

Instrumental Activation of Bid by Caspase-1 in a Transgenic Mouse Model of ALS

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Transgenic expression of mutant superoxide dismutase-1 (SOD1) produces an animal model of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder. We have previously shown that the mitochondrial-dependent programmed cell death (PCD) pathway, including the redistribution of Bax, the cytosolic release of cytochrome c, and the activation of caspase-9, is recruited during neurodegeneration in spinal cords of transgenic mutant SOD1 mice. Herein, we show that the pro-PCD protein Bid is highly expressed in spinal cords of both wild-type and transgenic mutant SOD1 mice. While full-length Bid is found in the spinal cord of the two groups of mice, its cleaved form is only seen in transgenic mutant SOD1 mice, as early as the beginning of symptoms. In contrast, activated caspase-8, which is known to cleave Bid, is detected only at the end-stage of the disease. We also found that the expression of a dominant negative mutant of caspase-1 attenuates Bid cleavage as well as the mitochondrial release of cytochrome c, and the ensuing activation of caspase-9 and -3 in spinal cords of transgenic mutant SOD1 mice. These findings suggest that Bid cleavage may occur in this model by a pathway other than caspase-8 and shed light onto the molecular correlates of the previously reported beneficial effect of caspase-1 inhibition in transgenic mutant SOD1 mice.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurological disease characterized by a progressive loss of motor

neurons (Rowland, 1995). To date, mechanical ventilation and, to a lesser extent, riluzole, a glutamate inhibitor, are the only approved therapies that prolong the lives of ALS patients (Rowland, 1995). Important insights into ALS pathogenesis come from the discovery that missense mutations in superoxide dismutase-1 (SOD1) are linked to familial ALS (Deng *et al.*, 1993; Rosen *et al.*, 1993) and that overexpression of different SOD1 mutants in mice replicate the clinical and pathological hallmarks of ALS (Julien, 2001). Mutant SOD1 (mSOD1) cytotoxicity is not triggered by a loss of enzymatic activity or by a dominant negative mechanism, but by a gain of function of unknown nature (Julien, 2001). Mounting evidence indicates that mSOD1-induced demise of spinal cord motor neurons involves, at least in part, programmed cell death (PCD) (Julien, 2001). Indeed, several studies, including our own, show that the mSOD1-mediated death signal upsets the balance between cell death antagonists (e.g., Bcl-2, Bcl-x_L) and cell death agonists (e.g., Bax, Bak) of the Bcl-2 family (Martin, 1999; Vukosavic *et al.*, 1999). In response to these changes, mitochondrial cytochrome c is released and caspase-9 is activated in spinal cords of transgenic mice expressing mSOD1 G93A (SOD1^{G93A}) (Guégan *et al.*, 2001); these are two key molecular events of the mitochondrial-dependent PCD pathway (Gottlieb, 2000), which occur in many pathological situations. The recruitment of this mitochondrial-dependent PCD pathway in transgenic mSOD1 mice is associated with the subsequent activation of effector caspase-3 and caspase-7 (Guégan *et al.*, 2001; Li *et al.*, 2000; Vukosavic *et al.*, 2000), which ultimately may participate in the actual demise of spinal cord motor neurons. The rele-

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vance of this proposed scenario in the pathogenesis of ALS is supported by the demonstrations that Bcl-2 overexpression and caspase inhibition, by targeting key steps of PCD, prolong survival in transgenic mSOD1 mice and mitigate neurodegeneration in these animals (Kostic *et al.*, 1997; Li *et al.*, 2000).

In keeping with the above outlined cascade of events, it must be added that Bid, a BH3-domain-only Bcl-2 family member, has emerged as indispensable in the release of mitochondrial cytochrome c and in the ensuing activation of downstream caspases and cell death in many pathological settings (Eskes *et al.*, 2000; Korsmeyer *et al.*, 2000; Wei *et al.*, 2001; Zhao *et al.*, 2001). Herein, we show that the pivotal pro-PCD protein Bid is highly expressed and cleaved in spinal cords of affected transgenic SOD1^{G93A} mice contemporaneously to the translocation of cytochrome c and the activation of caspase-9. We also present data suggesting that, in contrast to other model systems, caspase-1 and not caspase-8 may be the primary activator of Bid in transgenic SOD1^{G93A} mice. These findings shed light onto the molecular correlates of the previously reported beneficial effect of caspase-1 inhibition in transgenic SOD1^{G93A} mice (Friedlander *et al.*, 1997a; Li *et al.*, 2000) and advocate for an instrumental role of caspase-1-mediated Bid activation in ALS neurodegenerative process.

RESULTS

Bid Expression and Cleavage in Transgenic mSOD1 Mice

To determine the role of Bid in mSOD1-mediated neurodegeneration, we first assessed *bid* mRNA and Bid protein expression in spinal cords of transgenic SOD1^{G93A} mice. Compared to nontransgenic littermates, transgenic SOD1^{G93A} mice exhibited similar spinal cord *bid* mRNA content prior to any symptoms, but the content steadily rose thereafter, reaching a maximum in severely paralyzed mice (i.e., ~5-month-old end-stage mice) (Figs. 1A and 1B). Contrasting with *bid* mRNA alterations, its translational product, i.e., full-length Bid protein, detected by Western blot as a single band of ~25 kDa did not show any obvious difference between spinal cords of nontransgenic and transgenic SOD1^{G93A} mice at the different disease stages studied (Fig. 1C). However, truncated Bid (t-Bid), which resolves with an apparent molecular mass of ~16 kDa and represents the most biologically active form of Bid, was unequivocally observed in spinal cords of early symptomatic (i.e., by ~3 months of age) and end-stage transgenic

