

Noxa Is a Critical Mediator of p53-Dependent Motor Neuron Death after Nerve Injury in Adult Mouse

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Axotomy-induced motor neuron death occurs within a week in the neonatal rat and mouse. However, slowly progressive motor neuron death, which takes more than a month, occurs after axotomy in the adult mouse (C57BL/6) but not in the adult rat (Wistar). Here we demonstrate that expression of a p53-inducible Bcl-2 homology domain 3 (BH3)-only protein, Noxa, is enhanced in axotomized neurons of the adult mouse but not in the adult rat. In p53-deficient mice, slowly progressive neuronal death was suppressed and accompanied by reduced Noxa expression after axotomy. However, a minor response of Noxa expression was still observed after axotomy in p53-deficient mice, suggesting that p53-independent Noxa expression occurs to a minor extent. Noxa-deficient mice were used to confirm the consequence of Noxa expression in nerve-injured mouse motor neurons. In Noxa-deficient mice, axotomy-induced motor neuron death was suppressed. Furthermore, among the BH3-only protein members examined, Noxa exhibited the most marked upregulation after axotomy in the mouse. In conclusion, motor neuron death seen in the adult mouse is mainly p53 dependent, and Noxa is a major executor for axotomy-induced motor neuron death in the adult mouse, as a mediator located downstream of p53.

Key words: Noxa; BH3-only protein; nerve injury; motor neuron; axotomy; neuronal death

Introduction

Rats and mice are concordant animals, and most of their genotypes and phenotypes can be considered similar. However, we observed that this is not the case for motor neuron regeneration. Nerve-injured motor neurons of the adult rat can survive, whereas similar axotomy leads to gradual cell death of the injured motor neurons in the adult mouse. Motor neuron death seen in the adult mouse progresses much slower than that seen in the neonatal mouse and rat (Snider et al., 1992) (S. Kiryu-Seo and H. Kiyama, unpublished data). Therefore, the cell death seen in the adult mouse could be a more appropriate model for neurodegenerative diseases such as amyotrophic lateral sclerosis than the cell death seen in neonates. Because several studies highlighted that the tumor suppressor p53 is activated in damaged neurons and that subsequent caspase-3 activation occurs in a model of CNS injury such as focal ischemia, kainate excitotoxicity, and peripheral nerve injury (Miller et al., 2000; Morrison and Kinoshita, 2000; Martin and Liu, 2002), the slow progressive motor neuron death after axotomy, which is seen in the adult mouse, is also likely to be triggered by a p53-dependent mechanism. However,

the linkage between p53 and activation of the caspase cascade is still elusive, at least in motor neuron death in the adult mouse.

In general, p53 regulates cellular responses to stress through transcriptional regulation of genes such as Bax, p21^{WAF1}, and MDM2 (Vousden and Lu, 2002). Apoptosis stimulated by p53 was associated with disturbance of mitochondrial membrane potential, accumulation of reactive oxygen species, stimulation of caspase-9, and subsequent activation of the caspase cascade. In this context, several p53-transactivated target genes have been proposed to mediate apoptosis, although transcription-independent activities of p53 have been described recently (Baptiste and Prives, 2004). Among those p53 targets, mitochondrial proteins are particularly attractive, because p53-dependent apoptosis is mainly elicited through a mitochondria-mediated pathway (Polyak et al., 1997). The proapoptotic protein Bax is the most characterized and well established molecule as a mediator for the mitochondria-mediated apoptotic cascade (Miyashita and Reed, 1995). Motor neurons in Bax-deficient neonatal mice fail to degenerate after axotomy, and it was concluded that trophic factor deprivation-induced death of motor neurons is dependent on Bax (Deckwerth et al., 1996). In this respect, Bax could be a major death executor located downstream of p53 in the neonate. However, recently, other p53-inducible target genes, Noxa and Puma, have been identified (Oda et al., 2000; Nakano and Vousden, 2001). They belong to one of the Bcl-2 family proteins, Bcl-2 homology domain 3 (BH3)-only proteins, which are crucial death regulators that reside immediately upstream of the mitochondria and induce mitochondrial dysfunction (Seo et al., 2003), although their implications in nerve injury-induced motor neuron death are unknown. In this paper, we demonstrate

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that the p53-mediated death pathway contributes to progressive motor neuron death in the adult mouse and that Noxa is a critical mediator of the p53-mediated death pathway in the adult mouse.

