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# The BCL-2 family protein Bid is critical for pro-inflammatory signaling in astrocytes

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the loss of motoneurons in the spinal cord, brainstem and motor cortex. Mutations in the superoxide dismutase 1 (*SOD1*) gene represent a frequent genetic determinant and recapitulate a disease phenotype similar to ALS when expressed in mice. Previous studies using *SOD1*$^{G93A}$ transgenic mice have suggested a paracrine mechanism of neuronal loss, in which cytokines and other toxic factors released from astroglia or microglia trigger motoneuron degeneration. Several pro-inflammatory cytokines activate death receptors and may downstream from this activate the Bcl-2 family protein, Bid. We here sought to investigate the role of Bid in astrocyte activation and non-cell autonomous motoneuron degeneration. We found that spinal cord Bid protein levels increased significantly during disease progression in *SOD1*$^{G93A}$ mice. Subsequent experiments *in vitro* indicated that Bid was expressed at relatively low levels in motoneurons, but was enriched in astrocytes and microglia. Bid was strongly induced in astrocytes in response to pro-inflammatory cytokines or exposure to lipopolysaccharide. Experiments in *bid*-deficient astrocytes or astrocytes treated with a small molecule Bid inhibitor demonstrated that Bid was required for the efficient activation of transcription factor nuclear factor-κB in response to these pro-inflammatory stimuli. Finally, we found that conditioned medium from wild-type astrocytes, but not from *bid*-deficient astrocytes, was toxic when applied to primary motoneuron cultures. Collectively, our data demonstrate a new role for the Bcl-2 family protein Bid as a mediator of astrocyte activation during neuroinflammation, and suggest that Bid activation may contribute to non-cell autonomous motoneuron degeneration in ALS.



## Introduction

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset, progressive motoneuron disease characterized by degeneration of motoneurons in the motor cortex, brainstem and spinal cord ventral horns. Mutations in the copper/zinc superoxide dismutase gene (*SOD1*) account for approximately 20–25% of familial ALS patients (Deng et al., 1993; Rosen, 1993). Over-expression of the human familial ALS-linked *SOD1*$^{G93A}$ mutation in transgenic mouse models (*mtSOD1*) confers a phenotype similar to that of ALS patients with motor function deficits and a reduced lifespan (Gurney et al., 1994). Although the precise mechanisms of motoneuron degeneration in ALS remain largely unidentified, proteotoxicity, endoplasmic reticulum stress, glutamate excitotoxicity, oxidative stress, and activation of apoptosis have been shown to facilitate motoneuron death in *mtSOD1* mice and other ALS disease models (Bruijn et al., 2004; Pasinelli and Brown, 2006).

Apoptosis is a genetically controlled cell death process that is activated by multiple stress stimuli. Most forms of stress-induced apoptosis engage the so-called mitochondrial apoptosis pathway. This pathway is controlled by the Bcl-2 protein family (Youle and Strasser, 2008). The Bcl-2 homology domain 3 (BH3)-only proteins are pro-apoptotic members of this family. BH3-only proteins are transcriptionally and post-translationally activated in neurons in response to stress (Bruijn et al., 2004; Ward et al., 2004), and induce apoptosis due to their ability to bind and neutralize anti-apoptotic Bcl-2 family proteins (Youle and Strasser, 2008). Activation of BH3-only proteins leads to the mitochondrial membrane insertion and oligomerization of Bax and Bak (Lovell et al., 2008; Tait and Green, 2010). The channels formed by these oligomers constitute release

*Abbreviations:* ALS, Amyotrophic Lateral Sclerosis; APAF1, Apoptotic protease activating factor 1; BH3-only protein, Bcl-2 homology domain 3-only protein; *bid*, BH3 interacting domain death agonist; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; IFNγ, interferon-γ; IKK, inhibitor of kappaB kinase; IL-1β, interleukin-1β; mtSOD1, mutant superoxide dismutase 1; NEMO, NF-kappaB essential modifier; NF-κB, nuclear factor-kappaB; NOD1, nucleotide-binding oligomerization domain containing 1; SOD1, superoxide dismutase 1; PND, post-natal day; SMI-32, Sternberger monoclonal-incorporated antibody 32; tg, transgenic; wt, wild-type.

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channels in the mitochondrial outer membrane, enabling the release of pro-apoptotic factors that trigger caspase-dependent and caspase-independent apoptosis. Bax was shown to accumulate in mitochondria in animal models of ALS (Guegan et al., 2001), and deletion of the *bax* gene in *mtSOD1* transgenic mice inhibited motoneuron death (Gould et al., 2006). Recently it was shown that conditional, combined deletion of *bax* and *bak* potently delayed disease onset and progression in the $SOD1^{G93A}$ mouse model of ALS (Reyes et al., 2010). BH3-only proteins responsible for the activation of Bax (and potentially Bak) in animal models of ALS were also recently identified. The BH3-only protein Bim was shown to be transcriptionally up-regulated in response to *mtSOD1* overexpression, and deletion of *bim* protected against motoneuron loss in *mtSOD1* mice *in vivo* (Hetz et al., 2007). We showed recently that endoplasmic reticulum stress was able to activate the BH3-only protein Puma in motoneurons, and that deletion of *puma* protected motoneurons against cell death *in vitro* and in *mtSOD1* mice (Kieran et al., 2007).

Interestingly, several studies also suggest a non-cell autonomous mechanism of motoneuron loss in ALS (Ilieva et al., 2009). One possible explanation for a paracrine mechanism of motoneuron death is the release of pro-inflammatory or cell death-inducing cytokines from non-neuronal cells. Interleukin-1β (IL-1β) and interferon-γ (IFNγ) have been implicated in ALS disease progression (Friedlander et al., 1997; Meissner et al., 2010; Wang et al., 2011). Motoneuron apoptosis in ALS was also shown to involve Fas ligand up-regulation and activation of Fas death receptors (Locatelli et al., 2007; Raoul et al., 2002). Release of pro-inflammatory cytokines may lead to death receptor and caspase-8 activation, which is able to directly activate executioner caspases such as caspase-3 (Locatelli et al., 2007; Raoul et al., 2002). However, in most cell types, this direct activation pathway is not sufficient to activate apoptosis, and an amplification loop is required for cell death execution that involves the BH3-only protein Bid, and hence engages the mitochondrial apoptosis pathway (Lovell et al., 2008; Luo et al., 1998). Indeed, increased expression of Bid in both neurons and astrocytes as well as increased levels of Bid cleavage were observed in symptomatic and late stage $SOD1^{G93A}$ mouse spinal cords (Guegan et al., 2002). Interestingly, Bid was recently implicated in the production of pro-inflammatory cytokines in macrophages and microglia (Mayo et al., 2011) and was reported to be involved in the activation of the transcription factor nuclear factor-κB (NF-κB) in response to stimulation of pattern recognition receptors in intestinal epithelial cells, independent of its direct apoptosis-regulating function (Yeretssian et al., 2011). Therefore, in the present study we sought to clarify the role of Bid in the context of neurodegeneration and neuroinflammation relevant to ALS.