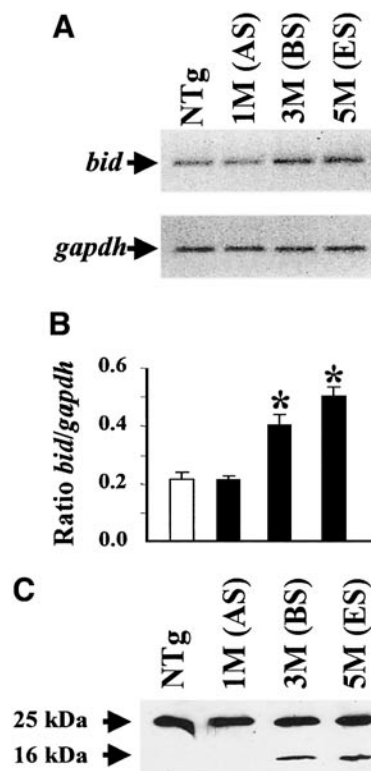


FIG. 1. Expression and cleavage of Bid in transgenic mSOD1 mice. (A and B) *bid* expression was analyzed by RT-PCR and compared with *gapdh* as internal control in whole spinal cords obtained from transgenic mutant SOD1 (mSOD1) mice (black bars) at asymptomatic stage (AS, ~1-month (M)-old), at early symptomatic stage (BS, beginning of symptoms, ~3-month-old), at end-stage (ES, ~5-month-old) and from their age-matched littermates (NTg, nontransgenic mice, white bars). Top panel (A) is representative of 4 to 6 animals per group. (B) The results were obtained from three independent PCR reactions, performed with two different RT. Values correspond to mean \pm SEM. * $P < 0.01$ using Student's *t* test versus age-matched nontransgenic mice. (C) The expression of Bid protein (full-length at 25 kDa) and of its cleavage product (at 16 kDa) was analyzed by Western blot using protein extracts of whole spinal cords obtained from transgenic mSOD1 mice at different ages and their age-matched nontransgenic littermates. Each lane corresponds to a different animal and is representative of four to six animals per group.

SOD1^{G93A} mice (Fig. 1C), but not in asymptomatic transgenic SOD1^{G93A} or in age-matched nontransgenic mice.

Spinal Cord Location of Bid in Transgenic mSOD1 Mice and Wild-Type Littermates

To provide more detailed information regarding the cellular localization of Bid, we examined spinal cords of both end-stage transgenic mSOD1 and age-matched wild-type mice by immunohistochemistry using an an-

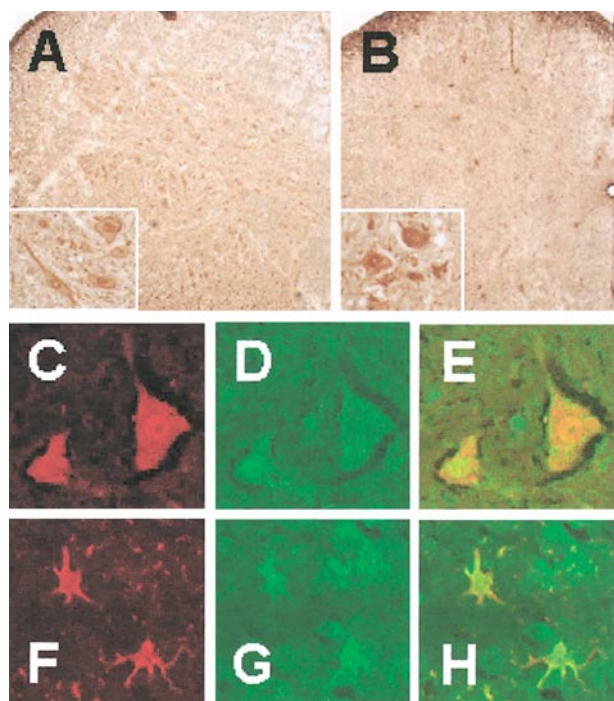


FIG. 2. Bid protein immunostaining was performed on spinal cord sections obtained from age-matched nontransgenic mice (A) and end-stage transgenic mSOD1 mice (B–H). In nontransgenic controls, Bid was expressed in the spinal cord essentially in neurons including large anterior horn neurons (A, see inset). In end-stage transgenic, Bid immunoreactivity is found in neurons and in smaller cells (B, see inset). This interpretation is confirmed by double immunofluorescence showing colocalization of the neuronal marker NNeu (C) and Bid (D) in the same neuron (E) and of the glial marker GFAP (F) and Bid (G) in the same reactive astrocyte (H).

tiserum directed against both full-length and cleaved Bid. In nontransgenic mice, numerous neurons immunoreactive for Bid were observed throughout the gray matter of the spinal cord (Fig. 2A). In end-stage transgenic mSOD1 mice, the greater the loss of motor neurons, the smaller the number of Bid immunoreactive neurons (Fig. 2B), but many remaining spinal cord neurons in the anterior horn showed a more robust immunoreactivity (Fig. 2B, inset). In the transgenic mice, Bid immunostaining was also seen in smaller cells with a glial morphology (Fig. 2B). Although the number of neurons in the anterior horn was decreased and that of glial cells was increased, we found, using double immunostaining procedures, that the majority of the Bid-positive cells were neurons (Figs. 2C–2E), while some were reactive astrocytes (Figs. 2F–2H) and none were activated microglia (not shown).

Activation of Caspase-8 in Transgenic mSOD1 Mice

Because caspase-8 is the established main mediator of Bid cleavage (Li *et al.*, 1998), we assessed the potential participation of this specific caspase in the mSOD1-induced neurodegenerative process and its temporal relationship with Bid activation in transgenic SOD1^{G93A} mice. Pro-caspase-8, the full-length inactive peptide, was detected in all nontransgenic and transgenic SOD1^{G93A} spinal cord samples (Fig. 3A). Still, spinal cord levels of pro-caspase-8 were markedly lower in end-stage transgenic SOD1^{G93A} mice than in any other group studied (Fig. 3A). Conversely, the activation of caspase-8, evidenced by the presence of the 43- and 26-kDa caspase-8 fragments, was detected in end-stage transgenic SOD1^{G93A} mice only (Fig. 3A) as was the rise in spinal cord caspase-8-related enzymatic activity (Fig. 3B). These results provide compelling evidence that, while caspase-8 is indeed activated in spinal cords of transgenic SOD1^{G93A} mice over the course of the disease, it occurs essentially near end-stage.