Materials and Methods

Animals. Seven-week-old male C57BL/6 mice ($n = 140$) and Wistar rats ($n = 62$) were anesthetized with pentobarbital (45 mg/kg, i.p.) and positioned supine; their right hypoglossal nerve was then cut with scissors. Homozygous p53-deficient mice ($n = 48$) were purchased from The Jackson Laboratory (Bar Harbor, ME). Noxa-deficient mice ($n = 15$) were established by Dr. T. Taniguchi and colleagues (Tokyo University, Tokyo, Japan) (Shibue et al., 2003). All mouse strains had an inbred C57BL/6 genetic background or had been backcrossed at least five times. All experiments were performed in compliance with institutional guidelines.

Histology. Animals were decapitated 1, 3, 7, 28, 35, and 56 d after the operation (eight animals at each point), and brains were removed quickly and frozen in powdered dry ice. Next, 18- μ m-thick sections were cut on a cryostat, thaw-mounted onto 3-aminopropyltriethoxysilane-coated slides, and stored at -80°C until use. The level of hypoglossal nucleus was serially sectioned, and every fifth section was attached on the same group of slides. The adjacent section was attached on the slide for the next group, and accordingly, five groups of slides from one animal were prepared. Cell counts were done blind as to the treatment condition using a well established counting method that effectively eliminates the possibility of counting the same cell twice (Clarke and Oppenheim, 1995). According to the literature, the following criteria were used for the counting: large soma, a clear nucleus with intact nuclear membrane, and at least one large clump of nucleolar material. For quantification of motor neuron survival after unilateral axotomy, thionine-stained hypoglossal motor neurons in injured and control sides were counted separately in every fifth section of each animal examined. The counts were done using three independent groups of slides per animal. Data were presented as the percentage of surviving neurons on the injured and control side. Statistical significance (p value) was calculated by two-tailed Student's t test. As for the comparison of total number of motor neurons between the knock-out (Noxa $^{-/-}$ and p53 $^{-/-}$) and the wild-type mice, motor neurons were counted following the criteria at the identical level between animals in every fifth section of the population examined, and the raw count of motor neurons was multiplied by five to give an estimate of total cell numbers and corrected with a correction factor that took into account the thickness of the section and the average nucleolar diameters (Abercrombie, 1946).

All procedures for *in situ* hybridization were performed as described previously (Kiryu et al., 1995). The fragments amplified by PCR (supplemental material, available at www.jneurosci.org) using cDNA isolated from rats and mice were inserted into pGEM-T Easy vector (Promega, Madison, WI) and used as probes. Data are representative of three independent experiments using at least seven animals.

Western blotting. Samples were collected from control and operated hypoglossal nuclei of 10 mice at 7 d after axotomy and homogenized in lysis buffer (20 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 5 μ g/ml aprotinin, 1 mM PMSF, and 1 μ g/ml leupeptin). The homogenate was centrifuged at 4°C for 15 min at 15,000 rpm. The supernatant (50 μ g) was loaded and immunoblotted with anti-Noxa antibody (generously provided by Dr. Taniguchi and colleagues, Tokyo University) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Ambion, Austin, TX).

Reverse transcription-PCR analysis. For details of the reverse transcription (RT)-PCR method, see the supplemental material (available at www.jneurosci.org). Quantification was performed using an image scanner (GT-8200UF; Epson) and image analysis software (Photoshop 6.0; Adobe Systems, San Jose, CA). The density was measured at five points for each band and normalized against that of GAPDH. The magnitude of the change in relative transcriptional level was expressed as operated side/control side. Statistical significance of differences was assessed by ANOVA followed by Fisher's PLSD test.