## Materials and methods

### Animals

All experiments described in this study were performed under a license from the Department of Health and Children in Ireland (B100/3985) in accordance with the European Communities regulations 2010 (2010/63/EU). All procedures were previously approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland. Transgenic SOD1 mice (*Mus musculus*), IMSR: B6.Cg-Tg ($SOD1^{*G93A}$) 1Gur/J, with the incorporation of the G93A mutant form of human superoxide dismutase (*SOD1*), were purchased from The Jackson Laboratories (JAX, Bar Harbor, Maine, USA). $bid^{-/-}$ mice (*M. musculus*) were generated in the laboratory of Prof. Andreas Strasser, WEHI, Melbourne, Australia (Kaufmann et al., 2007). After weaning on postnatal day (PND) 28, all pups from litters of the same generation and colony were housed in groups of three to five per cage and maintained at 21 ± 1 °C on a 12 h light/dark cycle, (07:00 h on; 19:00 h off) with *ad libitum* access to food and water.

### Reagents and chemicals

Unless otherwise stated, chemicals were purchased from Sigma-Aldrich (Arklow, Ireland) or Merck Chemicals (Nottingham, UK). Cell culture media were purchased from Gibco-Life Technologies (Dun Laoghaire, Ireland). BI-6C9 was from Sigma-Aldrich (Pubchem: ID 24724408).

### Primary motoneuron cultures

Primary motoneuron cultures (mixed cultures enriched for motoneurons) were prepared from wild-type and *bid* gene deficient ($bid^{-/-}$) E12 mouse embryos as described previously (Sebastia et al., 2009). Briefly, the spinal cord ventral horns were dissected and the tissue was removed and incubated for 10 min in 0.025% trypsin in Neurobasal® media. Cells were transferred into a 0.1 mg/ml DNase1 solution, and gently dissociated. Dissociated motoneurons were counted using a hemocytometer and seeded at a density of $10^5$ cells/ml onto poly-D,L-ornithine/laminin-coated cell culture wells and maintained at 37 °C and 5% $CO_2$. Motoneurons were maintained in complete Neurobasal® media supplemented with 2% horse serum, 2% B27, 2 mM GlutaMAX™, GDNF (Promega, Cat#2781; 2 ng/ml), CNTF (R&D Systems, Cat#557-NT-10, 1 ng/ml), 100 U/ml penicillin and 100 μg/ml streptomycin. Medium was changed after 1, 3 and 6 days *in vitro* (DIV). Cells were cultured up to 14 days. Primary motoneuron cultures yielded mixed populations of cells with glial (ca. 40–60%), neuronal (ca. 30–40%) and non-neuronal morphology (5–10%); approximately 30–50% of the neuronal population were motoneurons positive for the motoneuron marker SMI-32 (Suppl. Figs. 3A–A″).

### Isolation and culturing of primary astrocytes

Mixed glial cultures were prepared from the cortices of P2 $bid^{-/-}$ and wild-type mice. In brief, the cortices were dissected and the meninges were removed before incubation in Minimum Essential Medium containing 0.025% trypsin and 0.1 mg/ml DNase I for 15 min at 37 °C. The tissue was triturated mechanically in DMEM to dissociate the cells, passed through a 40 μm nylon cell strainer (BD Falcon, Oxford, UK), and spun at 2000 rpm for 5 min. The pellet was resuspended and plated in T75 flasks in DMEM-F12/L-glutamine containing 1% penicillin/streptomycin and 10% Fetal Bovine Serum (Sigma-Aldrich). Cells were cultured for a minimum of 14 days before being passaged and cultured as astrocytes. For astrocyte-enriched cultures, astrocyte flasks were placed on the orbital shaker at 600 rpm to shake off microglia; the remaining adherent cells were trypsinized and replated for the experiment at $5 \times 10^5$ cells per 6 well. Motoneuron media were used to generate astrocyte conditioned media (ACM) for motoneuron toxicity experiments (Neurobasal media with the abovementioned supplements).

### Isolation of primary microglia

Microglia were isolated from mixed glial cultures (as above). Following two weeks of cell culture, the T75 flasks of confluent mixed glial cultures were shaken in DMEM/F12 at 600 rpm on a plate shaker for 8 h at room temperature. The supernatant was collected and spun down at 2000 rpm for 5 min. The resuspended pellet was plated at a density of $1 \times 10^5$ cells/well of a 24 well plate. The isolated microglia were cultured for 2 days in well plates or Millicell-CM inserts (0.4 μm pore size, Fisher Scientific, Dublin, Ireland) in DMEM-F12/L-glutamine, 10% Fetal Bovine Serum and 1% Pen/Strep (Sigma-Aldrich) before treatment.

### Real-time quantitative PCR (qPCR)

RNA was extracted using Qiazol extraction and/or RLT-buffer lysis and RNeasy processing according to the manufacturer's guidelines (Qiagen, Sussex, UK). cDNA synthesis was performed based on equal amounts of RNA using the Superscript™ II Reverse Transcriptase

(Invitrogen, California, USA). qPCR analysis was performed using the LightCycler (Roche Diagnostics, Basel, Switzerland) and the QuantiTech SYBR Green PCR kit (Qiagen) following the manufacturer's recommendations and standard cycles and melting temperatures. The sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were sense 5′-AAC TTT GGC ATT GTG GAA GG-3′, antisense 5′-ACA CAT TGG GGG TAG GAA CA-3′; for *bid* 5′-TCC CCA GAG ACA TGG AGA AC-3′ and 5′-GTC GTG TGG AAG ACA TCA CG-3′. RNA levels were normalized to *gapdh* mRNA expression and expressed as n-fold expression over control. For multiplex-genotyping of human $SOD1^{G93A}$ transgenic mice, primer sequences as described by the supplier (Jackson Laboratories, stock# 004435) were used. For *bid* genotyping, wild-type and deleted allele-specific primers using a three-primer strategy as described by Kaufmann et al. (2007) were employed.

