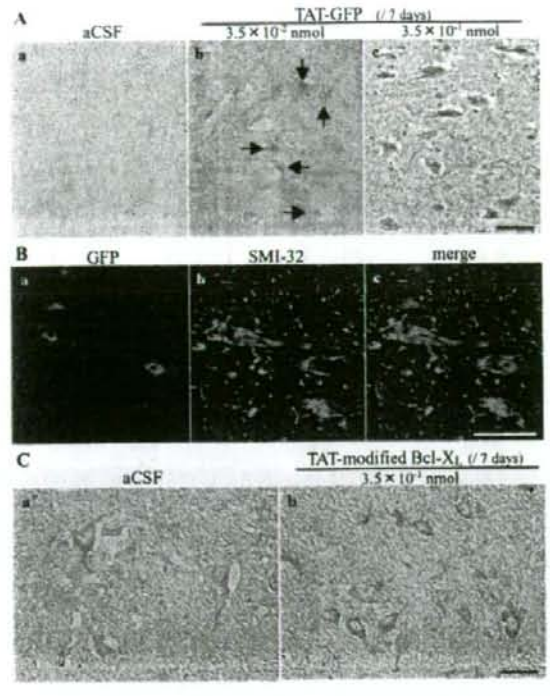

Fig. 2. Transfer of TAT-GFP and TAT-modified Bcl-X_L intrathecally infused into motor neurons of the ventral horn in the lumbar cord. **A:** Representative pictures show the transfer of TAT-GFP intrathecally infused into large cells with a diameter greater than 20 μm, in the ventral horns of WT mice, after 7 days of continuous intrathecal infusion of TAT-GFP into a lumbar site using an osmotic minipump. Compared with the ventral horn infused with aCSF (a), note the immunoreactivity in large cells in the ventral horn infused with 3.5×10^{-2} nmol (administered over 7 days) of TAT-GFP (b, arrows), and the even stronger immunoreactivity in similar cells in the ventral horn infused with 3.5×10^{-1} nmol (administered over 7 days) of TAT-GFP (c), as determined using anti-GFP antibody. **B:** Representative pictures show the transfer of TAT-GFP (3.5×10^{-2} nmol, administered over 7 days) into large neurons of the ventral horn, suggesting transfer of TAT-GFP into motor neurons. Note that the immunofluorescence signals for GFP (a, green) and SMI32 (b, red) are colocalized (c, overlay). **C:** Representative pictures show the transfer of TAT-modified Bcl-X_L intrathecally infused into large cells with a diameter greater than 20 μm, in the ventral horns of WT mice, after 7 days of continuous intrathecal infusion of TAT-modified Bcl-X_L into a lumbar site using an osmotic minipump. Compared with the ventral horn infused with aCSF (a), note the stronger immunoreactivity in large cells in the ventral horn infused with 3.5×10^{-1} nmol (administered over 7 days) of TAT-modified Bcl-X_L (b), as determined using anti-Bcl-X_L antibody. Scale bars = 50 μm.