Results

Axotomized motor neurons have different fates in rats and mice

We first assessed how the fate of injured motor neurons differs between the Wistar rat and C57BL/6 mouse (Fig. 1A–C). At \sim 2 months after hypoglossal nerve injury, adult C57BL/6 mice displayed extensive loss of motor neurons compared with Wistar rats. In both rats and mice, the number of surviving motor neurons in the hypoglossal nuclei showed no change at 7 d after nerve injury. However, from that time on, the number of surviving motor neurons in mice gradually decreased, whereas that in rats showed no change. At 8 weeks after nerve injury, only 20% of injured motor neurons of mice survived, whereas almost all motor neurons of rats survived.

Next, we explored the molecular basis of this difference. In our previous molecular screening work using differential display, random cloning, and DNA microarray, we identified a collection of rat genes, the expression of which was upregulated after rat hypoglossal nerve transection (Kiryu et al., 1995; Tanabe et al., 1999). We therefore subcloned those gene fragments from a mouse brain cDNA library by RT-PCR. Using these obtained mouse cDNA fragments, we examined whether the gene response pattern was identical between rats and mice by *in situ* hybridization. Although neuronal death seen in mice progressed slowly over weeks, all of the injured neurons still survived and did not demonstrate any morphological changes at 7 d after axotomy. At this time point, almost all of the genes examined, including major survival signal-associated genes [for instance, glial cell line-derived neurotrophic factor receptors (GFR- α 1 and c-Ret) (Honma et al., 2002), Akt, and Erk] showed a similar response pattern in rats and mice after axotomy (Fig. 1D). This suggests that antiapoptotic or defensive mechanisms seem to function in the adult mouse as well as the adult rat. This histological screening further demonstrated that axotomized motor neurons in both the adult rat and mouse showed enhanced mRNA expression of p53 transactivation genes such as Bax, MDM2, and p21^{WAF1} (Fig. 1E), suggesting that p53 activation occurs in both rats and mice. However, a marked increase in Noxa, which is a BH3-only protein and a p53 target gene, was observed in mice but not in rats (Fig. 1E). The increase in Noxa protein was also observed in injured neurons of mice (Fig. 1F). In contrast, another p53 target gene, Puma, which is also a BH3-only protein, did not show any detectable mRNA signal in both rats and mice. This suggests that Noxa may be a molecule that is responsible for p53-mediated motor neuron death seen in the adult mouse, whereas the rat could somehow succeed in escaping from the cell death as a result of lack of Noxa expression.

Noxa expression in response to nerve injury is suppressed in p53-deficient mice

The above finding suggests that the slow motor neuron death seen in the adult mouse is p53 dependent and that Noxa is a possible mediator of p53-dependent death in the mouse. To elucidate the involvement of p53 in this slow death seen in mice, we compared the viability of hypoglossal motor neurons after axotomy between wild-type and p53-deficient (p53 $^{-/-}$) mice. At 8 weeks after hypoglossal nerve injury, the number of surviving motor neurons in p53-deficient mice was apparently increased (\sim 80% were surviving), whereas $>$ 80% of injured motor neurons disappeared in wild-type mice (Fig. 2A,B). The number of uninjured motor neurons was similar in both p53-deficient and wild-type mice (Fig. 2C). We then confirmed whether Noxa expression in response to nerve injury is changed in p53-deficient