*Western blotting and immunoprecipitation*

Lumbar spinal cord lysates were assessed for Bid protein levels by Western blotting following dounce homogenization in RIPA-lysis buffer (Sigma-Aldrich). For Western-blot analyses of cell cultures, cells were rinsed with ice-cold PBS and lysed in RIPA lysis buffer containing protease inhibitor mix (Sigma-Aldrich). Protein content was determined using the BCA Micro Protein Assay kit (Pierce) and samples were supplemented with Laemmli sample buffer with 2-mercaptoethanol and denaturated at 95 °C for 5 min, followed by electrophoresis and Western-blot by standard protocols. Blots were incubated with a rabbit polyclonal anti-Bid antibody (1:1000, AR-52, Enzo Life Sciences, Exeter, UK; Cat# ALX-210-007-R050, Antibody Registry (RIID): AB_2259218), a mouse monoclonal anti-GFAP (1:1000, Sigma-Aldrich Cat# G3893, RIID: AB_477010), a goat polyclonal anti-Iba-1 (1:500, Abcam, Cat# ab5076, RIID: AB_2224402), a rat monoclonal anti-CD11b (1:1000, Abcam Cat# ab8878, RIID: AB_306831), a rabbit polyclonal anti-MAP2 (1:1000, Santa Cruz Biotechnology, Cat# sc-20172, RIID: AB_2250101), rabbit anti-phosphorylated IKKα/β (1:500, 16A6, Cell Signaling Technology, Hitchin, UK, Cat# 2697L, RIID: AB_2291699) and anti-IKKα/β (1:500, H470, Santa Cruz Biotechnology, Inc., Cat# sc-7607, RIID: AB_675667), a rabbit polyclonal anti-PARP antibody (1:1000, Cell Signaling Technology Cat# 9542, RIID: AB_2160739), a rabbit polyclonal anti-caspase-3 (1:1000, Cell Signaling Technology Cat# 9662S, RIID: AB_10694681), mouse monoclonal α-tubulin (Sigma-Aldrich, Cat# T6199, RIID: AB_477583), and β-Actin (clone AC-40, Sigma-Aldrich, Cat# A3853, RIID: AB_262137) antibodies (both 1:5000), a rabbit monoclonal phospho-Ser536p65 (clone 93H1, CST, Cat# 3033S, RRID: AB_331284), and a rabbit monoclonal pan-p65 (clone D12E12, Cell Signaling Technology Cat# 8242P, RRID: AB_10859369). A goat polyclonal anti-Bid (AF860, R&D Systems, Cat# AF860, RIID: AB_2065622) or normal goat-IgG (Santa Cruz) was used for immunoprecipitation analyses and captured by protein A/G agarose bead slurry (Santa Cruz Biotechnology) following standard protocols. Here, following Laemmli-buffer lysis and electrophoreses a rabbit polyclonal anti-IKKγ (1:500, Abcam Cat# ab77750, RIID: AB_156633) was used for immunodetection. Membranes were incubated with the species-specific peroxidase-labeled secondary antibodies diluted 1:1000–10,000 (Pierce, Northumberland, UK). Bands were detected using Immobilon Western Chemiluminescent HRP Substrate (Fisher Scientific) and imaged using a Fuji Film LAS-3000 imaging system (Fuji, Sheffield, UK). Western-blot optical densities were determined using standard procedures using ImageJ (NIH; imagej.nih.gov/ij).

*Immunofluorescence and microscopy*

Primary cultured neurons were exposed to propidium iodide (2 μg/ml, Sigma Cat#P4864) or trypan-blue solution (0.4%, Life Technologies Cat#15250061) prior to fixation in 3% paraformaldehyde (PFA, Polyscience, Eppelheim, Germany, Cat#18814-20) diluted in cytoskeletal buffer (CB-buffer; 10 mM PIPES pH 6.8, 300 mM NaCl, 10 mM EGTA, 10 mM glucose, 10 mM $MgCl_2$) for 12 min. Fixed cells were permeabilized by incubation with ice-cold PBS containing 0.1% (w/v) Triton X-100 and blocked with 0.3% (w/v) Triton X-100 and 5% (v/v) horse serum in PBS. They were incubated overnight in primary antibody at 4 °C diluted in PBS/0.3% (w/v) Triton X-100/3% (v/v) horse serum. We used a mouse monoclonal anti-neurofilament heavy polypeptide (SMI32, 1:1000, Covance, Maidenhead, UK, Cat# SMI-32P-100, RIID: AB_10719742), as well as the abovementioned p-IKK and GFAP/S100b-antibodies. Secondary Alexa-Fluor-488 or -568 coupled secondary antibody (1:500, Life Technologies) was incubated for 1 h at room temperature following extensive washes. Cell counts of trypan-blue/PI and SMI-32 labeling were performed manually by two experienced experimenters (blinded). Photomicrographs were taken using a SPOT RT SE 6 Camera (Diagnostic Instruments, Sterling Heights, MI, USA) on an Eclipse TE 300 inverted microscope (Nikon, Kingston upon Thames, UK) with Mercury-arc excitation and appropriate filter settings.

*Reporter assays*

Astrocyte cultures were transfected using Roche X-tremeGENE HP reagent (Roche, Dublin, Ireland) using standard protocols with a mixture of pGL4.32[luc2P/NF-κB-RE/Hygro] vector (NF-κB-RE, firefly reporter vector, Promega, Southampton, UK, Cat#E8491, GenBank: EU581860.1) and a Renilla-luciferase expressing construct under constitutive thymidine-kinase promoter control (phRL-TK-luc, Promega, Cat#E6241) at a ratio of 14:1 for normalization. Cultures were washed extensively and transferred back into their respective conditioned media for cytokine treatments on the subsequent day. Cells were lysed in passive-lysis buffer (PLB, Promega) following the indicated periods of time of cytokine or lipopolysaccharide (LPS) treatment in their respective conditioned media, and assayed by Dual-luciferase® assay (Promega, Cat#E19160) according to the manufacturer's specifications.

*Statistical analysis*

Statistical analyses were performed using PASW statistics 17 software (SPSS, IBM, Dublin, Ireland) or GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Significance was determined using two-tailed Student t-test, or Mann–Whitney U test for non-parametric data. For multiple comparisons, one-/two-way ANOVA followed by Holm–Sidak or Tukey's post-hoc test was used. For non-parametric data, Kruskal–Wallis H test, or Friedman test for matched data was used, and Dunn's *post-hoc* test. Grubbs test was used to test for outliers. All data are represented as mean ± range or mean ± SEM. p values ≤0.05 were considered to be significantly different and marked by an asterisk.

## Results

*Bid protein levels increase in the $SOD1^{G93A}$ mouse during disease progression*