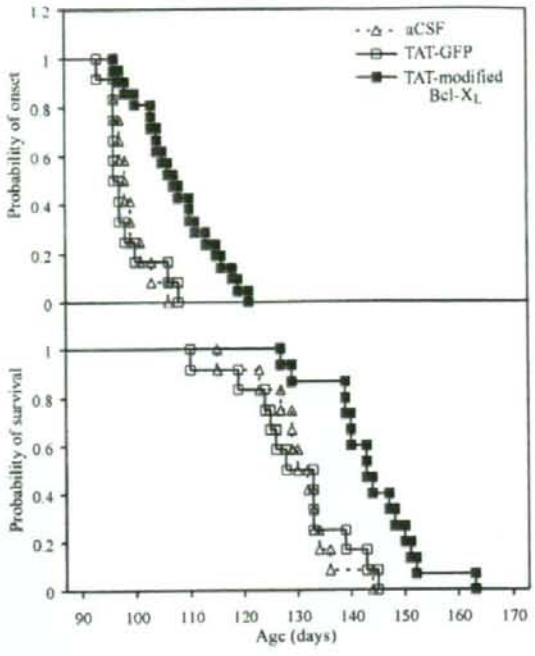

Fig. 3. Delayed disease onset and prolonged survival in G93A SOD1 Tg mice intrathecally infused with TAT-modified Bcl-X_L for 28 days. The cumulative probability of disease onset and survival for G93A mice beginning at 91 days of age intrathecally infused with aCSF (n = 13), TAT-GFP (1.4 nmol, administered over 28 days; n = 12), or TAT-modified Bcl-X_L (1.4 nmol, administered over 28 days, n = 21) into a lumbar site using an osmotic minipump is shown by a Kaplan-Meier curve. Intrathecal infusion of TAT-modified Bcl-X_L delays disease onset compared with aCSF ($P < 0.0002$, log-rank test) and TAT-GFP ($P < 0.0008$, log-rank test; upper panel). TAT-modified Bcl-X_L treatment (n = 15 for TAT-modified Bcl-X_L-treated mice; six mice were sacrificed at 133 days of age for histological analysis) also prolongs survival compared with aCSF (n = 13; $P < 0.0008$, log-rank test) and TAT-GFP (n = 12; $P < 0.004$, log-rank test; lower panel).

Intrathecal Infusion of TAT-Modified Bcl-X_L Delays Disease Onset and Prolongs Survival in G93A SOD1 Tg Mice

To examine the clinical benefits of TAT-modified Bcl-X_L treatment, aCSF, TAT-GFP (1.4 nmol, administered over 28 days), or TAT-modified Bcl-X_L (1.4 nmol, administered over 28 days) was intrathecally infused into a lumbar site of G93A SOD1 Tg male mice at 91 days of age, for 28 days, using a 200-μl-volume osmotic minipump, which was designed for a nominal infusion rate of 0.25 μl/hr for 28 days. Disease onset, as defined by showing a fine tremor in at least one hindlimb over a 20-sec period when hanging from the tail, of G93A mice treated with aCSF and TAT-GFP, occurred at 98.9 ± 3.0 days (means ± SD) of age (n = 13) and 98.3 ± 4.4 days of age (n = 12), respectively (Fig. 3). The disease onset of G93A mice treated with TAT-modified Bcl-X_L was delayed until 107.8 ± 7.3 days of age (n = 21), later by 9.0% ($P < 0.0002$) and 9.7% ($P < 0.0008$) compared with aCSF- and TAT-GFP-treated mice, respectively (Fig. 3). The survival of G93A mice treated with aCSF and TAT-GFP was

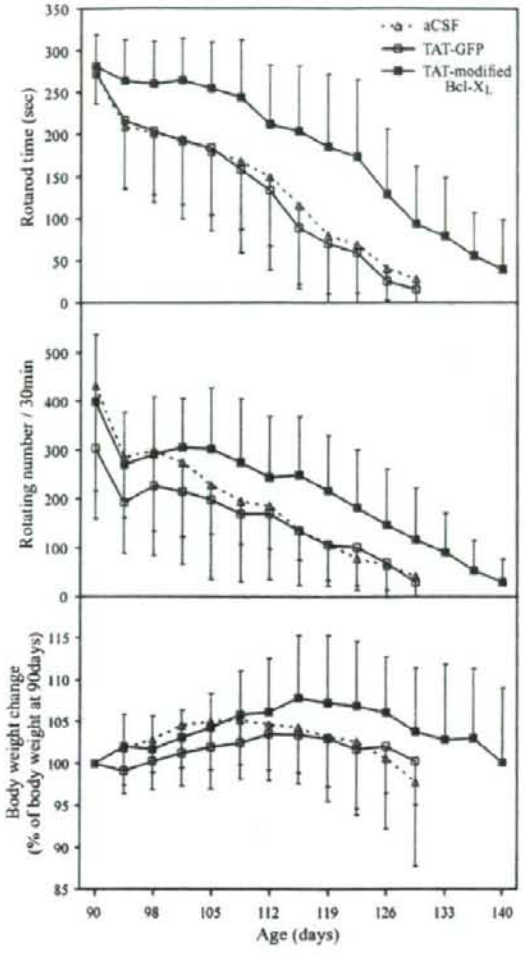

Fig. 4. Improvement in clinical scores in G93A SOD1 Tg mice intrathecally infused with TAT-modified Bcl-X_L for 28 days. Intrathecal infusion of TAT-modified Bcl-X_L (n = 21 for TAT-modified Bcl-X_L-treated mice) remarkably improves motor performance on the rotarod test ($P < 0.01$ from 95 to 130 days of age, as determined by repeated-measures ANOVA followed by Tukey-Kramer post hoc comparison; upper panel), and improves wheel rotating number during a 30-min period ($P < 0.05$ from 102 to 130 days of age, as determined by repeated-measures ANOVA followed by Tukey-Kramer post hoc comparison; middle panel) compared with aCSF (n = 13) and TAT-GFP (n = 12). TAT-modified Bcl-X_L (n = 21) treatment results in clear delays in body weight decrease compared with aCSF (n = 13) and TAT-GFP (n = 12) treatment (lower panel). Data are mean ± SD.