Effect of Caspase-1 Inhibition on Bid Cleavage and Bax Translocation

The caspase-8 activation is in striking contrast to the much earlier detection of t-Bid and thus raises the possibility that Bid cleavage results from an alternative process in transgenic SOD1^{G93A} mice since Bid appears to be cleaved, at least *in vitro*, by several other caspases including caspase-1 (see Fig. 3A in Li *et al.* (1998)). This could be quite relevant to the present study, since neuronal activated caspase-1 is detected very early during the course of the disease in transgenic SOD1^{G93A} mice and constitutes a chronic initiator of death in this model (Li *et al.*, 2000; Pasinelli *et al.*, 1998, 2000; Vukosavic *et al.*, 2000). To test whether caspase-1 is operative in the cleavage of Bid in this mouse model of ALS, transgenic SOD1^{G93A} mice were crossed with transgenic mice expressing the dominant negative mutant M17Z of caspase-1 (Friedlander *et al.*, 1997b). To avoid potential confounding actions of caspase-8, the effect of caspase-1 inhibition on Bid cleavage was analyzed at the early symptomatic stage, that is, prior to any evidence of caspase-8 activation in these animals. Transgenic expression of mutant M17Z in mice is an effective inhibitor of caspase-1 activation *in vivo* as demonstrated in a variety of neurological disease models (Friedlander, 2000). Consistent with the above-presented results, t-Bid was detected in early symptomatic transgenic SOD1^{G93A}/- mice (Figs. 1C and 4A). In contrast, t-Bid

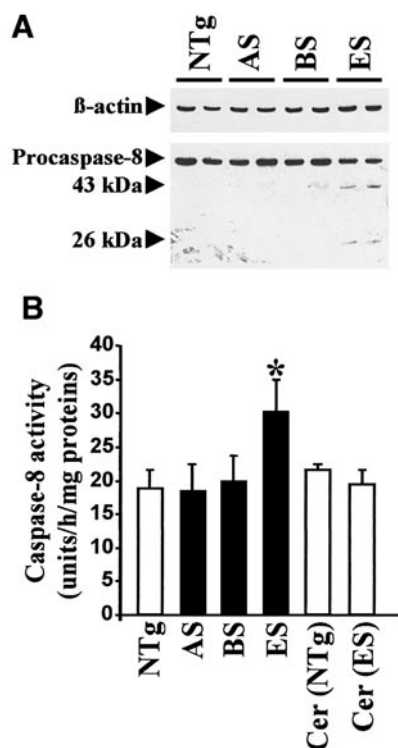


FIG. 3. Activation of caspase-8 in spinal cords of transgenic mSOD1 mice. (A) Using an antibody recognizing both forms of caspase-8, the expression of pro-caspase-8 (55 kDa) and its cleavage fragments (43 and 26 kDa) was analyzed by Western blot. Protein extracts were obtained from entire spinal cords of transgenic mSOD1 mice at asymptomatic stage (AS, ~1-month (M)-old), at the beginning of symptoms (BS, ~3-month-old), and at end-stage (ES, ~5-month-old) and of their age-matched nontransgenic littermates. The top panel corresponds to β -actin. Each lane corresponds to a different animal and is representative of 4 to 6 animals per group. (B) Caspase-8 activity, expressed in micromoles of AFC released per hour and mg of proteins, was evaluated *in vitro* using ac-IETD-AFC substrate in transgenic mSOD1 mice at different ages (black columns), in their age-matched nontransgenic littermates (NTg, white columns) and in cerebellum (Cer, gray columns) from NTg mice and from end-stage transgenic mSOD1 mice. Results are expressed as mean \pm SEM of three independent experiments with five animals in each group. * $P < 0.05$ using Student's *t* test versus age-matched nontransgenic mice.

was only barely seen in age-matched transgenic SOD1^{G93A}/M17Z mice (Fig. 4A), which indicates that inhibition of caspase-1 by mutant M17Z attenuates the cleavage of Bid.

t-Bid acts in concert with mitochondrially located Bax within the mitochondrial-dependent PCD pathway (Crompton 2000; Desagher et al., 1999; Eskes et al., 2000; Korsmeyer et al., 2000; Wei et al., 2001; Zhao et al., 2001). We thus wondered whether inhibition of caspase-1 could also hinder Bax redistribution from the cytosol to

the mitochondria. As we previously demonstrated (Guégan et al., 2001), cytosolic Bax content decreased whereas mitochondrial Bax content increased in spinal cords of transgenic SOD1^{G93A}/- mice over the course of the disease. Despite the chronic blockade of caspase-1, transgenic SOD1^{G93A}/M17Z mice showed the same time course and extent of spinal cord Bax translocation as transgenic SOD1^{G93A}/- mice (Figs. 4B and 4C). The