Bid is constitutively expressed in many central neurons including motoneurons (Krajewska et al., 2002), and its activation during apoptosis involves proteolytic cleavage rather than transcriptional activation (Li et al., 1998; Luo et al., 1998). However, there was also a report demonstrating increased full-length Bid protein levels during disease progression in $SOD1^{G93A}$ mice (Guegan et al., 2002). We therefore started our investigation to re-examine the role of Bid regulation during motoneuron degeneration. Western blotting analysis of glial-acidic fibrillary protein (GFAP) and Bid protein levels in lumbar spinal cord lysates of $SOD1^{G93A}$ mice showed moderately elevated full-length Bid protein levels in 'presymptomatic' postnatal day (PND) 50 mice, or symptomatic PND 90 mice, and a pronounced, significant up-regulation in late stage PND 120 $SOD1^{G93A}$ mice when compared to their non-transgenic counterparts (Figs. 1A, B; d120: transgenic increased by + 139.9% ± 84.62%). Concurrently, GFAP protein levels, likely including GFAP splice

variants (Kamphuis et al., 2012), increased significantly *in vivo* (Fig. 1A, d120: transgenic increased by +685.1% ± 51.39%). We also detected reduced immunoreactivity of the motoneuron marker SMI-32, and moderately up-regulated levels of the microglial marker Iba-1 in the lumbar spinal cord of these transgenic animals (Fig. 1A). A lack of commercially available antibodies that reliably recognize Bid protein levels by immunohistochemistry (*i.e.* that did not show significant non-specific staining in *bid*-deficient mice; Suppl. Figs. 2A–C, F) prevented us from exploring which cell types expressed Bid during disease progression. However, previous reports suggested significant Bid immunostaining in both neuronal and non-neuronal cells (Franz et al., 2002; Guegan et al., 2002). In addition, we failed to collect significant evidence for Bid cleavage in the spinal cord of *SOD1$^{G93A}$* transgenic animals between day50 and day120 (Suppl. Figs. 1A–D), or astrocytes exposed to a pro-inflammatory environment (Suppl. Fig. 1E).

### *Bid is expressed in primary astrocyte and microglia cultures*

To explore whether Bid is also expressed in non-neuronal cells, we investigated Bid protein levels by quantitative PCR and Western blotting in murine mixed motoneuron, primary astrocyte as well as microglial cultures that were generated using established isolation protocols. While motoneuron cultures exhibited moderate *bid* mRNA (Fig. 2A) and protein levels, we noted elevated Bid levels in cultured astrocytes, and an even stronger enrichment of Bid protein in microglia-enriched cell cultures (astrocyte over motoneuron lysates: increase of 279.5% ± 23.4%, microglia over motoneuron lysate: increase of 799.3% ± 43.5%; Figs. 2B & C). These results suggested that Bid may play a role in the regulation of neuroinflammation and astrocyte activation.

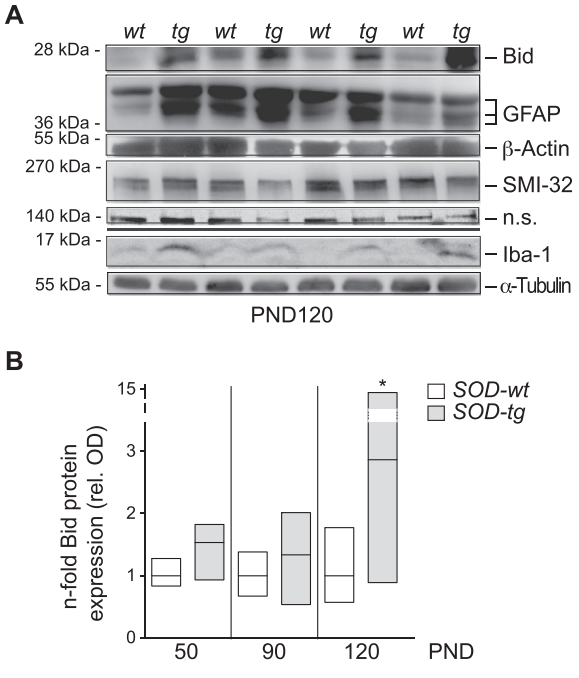


**Fig. 1.** Increased Bid protein levels during disease progression in SOD1$^{G93A}$ mice. (A) Western blot analysis of full length Bid, GFAP, SMI-32 and Iba-1 protein levels in wild-type (*wt*) and transgenic *SOD1$^{G93A}$* mice (*tg*) at PND120 during the symptomatic stage of ALS disease progression (marks highlight GFAP splice variants). (B) Quantification of Bid protein levels by Western-blot analyses of non-transgenic and transgenic *SOD1$^{G93A}$* spinal cords pre-symptomatically (PND50), at disease onset (PND90) and during disease (PND120). Data are normalized to respective wild-type as well as β-actin loading and represented as mean ± range (PND50 (n = 4/4), PND90 (6/6), PND (21/22),*p = 0.0462 (PND120) Mann–Whitney U test).

### *Bid is activated in cultured astrocytes exposed to pro-inflammatory stimuli*

Neuroinflammation is a common hallmark of many neurodegenerative disorders including ALS. To determine whether pro-inflammatory stimuli modulate Bid protein levels, we treated dissociated astrocyte-enriched cultures with the TLR-agonist lipopolysaccharide (LPS 100 ng/ml, 24 h). Western-blot analysis or immunofluorescence labeling revealed a remarkable induction of Bid-protein levels in astrocytes treated with LPS (Figs. 3A, B, Suppl. Figs. 2A, C, D). Spinal cord tissue of ALS mice were shown to have elevated levels of secreted interferon-γ (IFNγ, Wang et al., 2011) and interleukin-1β (IL-1β, Meissner et al., 2010) during pre-symptomatic, symptomatic and end stages. Treatment of mixed motoneuron cultures or astrocyte-enriched cultures with IFNγ and IL-1β resulted in an increase of Bid protein to levels comparable to those induced by LPS (Figs. 3C–F, Suppl. Fig. 2B), meanwhile, GFAP protein levels largely remained unaltered (Fig. 3E). Bid up-regulation occurred in the absence of astrocyte cell death (data not shown), and elevated levels of activated, cleaved caspase-3 or caspase-3-specific cleavage products of PARP-1 were not detectable, demonstrating the absence of an activation of the extrinsic or intrinsic apoptosis pathway in astrocytes (Figs. 3G & H). In contrast to our observations in astrocytes or mixed glial or mixed motoneuron cultures (Figs. 3C, I, J, Suppl. Figs. 2D & E), exposure of microglia-enriched cultures to LPS or IFNγ/IL-1β did not alter Bid protein levels (Figs. 3K–M, Suppl. Fig. 2D), suggesting that Bid is specifically induced in astrocyte cultures in response to TLR agonists and pro-inflammatory cytokines. This induction was similarly observed in Western-blot and immunofluorescence analyses in purified and mixed motoneuron and glial cultures, with the caveat of a high non-specific background immunofluorescence present in the *bid*-deficient astrocytes and tissue seen using three distinct full-length Bid-specific antibodies (Suppl. Figs. 2A–C, F).