130.8 ± 7.0 days (n = 13) and 129.8 ± 10.0 days (n = 12), respectively (Fig. 3). The survival of G93A mice treated with TAT-modified Bcl-X_L was prolonged to 143.7 ± 9.3 days (n = 15; six mice were sacrificed at 133 days of age for histological analysis), representing an increase in survival time by 9.9% ($P < 0.0008$) and 10.7% ($P < 0.004$) compared with aCSF- and TAT-GFP-treated mice, respectively (Fig. 3).

Intrathecal Infusion of TAT-Modified Bcl-X_L Improves Motor Performance in G93A SOD1 Tg Mice

In the rotarod test, G93A mice treated with TAT-modified Bcl-X_L had significantly better motor performance from 95 to 130 days of age than mice treated with aCSF or TAT-GFP ($P < 0.01$; Fig. 4). Although all of the mice showed a decrease in motor performance on a wheel-running test after minipump implantation, they had recovered by 95 days of age (Fig. 4). G93A mice treated with aCSF or TAT-GFP showed a marked decrease in motor activities after 98 days of age, whereas those treated with TAT-modified Bcl-X_L exhibited significant delays in their clinical deterioration ($P < 0.05$, from 102 to 130 days). G93A mice treated with aCSF or TAT-GFP showed a gradual decrease in body weight after 112 days of age, and those treated with TAT-modified Bcl-X_L exhibited clear delays in this decrease in body weight (Fig. 4).

Neuroprotective Effects of Intrathecal Infusion of TAT-Modified Bcl-X_L in G93A SOD1 Tg Mice

Because the majority (72.0%) of G93A mice treated with aCSF and TAT-GFP died before 133 days of age, and the majority (86.7%) of G93A mice treated with TAT-modified Bcl-X_L survived to this age (Fig. 3), in order to analyze the neuroprotective effects of TAT-modified Bcl-X_L in G93A mice, six G93A mice treated with TAT-modified Bcl-X_L and six WT mice were sacrificed at 133 days of age, and their spinal cords were compared with those of six G93A mice treated with aCSF or TAT-GFP that had died before 133 days of age. We counted the number of Nissl-stained motor neurons in L4 spinal cord sections of these mice. The number of surviving Nissl-stained neurons in the ventral horn was significantly higher in G93A mice treated with TAT-modified Bcl-X_L compared with those treated with aCSF or TAT-GFP ($P < 0.01$; Fig. 5). Additionally, to analyze the antiapoptotic effects of TAT-modified Bcl-X_L in G93A mice, we immunostained L4 spinal cord sections with anticleaved caspase 9 and anticleaved caspase 3 antibodies and then performed TUNEL staining. The number of cleaved caspase 9-, cleaved caspase 3-, and TUNEL-positive cells in the gray matter was significantly lower in G93A mice treated with TAT-modified Bcl-X_L compared with those treated with aCSF or TAT-GFP ($P < 0.01$; Fig. 6A, arrows, B). Upon closer analysis of the gray matter of G93A mice, the numbers of cleaved caspase 9-, cleaved caspase 3-, and TUNEL-positive cells were found to be increased much more in the ventral horn than in the dorsal horn (data not shown). To determine whether the cleaved caspase 9- and cleaved caspase 3-positive cells are neu-