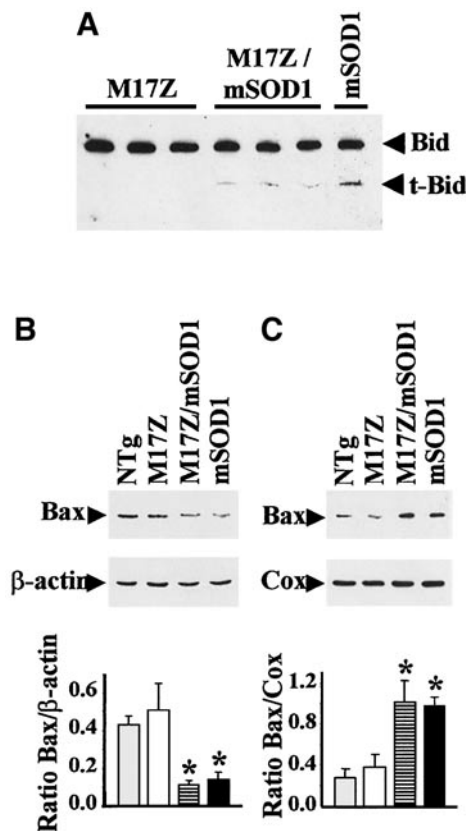


FIG. 4. Effect of caspase-1 inhibition on Bid cleavage and Bax translocation. (A) The expression of Bid protein and its cleavage product was analyzed by Western blot using protein extracts of whole spinal cords obtained from early symptomatic transgenic mutant SOD1 (mSOD1) mice, double transgenic M17Z/mSOD1 mice, and their age-matched littermates expressing the dominant negative caspase-1 mutant (M17Z). (B and C) Bax levels were analyzed by Western blot in cytosolic (B) and mitochondrial (C) fractions of spinal cords from nontransgenic mice (NTg, gray bars), transgenic M17Z mice (white bars), double transgenic M17Z/mSOD1 mice (hatched bars), and transgenic mSOD1 mice (black bars). Proteins of both mitochondrial and cytosolic fractions were extracted from fresh spinal cords as previously described (Guégan et al., 2001). The mean values (\pm SEM) of optical density (ratio Bax/ β -actin or Bax/Cox for cytosolic and mitochondrial fractions, respectively) are represented. * $P < 0.05$ using Student's *t* test versus nontransgenic mice or transgenic M17Z mice.

present data indicate that overexpression of mutant M17Z, while inhibiting Bid cleavage, does not interfere with Bax translocation in this mouse model of ALS.

Prevention of Cytochrome c Release by Caspase-1 Inhibition

Given the differential effects of caspase-1 inhibition on Bid cleavage and Bax translocation, we then examined whether, as in other cell systems (Wei *et al.*, 2001; Yin *et al.*, 1999), the reduction of Bid processing modulates the recruitment of the mitochondrial-dependent PCD pathway in transgenic SOD1^{G93A} mice, in particular the subcellular redistribution of cytochrome c. As previously reported, during the course of the disease, spinal cord mitochondrial cytochrome c translocates to the cytosol in symptomatic transgenic SOD1^{G93A} mice and the cytosolic cytochrome c contents reach a peak at early symptomatic stage (Fig. 5) (Guégan *et al.*, 2001).

Cytochrome c levels in the cytosolic (Figs. 5A and 5B) and mitochondrial (Figs. 5C and 5D) fractions were significantly respectively lower and higher in age-matched transgenic SOD1^{G93A}/M17Z mice compared to early symptomatic transgenic SOD1^{G93A} mice (Fig. 5) (Guégan *et al.*, 2001). Although no significant difference was detected in cytosolic and mitochondrial cytochrome c contents between 3-month-old transgenic M17Z mice and age-matched transgenic SOD1^{G93A}/M17Z, a slight mitochondrial translocation of cytochrome c to the cytosol was observed in transgenic SOD1^{G93A}/M17Z mice (Fig. 5).

Caspase-1 Inhibition Delays Caspase-9 and -3 Activation in Transgenic mSOD1 Mice

In light of the results presented above, we next assessed the effect of caspase-1 inhibition on caspase-9 and -3 activation in spinal cord of transgenic SOD1^{G93A} mice since caspase-9 is a key mediator of the mitochondrial-dependent PCD by which executioner caspase-3 is activated. As before (Guégan *et al.*, 2001; Vukosavic *et al.*, 2000), we found that caspase-9 and -3 are activated in spinal cords of end-stage transgenic SOD1^{G93A} mice (Fig. 6). Conversely, no caspase-9 and -3 activation was observed in age-matched transgenic SOD1^{G93A}/M17Z mice (Fig. 6). Because transgenic SOD1^{G93A}/M17Z mice live longer than SOD1^{G93A} mice (Friedlander *et al.*, 1997a), it is important to mention that there was a frank activation of caspase-9 and caspase-3 in end-stage transgenic SOD1^{G93A}/M17Z mice (Fig. 6). Accordingly, M17Z-mediated caspase-1 inhibition appears not to block but rather to delay the molecular cascade that

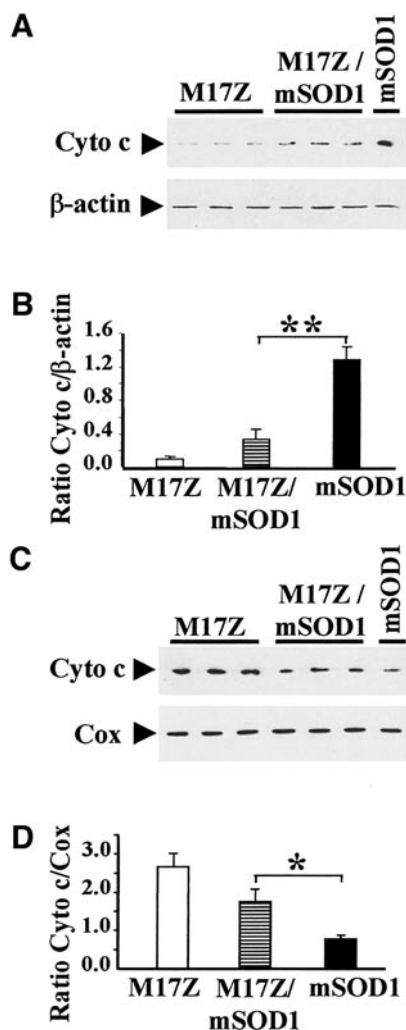


FIG. 5. Effect of caspase-1 inhibition on cytochrome c release. The cytochrome c level was analyzed by Western blot in cytosolic (A and B) and mitochondrial (C and D) fractions of spinal cords obtained from early symptomatic transgenic mSOD1 mice (black bars), double transgenic M17Z/mSOD1 transgenic mice (hatched bars), and their age-matched littermates expressing the dominant negative caspase-1 mutant (M17Z, white bars). Each lane corresponds to 3 different representative animals. The mean values (\pm SEM) of optical density (ratio cytochrome c/ β -actin and cytochrome c/Cox for cytosolic and mitochondrial fractions, respectively) are represented. * $P < 0.05$; ** $P < 0.01$ using Student's *t* test.

presumably underlies the neurodegenerative process in transgenic SOD1^{G93A} mice.