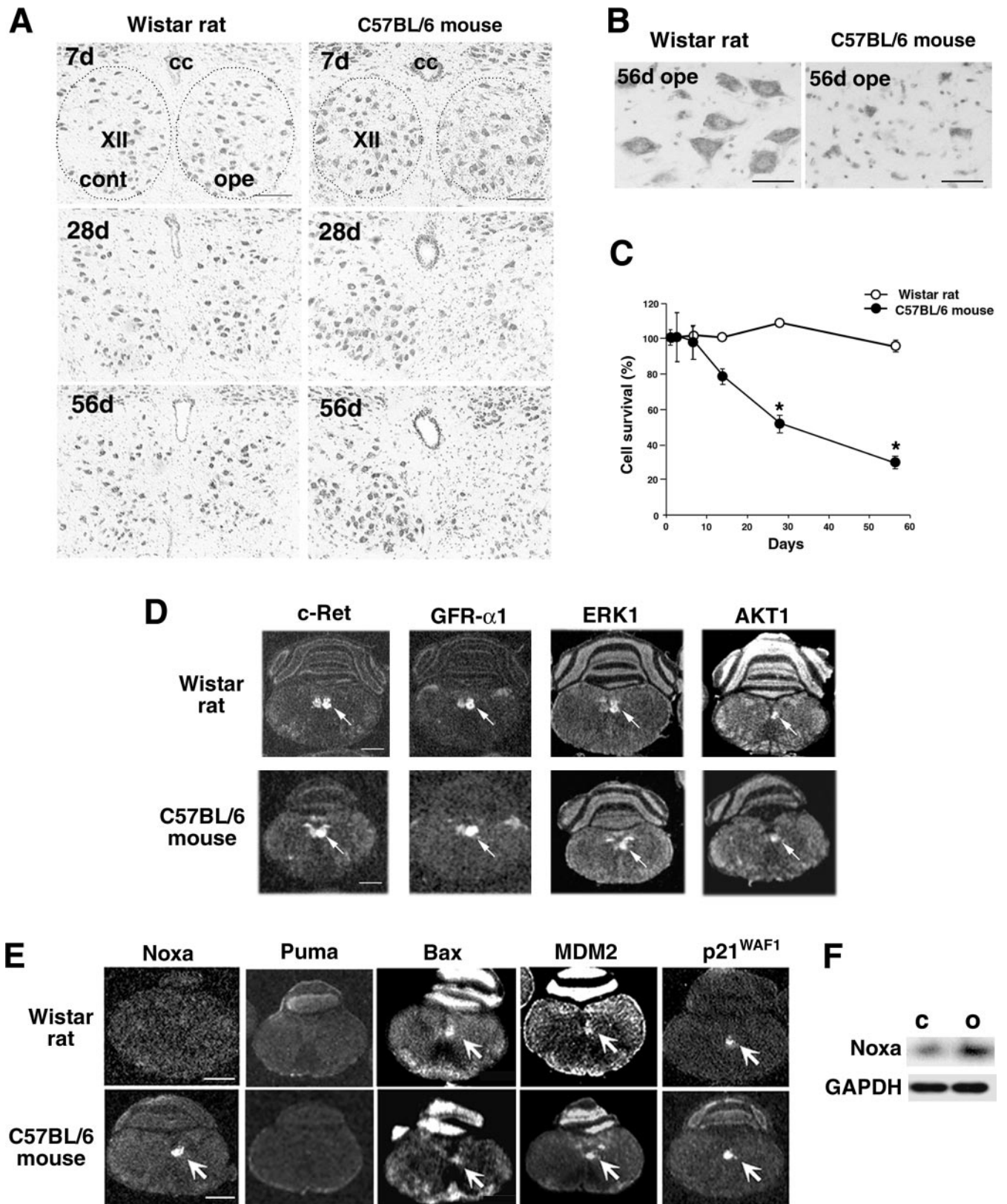


Figure 1. Different fates of axotomized motor neurons in adult rats and mice. *A*, Thionin staining of hypoglossal motor neurons. The hypoglossal nucleus is indicated shown by the dashed line. Adult C57BL/6 mice showed extensive motor neuron loss after hypoglossal nerve injury, whereas adult Wistar rats did not. cc, Central canal; XII, hypoglossal nucleus; cont, contralateral side; ope, operated side; Scale bars: left, 100 μ m; right, 50 μ m. *B*, High-power magnification of operated hypoglossal motor neurons in rats and mice 56 d after axotomy. Scale bars, 25 μ m. *C*, Hypoglossal motor neurons were counted in both Wistar rats (open circles) and C57BL/6 mice (filled circles). The percentage ratio of surviving motor neurons on the operated side compared with that on the control side was calculated. Each point shows the mean \pm SD ($n = 8$; $p < 0.01$ compared with the result of Wistar rat; t test). *D*, Expression of representative survival-associated molecules 7 d after axotomy was examined by *in situ* hybridization. Arrows indicate an increase in mRNA on the operated side (right side). ERK1, Extracellular signal-regulated kinase 1. *E*, Expression of p53-dependent transactivated genes 7 d after axotomy. Scale bars: *D*, *E*, top, 1.3 mm; bottom, 0.8 mm. *F*, Proteins extracted from control (c) and operated (o) hypoglossal nuclei of mice 7 d after axotomy were subjected to Western blot analysis using polyclonal anti-Noxa and anti-GAPDH antibodies.