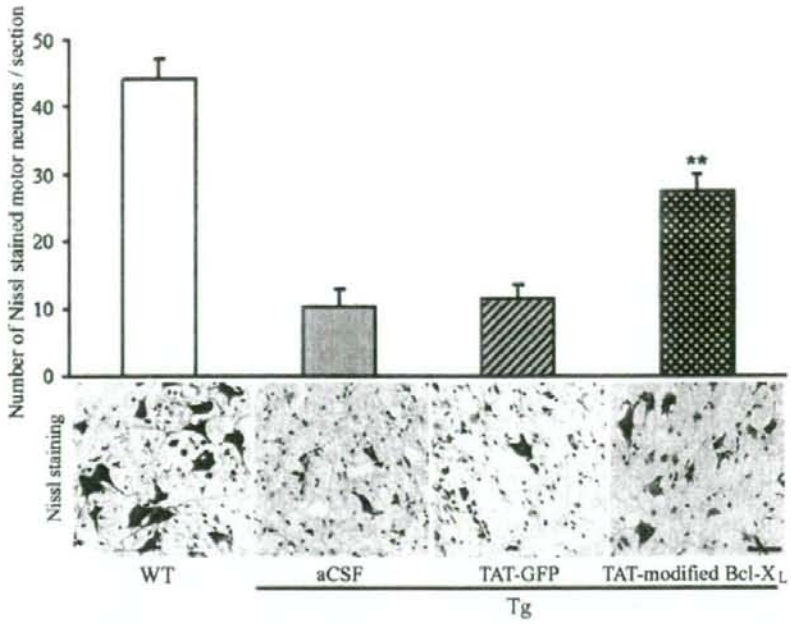

Fig. 5. Attenuation of motor neuron loss in L4 lumbar cords of G93A SOD1 Tg mice intrathecally infused with TAT-modified Bcl-X_L for 28 days. Note the increased number of surviving Nissl-stained motor neurons in TAT-modified Bcl-X_L-treated G93A mice sacrificed at 133 days of age compared with this number in aCSF- or TAT-GFP-treated mice that died before 133 days of age (upper panel). Data are mean ± SD. $^{**}P < 0.01$ compared with aCSF (one-factor ANOVA followed by Tukey-Kramer post hoc comparison). Representative photomicrographs of Nissl-stained motor neurons in the ventral horns of WT mice sacrificed at 133 days of age or G93A mice (lower panel). Scale bar = 50 μm.

rons or glia cells, we performed double-immunofluorescence studies using anticleaved caspase 9 or anticleaved caspase 3 antibodies plus anti-NeuN or anti-GFAP antibodies. The cleaved caspase 9- and cleaved caspase 3-positive cells, which include large cells of the ventral horn with a diameter greater than 20 μm, revealed an overlap with NeuN but not with GFAP (Fig. 7), suggesting that neurons, including motor neurons, expressed apoptotic markers.

DISCUSSION

In the present study, we demonstrated that a TAT-fused protein was effectively transferred into spinal cord neurons, including motor neurons, when delivered intrathecally (Fig. 2). We also showed that the intrathecal infusion of TAT-modified Bcl-X_L produces therapeutic benefits in G93A mice, delaying disease onset and prolonging survival with improved motor performance compared with aCSF- and TAT-GFP-injected mice (Figs. 3, 4). Furthermore, evidence of the therapeutic benefits of TAT-modified Bcl-X_L was provided by the attenuation of motor neuron loss and the reduced number of cells positive for cleaved caspase-9, cleaved caspase-3, and TUNEL in the lumbar cords of TAT-modified Bcl-X_L-treated G93A mice (Fig. 5, 6).

TAT-fused proteins can cross the blood–brain barrier and cell membranes and transfer into several tissues, including the brain, when infused into mice intraperitoneally (Schwarze et al., 1999; Cao et al., 2002). In mouse or gerbil models of focal ischemia or traumatic injury, full-length therapeutic proteins fused with TAT, including TAT-modified Bcl-X_L, show strong therapeutic effects when delivered intraperitoneally, intravenously, or intraocularly (Asoh et al., 2002; Cao et al., 2002; Dietz et al., 2002; Kilic et al., 2002, 2003, 2004; Kim et al., 2005); therefore, protein therapy using therapeutic proteins fused with TAT can be widely applicable for the treatment of various neurological disorders, including ALS. Because therapeutic proteins fused with TAT have to be efficiently delivered into the spinal cord in order to be used as therapy in ALS model mice, we first delivered TAT-fused GFP and modified Bcl-X_L into the spinal cord intrathecally, using an osmotic minipump, as described previously (Nagano et al., 2005; Ohta et al., 2006). A much smaller amount of TAT-GFP [3.5×10^{-2} nmol (1.3 μg)/7 days] and TAT-modified Bcl-X_L [3.5×10^{-1} nmol (10.2 μg)/7 days] was effectively transferred into spinal cord neurons, including