DISCUSSION

In the present study, we show that Bid is highly expressed in spinal cord neurons of wild-type mice and

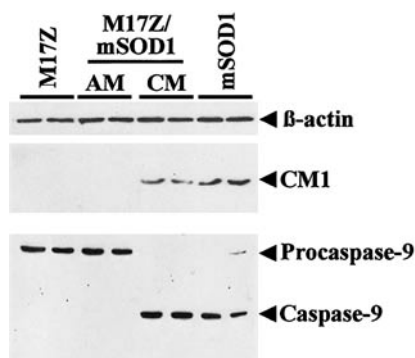


FIG. 6. Effect of caspase-1 inhibition on caspase-9 and -3 activation. Western blot analyses reveal that transgenic M17Z/mSOD1 mice age-matched (AM) with end-stage transgenic mSOD1 mice exhibit no active caspase-9 and -3, this latter being detected using the CM1 antibody. However, end-stage (clinical-matched, CM), double transgenic M17Z/mSOD1 mice exhibit activation of caspase-3 and -9 comparable with those detected in end-stage transgenic mSOD1 mice.

in neurons and reactive astrocytes of affected transgenic SOD1^{G93A} mice. By Western blot analysis, we found that both groups of mice had high spinal cord contents of full-length Bid whereas evidence of cleaved Bid was only seen in spinal cords of transgenic SOD1^{G93A} mice. We also found that Bid cleavage occurred in a time-dependent manner which paralleled the previously reported time course of Bax translocation, mitochondrial cytochrome c release, and caspase-9 activation in transgenic SOD1^{G93A} mice (Guégan *et al.*, 2001). To our knowledge, this is the first demonstration of the cleavage of Bid protein during a neurodegenerative disease, which, in light of the growing recognized importance of this molecule in PCD, may represent a novel finding with far reaching implications. Consistent with this view, herein, we also demonstrate that the attenuation of Bid cleavage is associated with the prevention of the recruitment of key mitochondrial-dependent PCD factors. The present data thus suggest a pivotal role or both Bid and mitochondrial-dependent PCD mechanism in the overall molecular cascade of events involved in the degenerative process in this mouse model of ALS. Along this line, it is interesting to note that, like activated caspase-3 (Pasinelli *et al.*, 2000), Bid is found not only in spinal cord neurons, but also in reactive astrocytes in affected transgenic SOD1^{G93A} mice. Given the dependence of motor neurons on astrocytes to prevent excitotoxicity by rapid uptake of synaptic glutamate by the EAAT2 glutamate transporter (Cleveland and Rothstein, 2001), it is possible that astrocytes are themselves direct targets for SOD1-mediated toxicity

and that the resulting injury of astrocytes promotes disease progression.

Bax translocation is regarded as necessary for the mitochondrial release of cytochrome c (Crompton, 2000; Desagher *et al.*, 1999). However, in this study we found that attenuation of Bid cleavage, in spite of conspicuous Bax translocation, appeared sufficient to impede mitochondrial release of cytochrome c and activation of caspase-9 and -3 in spinal cords of transgenic SOD1^{G93A}/M17Z mice. This *in vivo* finding is consistent with the demonstration that ablation of Bid protects the liver against mitochondrial-dependent PCD regardless of the presence of Bax (Yin *et al.*, 1999). So far, it is still uncertain how Bax and Bid initiate the release of cytochrome c from the mitochondria, but several lines of evidence indicate that in certain cell systems Bax depends on Bid for its subsequent insertion into the mitochondria (Eskes *et al.*, 2000; Korsmeyer *et al.*, 2000; Wei *et al.*, 2001; Zhao *et al.*, 2001). For instance, it has been shown that the association of Bid with Bax on the surface of the outer mitochondrial membrane triggers Bax conformational change which, in turn, facilitates Bax mitochondrial insertion and enhances its potency in releasing cytochrome c (Crompton, 2000; Desagher *et al.*, 1999).

In the present study we found that the apical caspase-8 is activated in spinal cords of transgenic SOD1^{G93A} mice, but only detected quite late in the course of the disease. This suggests that, while activated caspase-8 may still participate in the mutant SOD1-induced cell death process at this advanced stage of the disease, it is unlikely that it plays a major role in the apoptotic machinery at earlier stages. Activation of caspase-8 has been reported to occur primarily by the stimulation of the tumor necrosis factor (TNF) and Fas pathways (Juo *et al.*, 1998) which are believed to (Varfolomeev *et al.*, 1998) play an important role in initiating PCD in neurodegenerative paradigms such as ischemic shock (Martin-Villalba *et al.*, 1999) or spinal cord injury (Lee *et al.*, 2000). Therefore, should caspase-8 activation in transgenic SOD1^{G93A} mice be shown to be a faithful marker of TNF/Fas pathway involvement, then our data would argue that this pathway is not instrumental in the ignition of the apoptotic cascade in this mouse model of ALS. Alternatively, caspase-8 activation in transgenic SOD1^{G93A} mice may result from a feed-forward loop involving the mitochondrial/caspase-9 cell death pathway (Hakem *et al.*, 1998; Slee *et al.*, 1999) which may explain why caspase-8 activation appears as a late event in this model.

It is also worth noting that the late activation of caspase-8 in transgenic SOD1^{G93A} mice is in striking

contrast to previous reports including our own showing an earlier activation of caspase-1 in these animals (Pasinelli *et al.*, 1998). In connection with this it must be emphasized that in this model the time course of Bid cleavage is more consistent with that of caspase-1 activation than with that of caspase-8. This observation, together with the finding of an attenuated Bid cleavage in transgenic SOD1^{G93A}/M17Z mice, suggests an actual role for caspase-1 in the cleavage of Bid. Relevant to this is the *in vitro* demonstration by Li *et al.* (1998) in Fig. 3A that wild-type Bid can be completely cleaved not only by caspase-8 but also by caspase-1. Thus, while we cannot rule out that mutant caspase-1 M17Z interferes with other caspase family members, the inhibitory effect of M17Z on caspase-1 *in vivo* (Friedlander, 2000) and the ability of caspase-1 to cleave Bid *in vitro* (Li *et al.*, 1998) are consistent with our interpretation.