### *Bid negatively modulates nuclear factor-κB activity in cultured astrocytes*

Both, neuroprotective and neurotoxic activities have been attributed to the activation of astrocytes during ALS pathogenesis. Bid was recently implicated in the control of inflammation in intestinal epithelial cells by regulating the activation of the pro-inflammatory transcription factor nuclear factor-κB (NF-κB) through functional interaction with the IKK-complex (Yeretssian et al., 2011). We hence immunoprecipitated LPS-stimulated astrocyte lysates using Bid anti-serum. In activated cells, Bid markedly interacted with the NF-κB essential modulator (NEMO), a critical component of the IKK-complex (Fig. 4A). Notably, we observed increased levels of IKK-kinase (IKKβ) phosphorylation in astrocyte cultures exposed to IFNγ and IL-1β, and this was reduced by prior co-application of the Bid-inhibitor BI-6C9 (Fig. 4B). Concomitantly, we also observed increased levels of IKK-kinase (IKKβ) and p65 phosphorylation in astrocyte cultures exposed to IFNγ and IL-1β or LPS, which were not observed in *bid*-deficient astrocytes (Figs. 4B–D). We next investigated whether Bid was implicated in pro-inflammatory cytokine-induced NF-κB activation in astrocytes. We monitored the activity of the NF-κB transcription factor in reporter gene assays in wild-type and *bid*-deficient astrocyte cultures. Exposure of NF-κB-reporter-gene transfected astrocyte cultures to IL-1β and IFNγ demonstrated that pro-inflammatory cytokine-induced stimulation of NF-κB was significantly reduced by co-application of the small molecule Bid inhibitor BI-6C9 (Fig. 4E). Likewise, *bid*-deficiency significantly reduced pro-inflammatory cytokine-induced NF-κB activity (Fig. 4F). Furthermore, prolonged exposure of astrocyte cultures to a pro-inflammatory environment through application of IFNγ and IL-1β for three days to one week resulted in a substantial rise in the levels of the NF-κB target gene cyclooxygenase-II (COX-II), which was ablated in *bid*-deficient astrocyte cultures that were free from notable CD11b immunoreactivity (Figs. 4G–I).

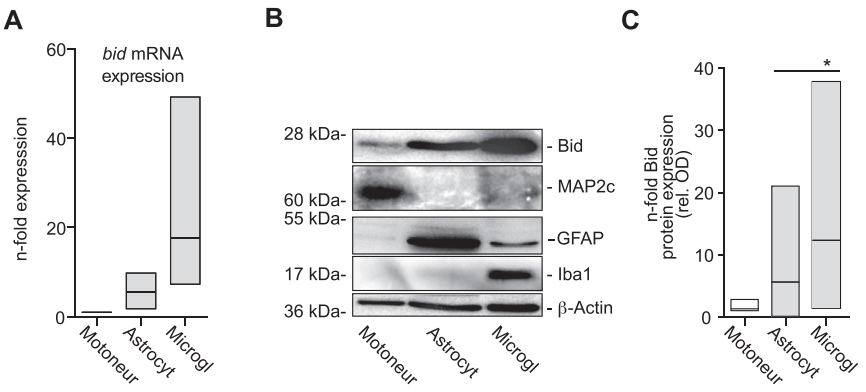


**Fig. 2.** Elevated Bid-protein levels in astroglial cells. (A, B) Dissociated murine motoneuron cultures (DIV7), astrocyte-enriched cultures and microglia-enriched cultures were lysed in RLT (A, n = 3) or RIPA-buffer (B) and mRNA reverse transcribed and subjected to real-time PCR analysis using *bid*- and *gapdh*-specific primers, while proteins were separated by gel-electrophoresis. Western-blots were exposed to a rabbit polyclonal Bid-antibody, as well as antibodies detecting the neuronal marker protein MAP2, the astroglial marker GFAP, and the microglial marker Iba-1. (C) Quantification of Bid protein levels compared to motoneuron content was derived from five Western-blot experiments (A, C: mean ± range).

*Bid-deficiency protects against non-cell autonomous motoneuron death*

To explore whether *bid*-deficiency had functional consequences in the context of non-cell autonomous motoneuron death, we treated either wild-type or *bid*-deficient primary astrocyte cultures with LPS or vehicle (saline). After wash-out of LPS, we collected the respective astrocyte conditioned media (ACM) and transferred them to wild-type motoneuron cultures (Fig. 5A). Motoneuron survival was evaluated by counting SMI-32 positive neurons that were also negative for propidium iodide and trypan-blue following 24 h of incubation (Fig. 5B). Following the assessment of mixed motoneuron cultures using the additional motoneuron marker HB9 (Arber et al., 1999, Suppl. Figs. 3B–B″), we determined that all SMI-32 positive cells in our cultures were NeuN as well as HB9-positive motoneurons (Suppl.

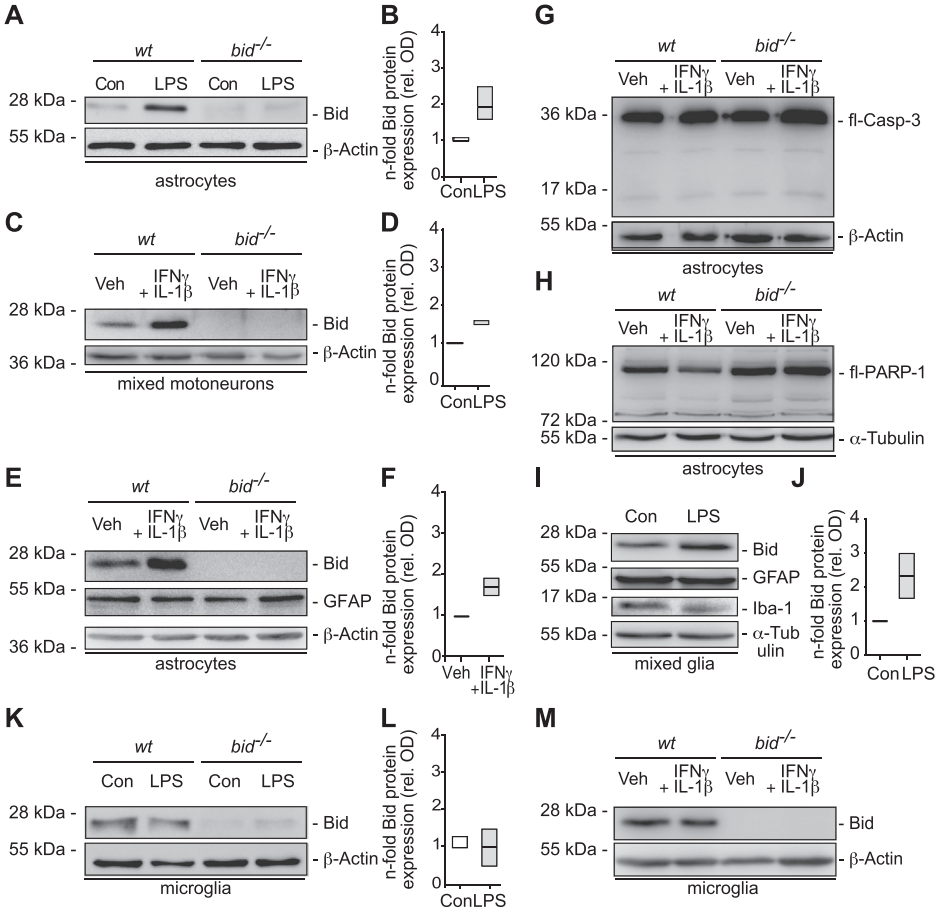


**Fig. 3.** Increased Bid levels in astroglial cultures following pro-inflammatory treatment. Mouse astroglial (A, B) or mixed glial cultures (composed from 50/50% microglial and astroglial cells; I, J) were treated with 100 ng/ml LPS for 24 h, lysed and subjected to Western-blot analyses. (C–F) Mouse motoneuron (C, D) or astroglial (E, F) cultures were treated for 24 h with IFNγ (500 U/ml) and IL-1β (50 ng/ml) or vehicle in cell culture media and lysates were subjected to gel-electrophoresis and Western-blot analyses using the antibodies as indicated. (K–M) Microglia-enriched cultures were cultured for one week and exposed to 100 ng/ml LPS (K, L) or the cytokine mix (M) as delineated above and subjected to gel-electrophoresis and Western-blot analyses. Quantification of Bid protein levels was derived by optical density analysis from Western-blot experiments and normalized to loading control (B, n = 3; D, n = 1, F, n = 2; J, n = 2; L, n = 4; mean ± range).