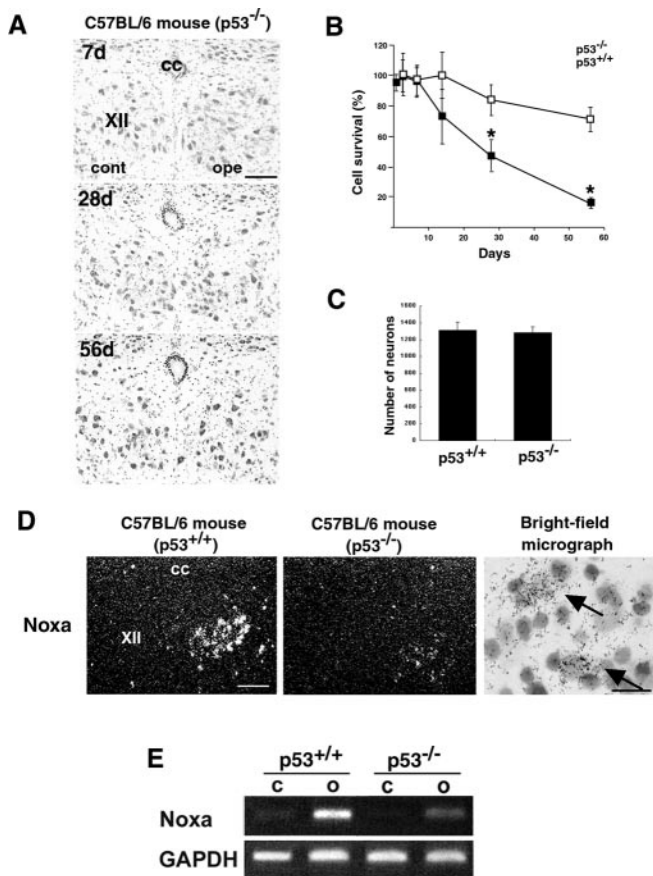


Figure 2. Motor neuron death of mice was associated with p53-dependent Noxa expression. *A*, p53 deficiency in mice ($p53^{-/-}$) effectively prevented axotomy-induced cell death. Scale bar, 50 μm . *B*, p53-deficient mice ($p53^{-/-}$, open squares) were rescued from motor neuron death compared with wild-type mice ($p53^{+/+}$, closed squares) after nerve injury. The percentage ratio of surviving motor neurons on the operated side (right side) compared with that on the control side (left side) was calculated. Each point shows the mean \pm SD ($n = 8$; $p < 0.01$ compared with the result of p53-deficient mouse; t test). *C*, Number of neurons in hypoglossal nuclei (uninjured) from $p53^{+/+}$ and $p53^{-/-}$ mice. Values represent means \pm SD ($n = 6$). *D*, Emulsion autoradiography of *in situ* hybridization showed that Noxa mRNA was more markedly increased on the operated side in wild-type mice than in p53-deficient mice. Scale bar, 50 μm . The right panel shows a bright-field micrograph of the injured side, in which the silver grain of Noxa mRNA signal accumulated in large-sized injured motor neurons (arrows). Scale bar, 25 μm . *E*, RT-PCR analysis of Noxa expression in control (c) and operated (o) hypoglossal nuclei obtained from wild-type ($p53^{+/+}$) and p53-deficient ($p53^{-/-}$) mice. Expression of GAPDH was used as an internal control. Consistent with *in situ* hybridization data, the expression of Noxa mRNA was suppressed in p53-deficient ($p53^{-/-}$) mice.