The actual position of caspase-1 within the apoptotic machinery remains a debated issue (Yuan and Yankner, 2000). The present study strengthens the apical location of caspase-1 within the PCD cascade, upstream to the central mitochondrial point (Slee *et al.*, 1999) in ALS neurodegenerative process and provides some hints into the molecular basis underlying the survival extension found previously in transgenic SOD1^{G93A}/M17Z mice (Friedlander *et al.*, 1997a). If, as shown here, caspase-1 inhibition seems to have dramatic molecular effects on PCD, its clinical translation, however, is limited to a prolonged survival, but does not halt the disease in these mice (Friedlander *et al.*, 1997a; Li *et al.*, 2000). It is thus likely that, while the disease progresses, the mSOD1-mediated death signal intensifies, which ultimately either overrides the reversible blockade of PCD imposed by caspase inhibition, or allows recruitment of alternative PCD pathways normally not in play in mSOD1-induced neurodegeneration. On the other hand, it is also possible that neurodegeneration in transgenic mSOD1 is multifactorial in nature, hence requiring the combination of effective strategies targeting PCD and other cellular perturbations (e.g., oxidative stress, energy crisis) to achieve optimal neuroprotection.

EXPERIMENTAL METHODS

Time-Course of Behavioral Abnormalities in Transgenic mSOD1 Mice

As previously described (Vukosavic *et al.*, 2000), the first behavioral abnormality appeared at about 3 months of age in transgenic mSOD1 mice and consisted

of a fine tremor and posturing of a least one limb when the animal was held in the air by the tail. Then, progressively, the animals became paralyzed, could not eat, drink, or move freely, and then were killed at about 5 months of age, corresponding to the end stage.

Animals

Two lines of hemizygous transgenic mice were used: (i) line G1H (Jackson Laboratory; Bar Harbor, ME), that carried a substitution of glycine by alanine at the codon 93 of the human SOD1 protein and expressed about 18 copies of human mutant SOD1 gene (Gurney *et al.*, 1994); and (ii) line M17Z that carried at codon 285 the substitution of cysteine by glycine in the active-site of caspase-1 protein. This transgenic mouse line expressed a dominant negative inhibitor of caspase-1 under the control of NSE promoter (NSE-M17Z) (Friedlander *et al.*, 1997b). Some transgenic mSOD1 mice were crossed with transgenic M17Z mice to produce double transgenic mSOD1/M17Z mice. On postnatal day 14, all mice were genotyped as performed previously (Kostic *et al.*, 1997).

Total RNA Extraction and RT-PCR

Total RNA from both whole spinal cords and cerebella obtained from mice at different stages of the disease ($n = 4-6$ per group) were prepared using RNeasy kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. Reverse transcriptase (RT) steps were performed using Superscript first-Strand Synthesis System (GibcoBRL, Life technologies, Rockville, MD). Normalization of cDNA amounts was performed by PCR on a thermal cycler (GeneAMP PCR system 9700) using primers for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*; forward primer: 5'-GTT TCT TAC TCC TTG GAG GCC AT-3'; reverse primer: 5'-TGA TGA CAT CAA GAA GTG GTG AA-3') for a total of 22 cycles. The *bid* primer sequences were: forward primer 5'-AGC TAG CCG CAC AGT TCA TG-3' and reverse primer 5'-AGC TGT TCT CTG GGA CCT GTC-3'. The PCR reaction mixture contained 1 μ l of cDNA template, 20 pmoles of each primer and 18 μ l of Supermix (GibcoBRL). The PCR condition for *bid* primers was 1 min at 94°C, 1 min at 55°C, 1 min 30s at 72°C for a total of 30 cycles; the conditions for each PCR amplification resulted in an exponential amplification range for quantification of each mRNA. PCR products were then separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The use of the *bid* primers yielded one band (358 bases) and corresponded

to *bid* expression as verified by sequencing. Negative control (PCR without added cDNA) was performed. The relative intensity of ethidium bromide-stained bands was determined using an AGFA densitometer, and data were analyzed using the NIH Image 1.62 software. The results were obtained from three independent PCR reactions, performed with 2 different RT.

Protein Extraction

Total protein was extracted from frozen whole spinal cords ($n = 4-6$ per group) of transgenic mSOD1 mice at asymptomatic stage (AS, ~1-month-old), at the beginning of symptoms (BS, ~3-month-old) and at the end stage (ES, ~5-month-old), and from their age-matched littermates. Tissues were homogenized in 10 vol (w/v) of cold buffer consisting of: 0.25% NP-40, 142.5 mM KCl, 5 mM MgCl₂, 10 mM Hepes, pH 7.2, and protease inhibitor cocktail (Complete mini, Boehringer-Mannheim, Indianapolis, IN). Homogenates were centrifuged (13,000g, 20 min, 4°C) and protein concentrations of the supernatants were determined using BCA protein assay (Pierce, Rockford, IL). Protein extraction of both mitochondrial and cytosolic fractions was performed using fresh spinal cords as previously described (Guégan *et al.*, 2001). Briefly, mitochondrial and cytosolic fractions were extracted from fresh spinal cords ($n = 4-6$ per group) obtained from transgenic mSOD1 mice and their non-transgenic littermates. Tissues were gently homogenized with a glass/glass homogenizer in 10 vol (w/v) of cold buffer consisting of: 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 2 mM EDTA, 20 mM HEPES and protease inhibitor cocktail. Homogenates were centrifuged (500g, 5 min, 25°C) and supernatants were collected and centrifuged (13,000g, 20 min, 4°C). Resulting pellets were designated mitochondrial fractions while supernatants were further centrifuged (100,000g, 60 min, 4°C). Resulting supernatants were designated cytosolic fractions. To verify the relative mitochondrial purification, each fraction was subjected to Western blotting for β -actin as a cytosolic marker using a mouse monoclonal antibody anti- β -actin (clone AC15, Sigma, St Louis, MO) and cytochrome c oxidase (COX) as a mitochondrial marker using a mouse monoclonal antibody anti-COX subunit IV (Molecular Probes, Eugene, OR).