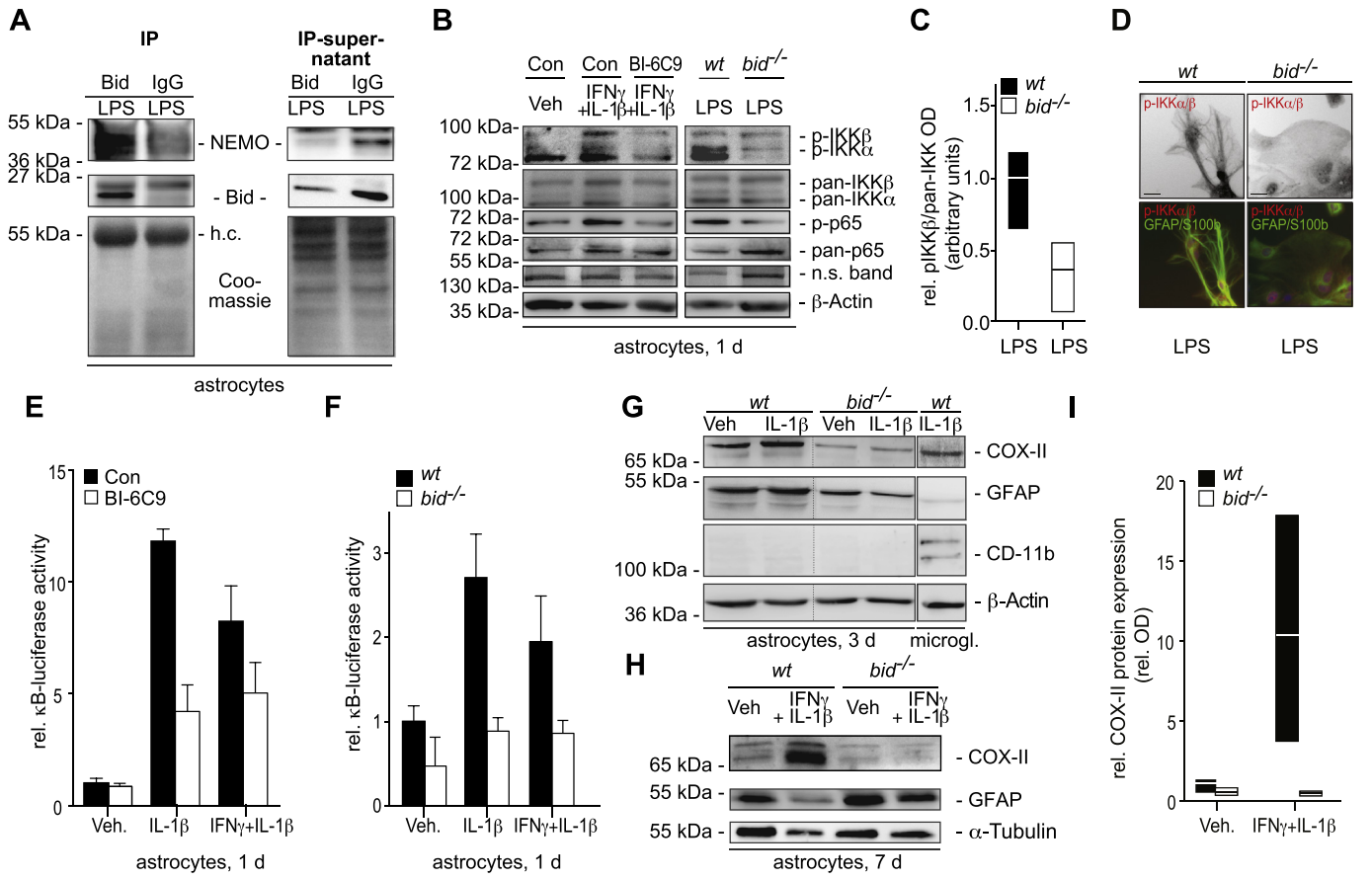


**Fig. 4.** Bid-protein functionally interacts with essential components of the IKK-signalosome and contributes to NF-κB target-gene induction. (A) Murine astrocytes were treated for 24 h with the TLR-agonist LPS (100 ng/ml), and cells were lysed and immunoprecipitated using rabbit-derived anti-Bid antiserum. A notable immunoreactivity against NEMO was noted in the immunoprecipitated samples and a concurrently decreased NEMO immunoreactivity in the supernatant. Bid immunoreactivity was detected using mouse anti-Bid antibodies in the immunoprecipitate (h.c., heavy-chain). (B) Cultured wild-type (*wt*) or *bid*-deficient astrocytes were exposed to either vehicle, LPS, or a combination of the Bid-inhibitor BI-6C9 (10 μM, 30 min pre-incubation) and LPS for 24 h, as indicated. Western-blot membranes derived from the RIPA cell lysates were exposed to phosphorylation-specific (p-)IKKα/β and (p-)p65 antibodies, and pan-IKKα/β, pan-p65 or loading control antibodies following membrane stripping. (C) The optical density data from three Western-blots of LPS-treated (1–7 days) wild-type (*wt*) or *bid*-deficient astrocytes directed at phosphorylated IKKβ was determined and normalized to total IKKβ levels (mean ± range). (D) wild-type or *bid*-deficient astrocytes were exposed for 24 h to LPS, fixed and immuno-labeled against phosphorylated-IKKα/β (red) and GFAP and S100b (green), as indicated. (E) Astrocyte cultures, transfected with the κB-dependent reporter gene vector, were treated with either IL-1β or IL-1β (50 ng/ml) + IFNγ (500 U/ml), with or without the Bid-inhibitor BI-6C9 (100 μM) or respective vehicle, as indicated. The bar graphs represent κB-dependent relative firefly activity (n = 3–8 from two pooled experiments, one outlier removed, data represents mean ± SEM). (F) Mixed wild-type or *bid*-deficient astroglial cultures were transfected with κB-response element luciferase reporter vectors and subsequently treated with IFNγ (500 U/ml) and/or IL-1β (50 ng/ml) for 24 h as indicated. A significantly decreased level of NF-κB activity in Bid-deficient cells was noted (n = 10–16 wells from three pooled experiments, 1 outlier removed, data represents mean ± SEM). (G, H) Astrocyte-cultures were grown to confluency, repeatedly stimulated with the pro-inflammatory cytokine mix every two days for a total of 3 days (G), or one week (H) and lysed. Following gel-electrophoresis and Western-blotting, blots were exposed to antibodies as indicated. The dotted line indicates that intervening lanes have been spliced out. (I) Experiments G & H were repeated and the quantified optical densities from a total of n = 4 biological replicates were normalized to loading control and graphed (mean ± range).