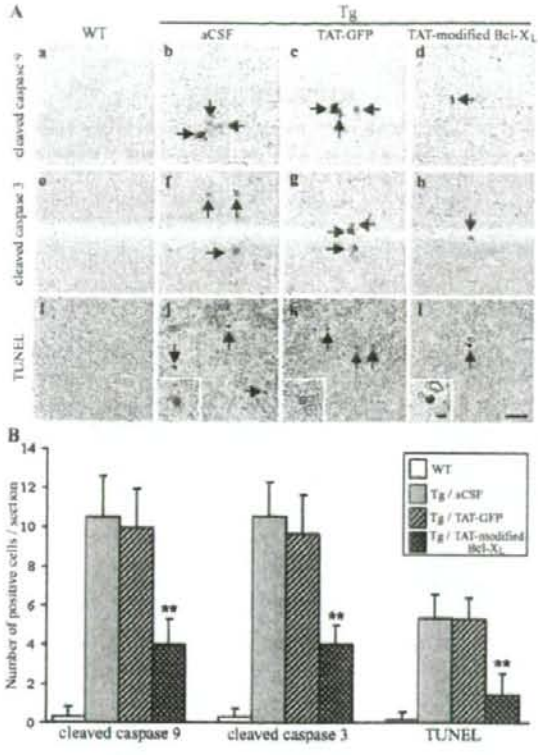

Fig. 6. **A,B:** Decreased number of cleaved caspase 9-, cleaved caspase 3-, and TUNEL-positive cells in the L4 lumbar cords of G93A SOD1 Tg mice intrathecally infused with TAT-modified Bcl-X_L for 28 days. Compared with WT mice (Aa,e,i, B), note the increased number of cleaved caspase 9-, cleaved caspase 3-, and TUNEL-positive cells in G93A mice treated with aCSF (Ab,f,j, arrows, B) or TAT-GFP (Ac,g,k, arrows, B), and the attenuation of this increase in mice treated with TAT-modified Bcl-X_L (Ad,h,l, arrows, B). Data are mean ± SD. $^{**}P < 0.01$ compared with aCSF (one-factor ANOVA followed by Tukey-Kramer post hoc comparison). Representative photomicrographs of cleaved caspase 9 (Aa–d, arrows)-, cleaved caspase 3 (Ae–h, arrows)-, and TUNEL (Ai–l, arrows)-positive cells in the ventral horns of WT or G93A mice. Larger scale bar in l = 50 μm for a–i; smaller scale bar in l = 10 μm for j–l.

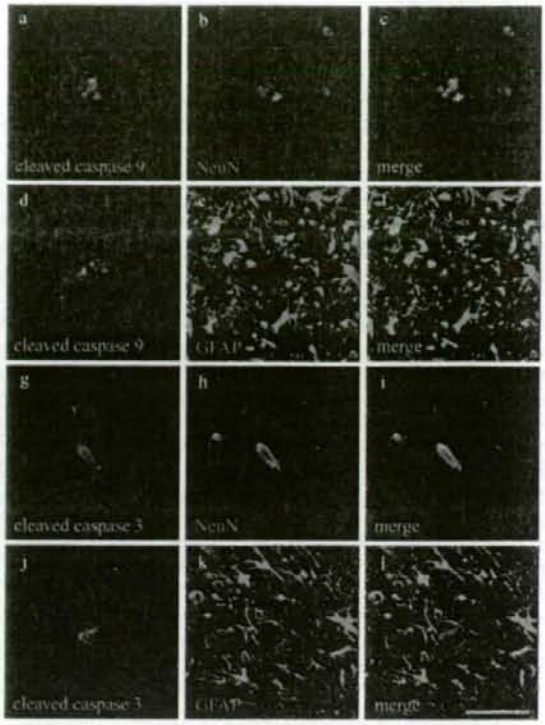

Fig. 7. Expression of cleaved caspase 9 and cleaved caspase 3 in motor neurons of the ventral horn in the lumbar cord of G93A SOD1 Tg mice. Representative pictures show the expression of cleaved caspase 9 and 3 in large neurons with a diameter greater than 20 μm in the ventral horns of G93A mice, suggesting the expression of these molecules in motor neurons. Note that the immunofluorescence signals for cleaved caspase 9 (a,d, green) or cleaved caspase 3 (g,j, green) and NeuN (b,h, red) are colocalized (c,i, overlay), but those for cleaved caspases and GFAP are not colocalized (e,k, red, f,l, overlay). Scale bar = 50 μm.

motor neurons (Fig. 2Ab, arrows, Cb), than the amounts previously reported for intraperitoneal or intravenous administration [1.7 nmol (Schwarze et al., 1999), 5 mg/kg (Asoh et al., 2002), 3 mg/kg (Cao et al., 2002), 0.6 nmol (Kilic et al., 2002), 0.6 nmol (Kilic et al., 2003), 10 mg/kg (Kim et al., 2005)]; thus, intrathecal infusion of TAT-fused proteins using an osmotic minipump is a better method of delivering therapeutic proteins into the spinal cord.