Western Blot Analysis

Proteins (30–80 μ g) were electrophoresed through a 10–15% SDS-polyacrylamide gel and blotted to nitrocellulose membrane. Blots were probed with the follow-

ing primary antibodies: a rabbit polyclonal caspase-8 antibody (SC-7890, Santa-Cruz Biotech Inc., Santa Cruz, CA; 1/200 final dilution), a goat polyclonal anti-Bid antibody (R&D Systems, Minneapolis, MI; 1/500 final dilution), a mouse monoclonal anti-Bax antibody (SC-7480, Santa-Cruz Biotech Inc., 1/250 final dilution for both fractions), a mouse monoclonal cytochrome c antibody (clone 7H8.2C12, Pharmingen, San Diego, CA; respectively 1/1,500 and 1/10,000 final dilutions for cytosolic and mitochondrial fractions), a mouse monoclonal antibody anti- β -actin (clone AC15, Sigma), a mouse monoclonal antibody anti-COX subunit IV (Molecular Probes), a rabbit polyclonal anti-caspase-9 antibody recognizing the pro- and cleaved forms (AAP-109, Stressgen Biotechnologies Corp., Victoria, BC, Canada; 1/1000 final dilution), and a rabbit polyclonal anti-active caspase-3 (CM1) antibody generously provided by Dr. Anu Srinivasan (IDUN Pharm., Inc., San Diego, CA; 1/1000 dilution). Bound primary antibody was detected using a horseradish-conjugated anti-IgG antibody (Amersham, Arlington Heights, IL) and the reaction was visualized by using enhanced chemiluminescent substrate (SuperSignal Ultra, Pierce). The film (Kodak BioMax MS) was scanned on a 1200U Epson, and bands were quantified by using the NIH-Image 1.62 software.

Bid Immunohistochemistry

This was performed following our standard protocol as previously described (Kostic *et al.*, 1997). In brief, after being fixed by perfusion, spinal cords were dissected out from the spine on ice, postfixed by immersion in 4% paraformaldehyde in phosphate buffer (PB; pH 7.1; 4 h, 4°C), cryoprotected in 20% (w/v) sucrose in PB, and frozen by immersion in isopentane cooled on dry ice. Frozen spinal cord samples were cut (40 μ m) in a cryostat and 10–20 serial sections from lumbar (L3) levels were collected in ice-cold PB free floating, and then successively rinsed (3 \times 5 min) in 0.1 M PB (pH 7.4) containing 9 gr/L NaCl (PBS), incubated in 3% normal serum (NS) in PBS (60 min, 25°C), and incubated for 48 h (4°C) in a 1:300 rabbit polyclonal anti-Bid antibody (Pharmingen) diluted in PBS containing 3% NS; this antibody was raised against a synthetic peptide corresponding to the amino acids 129–146 of Bid and thus recognizes both the full-length and the cleaved forms of Bid. After 3 \times 5-min rinses in PBS, sections were successively incubated (1 h, 25°C) in biotinylated-conjugated polyclonal anti-IgG antibody (1:200; Vector, Burlingame, CA), rinsed (3 \times 5 min) in PBS, incubated in horseradish-conjugated avidin/biotin complex (Vec-

tor), rinsed (3×5 min) in PBS, and incubated in diaminobenzidine/ H_2O_2 . Some sections were also coincubated with anti-Bid antibody and specific nuclear protein (NeuN; 1:5000; monoclonal; Chemicon, Temecula, CA), GFAP (1:500; monoclonal; Boehringer-Mannheim, Indianapolis, IN), or MAC-1 (1:250; monoclonal; Serotec, Raleigh, NC). Sections were then incubated with a Texas red-conjugated anti-mouse antibody and a biotinylated-conjugated anti-rabbit antibody with fluorescein-conjugated avidin (Vector) and examined by confocal microscopy.

Caspase-8 Activity Assay

Proteins were extracted from entire spinal cords and cerebella of nontransgenic mice, and transgenic SOD1^{G93A} mice at asymptomatic, early, and end stages ($n = 5$ for each group). Tissues were then homogenized in cold buffer consisting of: 25 mM Hepes pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA, 0.1% Triton X100, 2 mM dithiothreitol (DTT), 1 mM PMSF, 10 $\mu g/ml$ leupeptine, 2 $\mu g/ml$ aprotinin. Homogenates were centrifuged at 13,000g for 30 min at 4°C and supernatants collected. Proteins (100 μg) were incubated in caspase assay buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM DTT) and enzymatic reaction was started by addition of the fluorogenic substrate (0.2 mM of Ac-IETD-AFC, Biomol Research Laboratories, Plymouth Meeting, PA). After incubation at 37°C for 2 h, the release of AFC that reflects the substrate cleavage was measured with a Perkin-Elmer LS 50B spectrofluorometer (excitation 400 nm, emission 505 nm). Fluorescent arbitrary units were converted into $\mu moles$ of AFC released per hour and per mg protein using a standard curve of free AFC (Sigma, A8401).