Figs. 3A″, B″) and that SMI-32 represents a stringent motoneuron marker in mixed motoneuron cultures (Carriedo et al., 1996). A significant decrease in surviving, SMI-32 positive cells was noted following incubation of motoneurons with wild-type ACM medium (decreased by 26 ± 19%), but not following their exposure to a medium generated by *bid*-deficient astrocytes (increased by 6 ± 15%; Fig. 5C). Similarly, we observed a significant increase in survival when motoneurons were exposed to the glial-conditioned media (GCM) from *bid*-deficient astrocytes co-cultured with wild-type microglia, when compared to wild-type astrocytes co-cultured with wild-type microglia (increased by 1.26 ± 0.22 fold, Figs. 5D, E).

## Discussion

Mutant SOD1 (*mtSOD1*) was suggested to cause motoneuron degeneration by impairing axonal transport, facilitating protein aggregate production, and sequestering heat-shock proteins and anti-apoptotic proteins (Pasinelli and Brown, 2006). The toxicity of *mtSOD1* was also shown to involve non-cell autonomous cell death pathways (Ilieva et al., 2009; Lee et al., 2012). Astrocytes expressing *mtSOD1* were shown to be a primary source of the pro-inflammatory cytokine IFNγ which may directly or indirectly kill neighboring motoneurons (Aebischer et al., 2011; Siegmund et al., 2005; Wang et al., 2011). Increased expression of pro-inflammatory cytokines, including IL-1β, IFNγ and IL-6, was a common pathological hallmark seen in both transgenic $SOD1^{G93A}$ mice and ALS patients (Lasiene and Yamanaka, 2011; Li et al., 2000; Wang et al., 2011). Direct administration of IFNγ, or IFNγ derived from SOD1 mutant astrocytes, induced motoneuron degeneration *in vitro* in a selective and dose-dependent manner (Aebischer et al., 2011; Beers et al., 2006; Boillee et al., 2006; Siegmund et al., 2005). On the other hand, neuroinflammatory processes may also stimulate tissue regeneration and exert beneficial effects during neurodegeneration (Lalancette-Hébert et al., 2007; Morganti-Kossman et al., 1997; Wyss-Coray et al., 2002; Zhao et al., 2006). Our data indicate that Bid is induced during neuroinflammation in ALS. We observed a marked induction of Bid protein at the symptomatic stage in $SOD1^{G93A}$ mice that occurred in parallel with a markedly upregulated GFAP and moderately elevated levels of the microglial marker Iba-1. In line with our results, Bid expression was previously shown to be markedly elevated in both neuronal and non-neuronal cells in the $SOD1^{G93A}$ mouse