mice. *In situ* hybridization revealed that Noxa expression was suppressed in injured motor neurons of p53-deficient mice compared with those of wild-type mice (Fig. 2*D*). Semiquantitative RT-PCR using control and operated hypoglossal nuclei also confirmed that Noxa expression was suppressed in p53-deficient mice after axotomy (Fig. 2*E*). These results clarified that Noxa expression is indeed regulated by p53 in injured motor neurons of adult mice. Slight but significant expression of Noxa mRNA was observed in nerve-injured motor neurons of p53-deficient mice (Fig. 2*D,E*), suggesting that p53-independent expression of Noxa exists to a minor extent. Nevertheless, the implication of p53 is significant in nerve injury-induced motor neuron death in the adult mouse, and a p53 target gene, Noxa, could be involved in this system.

To further clarify the consequence of Noxa expression in injured motor neurons, Noxa-deficient and littermate control mice

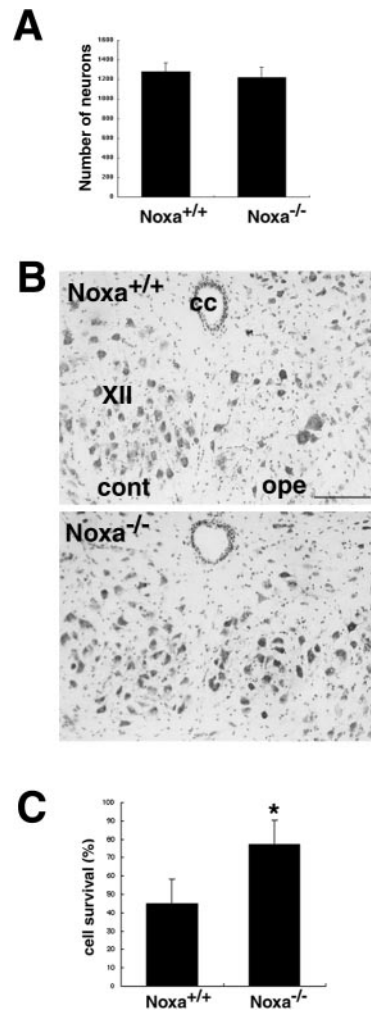


Figure 3. Noxa-deficient mice showed rescue from motor neuron death. *A*, Number of neurons in hypoglossal nuclei (uninjured) from littermate control ($Noxa^{+/+}$) and Noxa-deficient ($Noxa^{-/-}$) mice. Values represent means \pm SD ($n = 6$). *B*, Thionine staining of hypoglossal motor neurons 35 d after hypoglossal nerve injury in $Noxa^{+/+}$ and $Noxa^{-/-}$ mice. Scale bar, 50 μm . *C*, Survival of motor neurons from $Noxa^{+/+}$ and $Noxa^{-/-}$ mice 35 d after axotomy. Results represent the percentage ratio of surviving motor neurons on the operated side compared with those on the contralateral side. $Noxa^{+/+}$ ($n = 10$) and $Noxa^{-/-}$ ($n = 9$) were significantly different ($*p < 0.01$; t test).

underwent hypoglossal axotomy and were examined 5 weeks later. We confirmed that the number of uninjured motor neurons was similar in Noxa-deficient and wild-type mice (Fig. 3*A*). At 5 weeks after axotomy, the number of neurons was reduced by $\sim 45\%$ in wild-type mice when compared with the uninjured side of the same mice (Fig. 3*B,C*). In contrast, the number of surviving neurons was significantly higher in Noxa-deficient mice ($\sim 80\%$).

Noxa is a prominent BH3-only proapoptotic factor expressed in nerve-injured mouse motor neurons

Noxa is one of the BH3-only proteins, which have been proposed to act upstream of mitochondria to regulate caspase activation. Therefore, we examined the expression of other BH3-only proteins, such as DP5, Bim, Bid, and Blk, after nerve injury. Among those examined, DP5, Bim, Blk, and Bid were slightly upregulated after nerve injury both in the rat and mouse. Among those examined, Noxa expression was the most notable in injured motor neurons of the mouse, whereas it was hardly detected in the rat

(Fig. 4A). It should be emphasized that another p53 target gene, Puma, did not show any detectable signal in both the rat and mouse after axotomy. Semiquantitative RT-PCR analysis confirmed that a fourfold increase in Noxa mRNA was induced after nerve injury, although other BH3-only proteins showed an approximately twofold increase in mice (Fig. 4B,C).