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REFERENCES

- Cleveland, D. W., and Rothstein, J. D. (2001). From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat. Rev. Neurosci.* **2**: 806–819.
- Crompton, M. (2000). Bax, Bid and the permeabilization of the mitochondrial outer membrane in apoptosis. *Curr. Opin. Cell Biol.* **12**: 414–419.
- Deng, H.-X., Hentati, A., Tainer, J. A., Iqbal, Z., Cayabyab, A., Hung, W.-Y., Getzoff, E. D., Hu, P., Herzfeldt, B., Roos, R. P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H. E., Ahmed, A., Roses, A. D., Hallelwell, R. A., Pericak-Vance, M. A., and Siddique, T. (1993). Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. *Science* **261**: 1047–1051.
- Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maubdrell, K., Antonsson, B., and Martinou, J.-C. (1999). Bid-induced conformational changes of Bax are responsible for mitochondrial cytochrome c release during apoptosis. *J. Cell Biol.* **144**: 891–901.
- Eskes, R., Desagher, S., Antonsson, B., and Martinou, J.-C. (2000). Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol. Cell Biol.* **20**: 929–935.
- Friedlander, R. M. (2000). Role of caspase 1 in neurologic disease. *Arch. Neurol.* **57**: 1273–1276.
- Friedlander, R. M., Brown, R. H., Gagliardini, V., Wang, J., and Yuan, J. (1997a). Inhibition of ICE slows ALS in mice. *Nature* **388**: 31.
- Friedlander, R. M., Gagliardini, V., Hara, H., Fink, K. B., Li, W., Macdonald, G., Fishman, M. C., Greenberg, A. H., Moskowitz, M. A., and Yuan, J. (1997b). Expression of a dominant negative mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. *J. Exp. Med.* **185**: 933–940.
- Gottlieb, R. A. (2000). Mitochondria: Execution central. *FEBS Lett.* **482**: 6–12.
- Guégan, C., Vila, M., Rosoklija, G., Hays, A. P., and Przedborski, S. (2001). Recruitment of the mitochondrial-dependent apoptotic pathway in amyotrophic lateral sclerosis. *J. Neurosci.* **21**: 6569–6576.
- Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., Caliendo, J., Hentati, A., Kwon, Y. W., Deng, H.-X., Chen, W., Zhai, P., Sufit, R. L., and Siddique, T. (1994). Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science* **264**: 1772–1775.
- Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., De la Pompa, J. L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S. A., Lowe, S. W., Penninger, J. M., and Mak, T. W. (1998). Differential requirement for caspase-9 in apoptotic pathways *in vivo*. *Cell* **94**: 339–352.
- Julien, J. P. (2001). Amyotrophic lateral sclerosis: unfolding the toxicity of the misfolded. *Cell* **104**: 581–591.
- Juo, P., Kuo, C. J., Yuan, J. Y., and Blenis, J. (1998). Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. *Curr. Biol.* **8**: 1001–1008.
- Korsmeyer, S. J., Wei, M. C., Saito, M., Weiler, S., Oh, K. J., and Schlesinger, P. H. (2000). Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death. Differ.* **7**: 1166–1173.
- Kostic, V., Jackson-Lewis, V., De Bilbao, F., Dubois-Dauphin, M., and Przedborski, S. (1997). Bcl-2: Prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science* **277**: 559–562.
- Lee, Y. B., Yune, T. Y., Baik, S. Y., Shin, Y. H., Du, S., Rhim, H., Lee,

- E. B., Kim, Y. C., Shin, M. L., Markelonis, G. J., and Oh, T. H. (2000). Role of tumor necrosis factor-alpha in neuronal and glial apoptosis after spinal cord injury. *Exp. Neurol.* **166**: 190–195.
- Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**: 491–501.
- Li, M., Ona, V. O., Guegan, C., Chen, M., Jackson-Lewis, V., Andrews, L. J., Olszewski, A. J., Stieg, P. E., Lee, J. P., Przedborski, S., and Friedlander, R. M. (2000). Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. *Science* **288**: 335–339.
- Martin-Villalba, A., Herr, I., Jeremias, I., Hahne, M., Brandt, R., Vogel, J., Schenkel, J., Herdegen, T., and Debatin, K. M. (1999). CD95 ligand (Fas-L/APO-IL) and tumor necrosis factor-related apoptosis-inducing ligand mediate ischemia-induced apoptosis in neurons. *J. Neurosci.* **19**: 3809–3817.
- Martin, L. J. (1999). Neuronal death in amyotrophic lateral sclerosis is apoptosis: Possible contribution of a programmed cell death mechanism. *J. Neuropathol. Exp. Neurol.* **58**: 459–471.
- Pasinelli, P., Borchelt, D. R., Houseweart, M. K., Cleveland, D. W., and Brown, R. H. J. (1998). Caspase-1 is activated in neural cells and tissue with amyotrophic lateral sclerosis-associated mutations in copper-zinc superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **95**: 15763–15768.
- Pasinelli, P., Houseweart, M. K., Brown, R. H., Jr., and Cleveland, D. W. (2000). Caspase-1 and -3 are sequentially activated in motor neuron death in Cu,Zn superoxide dismutase-mediated familial amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. USA* **97**: 13901–13906.
- Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H.-X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herzfeldt, B., Van den Bergh, R., Hung, W.-Y., Bird, T., Deng, G., and Mulder, D. W. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**: 59–62.
- Rowland, L. P. (1995). Hereditary and acquired motor neuron diseases. In *Merritt's Textbook of Neurology* (L. P. Rowland, Ed.), pp. 742–749. Williams & Wilkins, Philadelphia.
- Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., and Martin, S. J. (1999). Ordering the cytochrome c-initiated caspase cascade: Hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J. Cell Biol.* **144**: 281–292.
- Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannikulchai, N., Beckmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K. B., Goncharov, T., Holtmann, H., Lonai, P., and Wallach, D. (1998). Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* **9**: 267–276.
- Vukosavic, S., Dubois-Dauphin, M., Romero, N., and Przedborski, S. (1999). Bax and Bcl-2 interaction in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J. Neurochem.* **73**: 2460–2468.
- Vukosavic, S., Stefanis, L., Jackson-Lewis, V., Guégan, C., Romero, N., Chen, C., Dubois-Dauphin, M., and Przedborski, S. (2000). Delaying caspase activation by Bcl-2: a clue to disease retardation in a transgenic mouse model of amyotrophic lateral sclerosis. *J. Neurosci.* **20**: 9119–9125.
- Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. *Science* **292**: 727–730.
- Yin, X. M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* **400**: 886–891.
- Yuan, J., and Yankner, B. A. (2000). Apoptosis in the nervous system. *Nature* **407**: 802–809.
- Zhao, Y., Li, S., Childs, E. E., Kuharsky, D. K., and Yin, X. M. (2001). Activation of pro-death bcl-2 family proteins and mitochondrial apoptosis pathway in tumor necrosis factor-alpha-induced liver injury. *J. Biol. Chem.* **276**: 27432–27440.

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