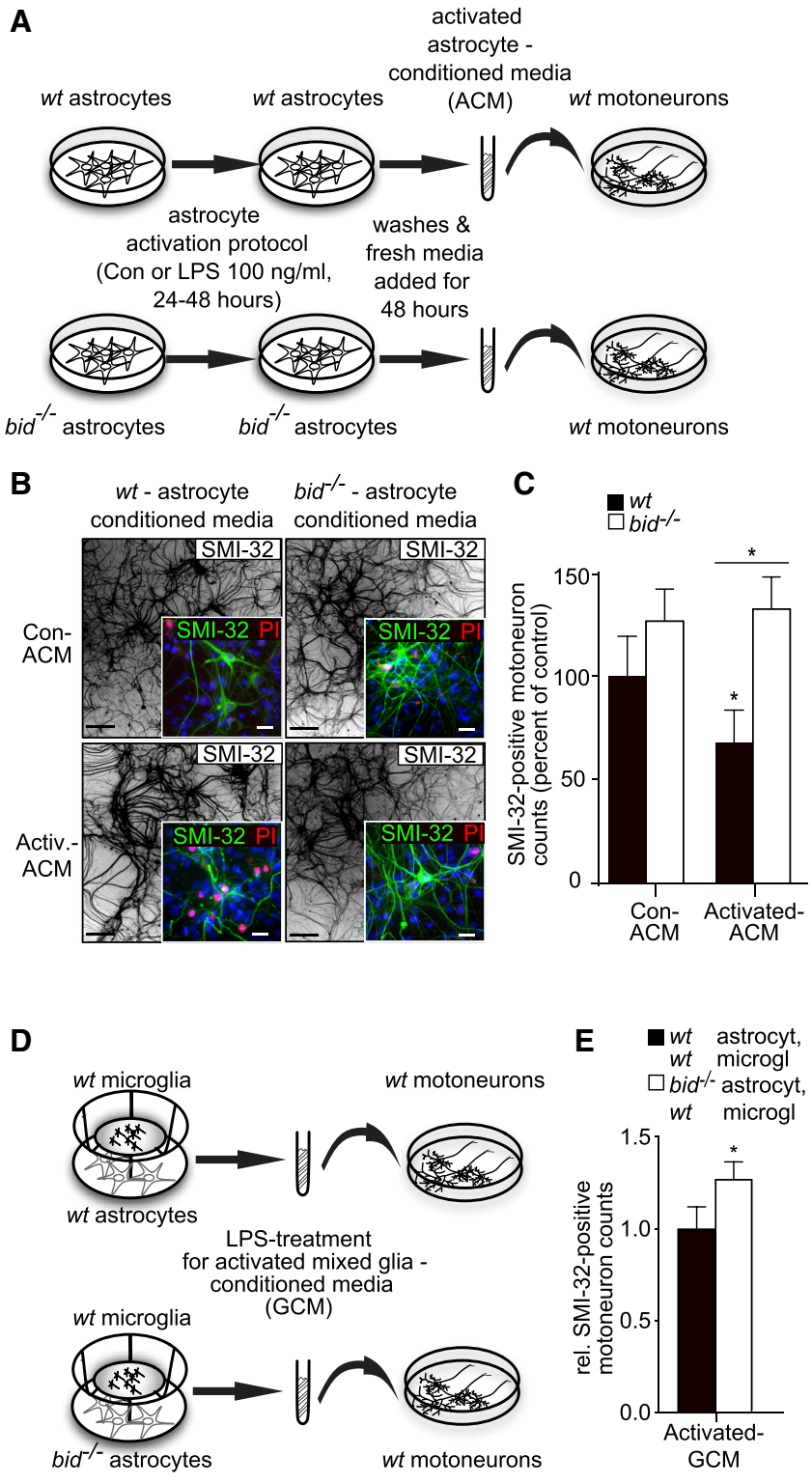


**Fig. 5.** Bid-protein deficiency ablates the potency of activated astrocytes to kill wild-type motoneurons. (A) describing the collection of activated astrocyte-conditioned media and their application to motoneuron cultures. (B, C) Wild-type or *bid*-deficient astrocytes were treated with 100 ng/ml LPS for 24 h in astrocyte media. Cells were washed extensively and motoneuron media were conditioned on thus activated astrocytes for 48 h without further cell stimulus. The media collected from these cells (ACM) were applied to wild-type motoneurons for 24 h, and cells were exposed to trypan-blue solution, fixed and SMI-32 (inset, green) and PI (inset, red) labeled. A potent decrease of SMI-32 positive motoneuron density to 74% was recorded following wild-type ACM exposure, but motoneurons were potently protected when exposed to $bid^{-/-}$ derived ACM (*, $p \leq 0.05$ for wild-type vs $bid^{-/-}$, two-way ANOVA, pooled from 2 experiments from 4 litters, $n = 8–10$ from 4 biological replicates, data represents mean ± SEM). Assessment of trypan-blue positive cells yielded significantly reduced cell death in *bid*-deficient cells *versus* wild-type ($p = 0.0009$ for wild-type vs $bid^{-/-}$, two-way ANOVA, data not shown). Scale bars 50 μm, 10 μm in insets. (D, E) wild-type or *bid*-deficient astrocytes were co-cultured with wild-type microglia as depicted (D) and these mixed cultures were treated with 100 ng/ml LPS for 24 h in astrocyte media. Cells were washed extensively and motoneuron media were conditioned on these activated astrocytes for 48 h without further cell stimulus. The media collected from these cells (GCM) were applied to wild-type motoneurons for 48 h, and cells were exposed to trypan-blue solution, fixed and SMI-32 and PI labeled. A significant protection was noted following exposure to $bid^{-/-}$ derived GCM (*, $p \leq 0.05$, one-sided t-test, $n = 6–7$ wells, pooled from 2 biologically separate experiments, data represents mean ± SEM).

model (Guegan et al., 2002), and to correlate with disease severity and progression in ALS patients, reaching a maximum at end stage (Guegan et al., 2002). In experiments using enriched motoneuron, astrocyte and microglia cultures, we demonstrated that Bid was preferentially expressed in astroglia and microglia. We further found it to be strongly induced in astrocytes upon exposure to pro-inflammatory stimuli, but was not induced in stimulated microglia. Bid was previously shown to be required for death receptor-induced apoptosis in numerous cell types, and couples death receptor signaling to the mitochondrial apoptosis pathway (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). While IFNγ and IL-1β themselves do not engage death receptors, it was shown that IFNγ-activated microglia induce the expression of the pro-inflammatory cytokine TNF-α and hence may indirectly activate the extrinsic apoptosis pathway. IFNγ may also co-operate with TNF-α to induce oxidative stress (Hanisch, 2002; Mir et al., 2009). Additionally, increased Fas ligand expression and activation were identified as key mediators of apoptosis in motoneurons during disease progression in the *SOD1*$^{G93A}$ mouse (Locatelli et al., 2007; Raoul et al., 2002). Our data suggest that Bid, in addition to a role in death receptor-induced apoptosis of motoneurons, may play a role in non-cell autonomous motoneuron death by controlling NF-κB activity in astroglia.

Our studies revealed that *bid*-deficiency or the application of a small molecule Bid inhibitor significantly reduced LPS-, IFNγ and IL-1β-induced NF-κB activity in astrocytes. Suppression of pathologically elevated NF-κB activity in astroglia rather than inhibition of death receptor signaling may indeed underlie the protection afforded by astrocyte *bid*-deficiency described in this study. The effects of Bid on NF-κB activation in astrocytes occurred in the absence of caspase-3 activation, suggesting a non-apoptotic role of Bid in this context. Interestingly, the suppression of pathologically elevated NF-κB activity has repeatedly been linked to improved outcome in CNS injury (Brambilla et al., 2009), sciatic nerve injury and ALS (Fu et al., 2010; Otsmane et al., 2013). A 'non-apoptotic' function of Bid may be in accordance with previous studies that demonstrated a direct influence of Bid on NF-κB activity (Luo et al., 2010; Yeretssian et al., 2011). The latter study suggested a biological link between the NOD1 (nucleotide-binding oligomerization domain containing 1) signalosome and Bid in intestinal epithelial cells, evidenced by co-immunoprecipitation between Bid protein, components of the inflammasome and the IκB kinase (IKK)-complex, and a marked deficiency of *bid* deficient cells to phosphorylate the canonical IKK target protein, IκBα. In accordance with these studies, we found that activated astrocytes expressed a protein complex comprising IKKγ and Bid. However, recently other results showed that Bid may not be essential for NOD signaling in other cell types (Nachbur et al., 2012), suggesting that Bid effects on NF-κB activation are highly context- and tissue-dependent.

The pro-inflammatory potency of glial Bid expression may be partially mediated by cyclooxygenase-2, a NF-κB-induced mediator of prostaglandin E2 output (Kaltschmidt et al., 2002; Roshak et al., 1996). Notably, COX-II enzyme is potently up-regulated in the ALS-diseased human spinal cord (Yiangou et al., 2006). These results are also in agreement with a study showing a non-apoptotic, Bid-dependent activation of COX-II in fibroblasts exposed to alkylating agents (Luo et al., 2010). Recently, it was found that *bid*-deficient macrophages and microglia also exhibited an impaired ability of pro-inflammatory cytokine production (Mayo et al., 2011). It is therefore conceivable that Bid plays a key role in the supply of pro-inflammatory cytokines from both microglia and astroglia (Nagai et al., 2007), thereby amplifying inflammation (Yamanaka et al., 2008). In line with a non-apoptotic role of Bid, we failed to detect the pro-apoptotic, cleaved form of Bid, tBid, by Western blot analyses during disease progression in *SOD1*$^{G93A}$ mice using three separate antibodies detecting either full-length (AF-860 (R&D Systems), AR-52 (Enzo Life Sciences); Fig. 1A, Suppl. Figs. 1A–C, E), or cleaved Bid (AB10002, EMD Millipore; Suppl. Fig. 1D). However tBid is a short-lived protein, which may be difficult to detect.

In summary, our study highlights a new, non-apoptotic role for astrocytic Bid in the control of neuroinflammation, and suggests that Bid activation may contribute to non-cell autonomous motoneuron degeneration in ALS.

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## Conflict of interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2014.06.008.

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