Discussion

The present study revealed that slow progressive death of adult mouse motor neurons after axotomy is primarily p53 dependent and that a p53 target gene, Noxa, is a crucial executor in p53-dependent neuronal death. Because expression of p53 target genes such as p21^{WAF1}, MDM2, and Bax was equally observed in both the rat and mouse after nerve injury, p53 activation is apparent in both the rat and mouse. However, motor neuron death occurs only in the mouse. The major difference is the exceptional upregulation of Noxa in the mouse, and this might affect the balance between proapoptotic and prosurvival molecules such as BH3-only proteins and Bcl-2 family members. Because the balance is not severely disrupted in adult mouse motor neurons, axotomy-induced motor neuron death may progress slowly compared with that observed in the neonatal rat and mouse, in which expression of many prosurvival molecules is suppressed (Honma et al., 2002). In this respect, Noxa expression would be highly responsible for determining the fate of injured motor neurons in the adult mouse.

The balance between proapoptotic and prosurvival molecules can be disturbed after nerve injury. For instance, Bax knock-out, Bcl-2 overexpression, or Bcl-XL overexpression succeeded in rescuing motor neurons from axotomy-induced death *in vivo* (Farlie et al., 1995; Deckwerth et al., 1996; Parsadanian et al., 1998). However, Bax can only execute cells in which essential initiators of cell death, such as BH3-only proteins, have been activated in response to stimuli, because newborn Bax transgenic mice did not show death of motor neurons after sciatic nerve axotomy (Bernard et al., 1998). One of the BH3-only protein members, Bim, was proved to accelerate death of sensory and autonomic ganglion neurons after NGF deprivation and nerve injury (Putcha et al., 2001; Whitfield et al., 2001; Napankangas et al., 2003). Similarly, mice deficient in DP5, another BH3-only member, were protected against nerve-injured motor neuron death after hypoglossal nerve injury, and SCG neurons were against delayed death by NGF deprivation (Imaizumi et al., 2004). It is possible that there might be cell-specific and/or stimulus-specific expression of BH3-only protein species, and thus, it would be intriguing to know which BH3-only protein participates in determining the fate of nerve-injured neurons. In injured motor neurons of the adult mouse, Noxa seemed to comprise a large proportion of BH3-only protein family members and to be enough to change the balance. Considering that p53 promotes cell death mainly through transcriptional activation, it can be concluded that Noxa is a crucial mediator of p53-dependent death. In con-