Antiapoptotic members of the Bcl-2 family, Bcl-2 and Bcl-X_L, block cytochrome c release from mitochondria (Reed, 1997; Halestrap et al., 2000), and this blockage interferes with the proapoptotic actions of BAX (Cheng et al., 2001; Sathasivam et al., 2001). In G93A mice, the levels of Bcl-2 and Bcl-X_L tend to decrease gradually from the asymptomatic stage, and the level of BAX increases slightly during the asymptomatic stage, but much more drastically during the early symptomatic stage (Vukosavic et al., 1999). Overexpression of Bcl-2 delays the activation of caspases (Vukosavic et al., 2000) and transgenic overexpression of Bcl-2 or intraspinal injection of adeno-associated virus encoding Bcl-2 during the asymptomatic stage produce neuroprotective effects (Kostic et al., 1997; Azzouz et al., 2000); thus, Bcl-2 and Bcl-X_L can be used as therapeutic proteins for the treatment of ALS. We constructed a modified Bcl-X_L with three amino acid substitutions in Phe, Asn, and Lys in place of Tyr^{22}, Gln^{26}, and Arg^{165}, respectively (Asoh et al., 2000). Because a modified Bcl-X_L has a more powerful antiapoptotic effect rather than original Bcl-X_L (Asoh et al., 2000, 2002) and G93A mice gener-

ally experience disease onset at about 100 days of age (Ohta et al., 2006), we intrathecally infused the modified Bcl-X_L protein fused with TAT, for 28 days, in G93A mice at 91 days of age. This treatment method successfully resulted in clinical benefits (delay of disease onset, longer survival, improvement in motor performance, and inhibition of motor neuron death; Figs. 3–5). The uptake of TAT-fused protein into the brain peaks at 4–8 hr after infusion, after which it gradually decreases (Cao et al., 2002). In the present study, infusion of TAT-modified Bcl-X_L stopped at 118 days of age (28 days after minipump placement), and the amount of TAT-modified Bcl-X_L reaching the spinal cord was expected to decrease gradually along with its antiapoptotic effects. However, at 133 days of age (15 days after the termination of TAT-modified Bcl-X_L injection), the numbers of cells positive for cleaved caspases 9 and 3, as well as TUNEL, were much lower in mice treated with TAT-modified Bcl-X_L than in those treated with aCSF or TAT-GFP (Fig. 6). This result suggests that the antiapoptotic effects of TAT-modified Bcl-X_L continued for at least 15 days after it stopped being infused. Double-immunofluorescence studies using anticleaved caspase 9 or 3 antibodies plus anti-NeuN or anti-GFAP antibodies revealed that neurons, including motor neurons, expressed apoptotic markers (Fig. 7), consistently with previous reports (Vukosavic et al., 2000; Inoue et al., 2003). Therefore, we considered TUNEL-positive cells to be neurons, including motor neurons, consistent with previous reports (Martin et al., 2007). Cao et al. (2002) reported that most of the TAT-Bcl-X_L was delivered into neurons but some into astrocytes. We considered that TAT-modified Bcl-X_L might have transduced in some astrocytes of spinal cord of G93A mice and that the astrocytes might act as inhibitors of apoptosis for motor neurons.

Although we showed that intrathecal infusion of TAT-modified Bcl-X_L results in therapeutic effects in ALS model mice, the therapeutic effectiveness we showed was consistent with previous data (Shoemaker et al., 2007); that is, blocking apoptotic pathways only is not fully sufficient to treat ALS (Migheli et al., 1999; Gould et al., 2006). However, we also showed that a TAT-fused protein was effectively transferred into spinal cord neurons, including motor neurons, when delivered intrathecally (Fig. 2). In the future, other potential therapeutic proteins for the treatment of ALS, which cannot usually cross cell membranes, can be used in therapy by making fusion proteins with TAT.

ACKNOWLEDGMENTS

We thank Dr. Steven F. Dowdy for the generous gift of pTAT-HA-GFP vectors. This work was partly supported by Grants-in-Aid for Scientific Research (B) 15390273 and (Hoga) 15659338 and the National Project on Protein Structural and Functional Analyses from the Ministry of Education, Science, Culture and Sports of Japan, and by grants (to Itoyama Y, Kimura I and Kuzuhara S) from the Ministry of Health and Welfare of Japan.

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