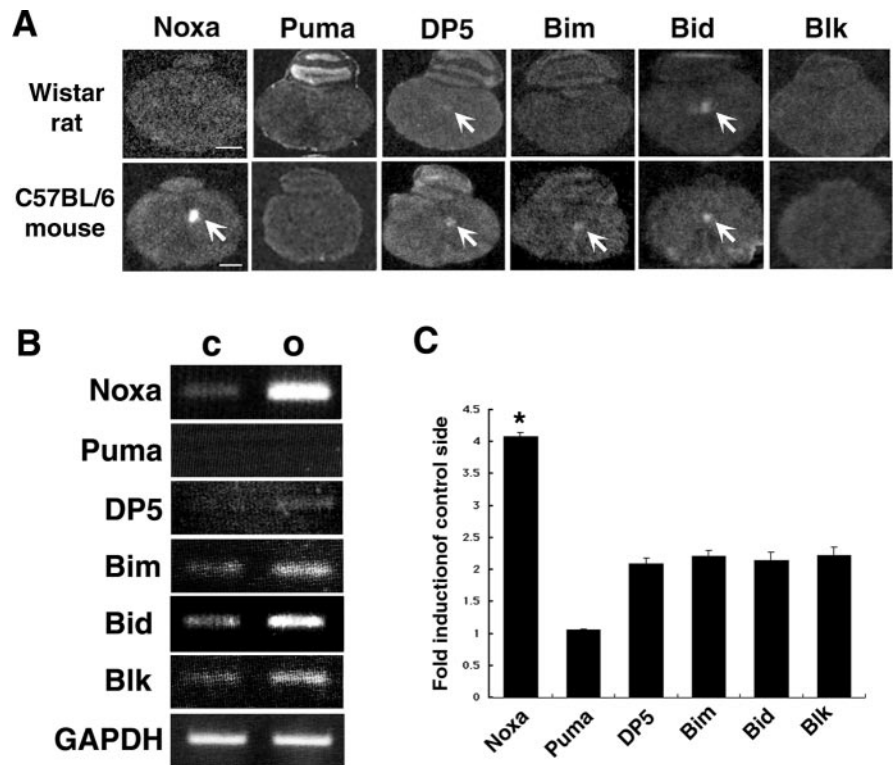


Figure 4. Expression of BH3-only proteins after axotomy. *A*, Expression of BH3-only protein family members 7 d after axotomy in rats and mice was examined by *in situ* hybridization (right is operated side; arrows). Scale bars: top, 1.2 mm; bottom, 0.7 mm. *B*, RT-PCR analysis of BH3-only protein members in control (c) and operated (o) hypoglossal nuclei of mice 7 d after nerve injury. Noxa appeared to comprise a large proportion of the BH3-only protein family members in injured motor neurons of mice. *C*, Quantification of results of the RT-PCR shown in *B*. The density of each band was normalized against that of GAPDH. Data represent mean values \pm SD from three independent experiments ($p < 0.001$ compared with the results of other BH3-only proteins; ANOVA).

trast, Puma, which was also identified as a p53-inducible gene, could not be detected in axotomized motor neurons of mice and rats, suggesting that Puma has a proapoptotic function in different types of neuronal death, such as ischemic death (Reimertz et al., 2003). However, additional regulatory mechanisms underlying Noxa expression might be also involved *in vivo*. Several findings suggest that E2F1 and HIF1 α also induce expression of Noxa, independent of p53 (Reimertz et al., 2003; Kim et al., 2004). Indeed, p53-deficient mice do not show absolutely abolished expression of Noxa after hypoglossal axotomy. Alternatively, another p53 family member, p73, which shares the same binding site as p53, might be involved.

In this study, the functional consequence of Noxa in injured motor neurons was clarified using Noxa-deficient mice. Noxa-deficient mice showed significant survival of axotomized motor neurons. However, Noxa knock-out mice showed slightly less protection against nerve injury-induced death than those with p53 deficiency, suggesting the possible existence of additional p53-dependent death pathways. Recent reports support the idea that p53 has cytoplasmic and transcription-independent functions in promoting apoptosis (Chipuk et al., 2003; Mihara et al., 2003). In this case, p53 protein directly interacts with Bcl-XL with higher affinity, and such binding can release BH3-only protein from the Bcl-XL/BH3 protein complex. Thereafter, free BH3-only protein might accelerate the Bax-dependent cell death pathway. Noxa-deficient mice could not show inhibition of such post-transcriptional activity of p53. In addition to p53-dependent BH3-only proteins, p53-independent BH3-only proteins are

likely to be implicated. In fact, significant rescue activity was also observed in another BH3-only protein, DP5-deficient mouse (Imaizumi et al., 2004). It is therefore likely that this death program relied on redundant activity of more than one BH3-only protein. In the absence of Noxa, other BH3-only proteins are transcriptionally regulated by p53, E2F1 and HIF1 α , and post-transcriptionally activated by signaling such as the c-Jun N-terminal kinase cascade. Loss of one BH3-only protein might be insufficient to rescue injured neurons completely (Imaizumi et al., 2004). Nevertheless, it is now conceivable that p53 could be a pivotal molecule as an intracellular death signal generator in nerve-injured motor neurons of the adult mouse. Noxa could function as a main executor of p53-dependent cell death seen in injured motor neurons of the adult mouse.

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