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GOSPEL: A novel neuroprotective protein that binds to GAPDH upon *S*-nitrosylation

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Abstract

We recently reported a novel cell death cascade whereby cellular stressors activate nitric oxide formation leading to *S*-nitrosylation of GAPDH that binds to Siah and translocates to the nucleus. The nuclear GAPDH/Siah complex augments p300/CBP-associated acetylation of nuclear proteins, including p53, which mediate cell death. We report a novel 52 kDa cytosolic protein, GOSPEL, which physiologically binds GAPDH, in competition with Siah, retaining GAPDH in the cytosol and preventing its nuclear translocation. GOSPEL is neuroprotective, as its overexpression prevents NMDA-glutamate excitotoxicity while its depletion enhances death in primary neuron cultures. *S*-nitrosylation of GOSPEL at cysteine-47 enhances GAPDH-GOSPEL binding and the neuroprotective actions of GOSPEL. In intact mice virally delivered GOSPEL selectively diminishes NMDA neurotoxicity. Thus, GOSPEL may physiologically regulate the viability of neurons and other cells.

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is generally regarded as a housekeeping glycolytic enzyme. However, several studies implicate GAPDH in cell signaling (Chuang et al., 2005; Hara et al., 2006a; Sirover, 1999, 2005). We recently described a signaling system influencing cell death whereby diverse cell stressors activate inducible or neuronal nitric oxide synthase (NOS) leading to the generation of nitric oxide (NO), which *S*-nitrosylates GAPDH (Benhar and Stamler, 2005; Hara et al., 2005; Hara et al., 2006a). *S*-nitrosylation abolishes catalytic activity of the enzyme and confers upon it the ability to bind to Siah, an E3-ubiquitin-ligase. The nuclear localization signal of Siah elicits nuclear translocation of GAPDH. In the nucleus GAPDH binds to the protein acetyltransferase p300/CBP, enhancing

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its acetylation of various targets, including p53, with subsequent augmentation of cytotoxicity (Sen et al., 2008).

This signaling cascade appears to mediate the cytoprotective actions of the monoamine oxidase inhibitor *R*-(-)-deprenyl (selegiline, hereafter designated deprenyl) as well as nonmonoamine oxidase inhibiting derivatives (Tatton et al., 2003; Waldmeier et al., 2000). At low nanomolar concentrations these drugs prevent the *S*-nitrosylation of GAPDH, its binding to Siah, and nuclear translocation, with neuroprotective actions in cells and rodent models of Parkinsonism at drug doses as low as 0.01 mg/kg (Hara et al., 2006b). Disruption of the NO-GAPDH-Siah cascade also blocks neurotoxicity elicited by excess activation of NMDA (*N*-methyl-D-aspartate) receptors and mutant Huntingtin (Bae et al., 2006; Hara et al., 2005).

In the present study we report a novel protein interactor of GAPDH, designated GOSPEL (GAPDH's competitor Of Siah Protein Enhances Life). Cell stress elicits *S*-nitrosylation of GOSPEL, enabling it to bind GAPDH in competition with Siah. The binding of GOSPEL to GAPDH prevents nuclear translocation of GAPDH and prevents cyto/neurotoxicity. In intact mice GOSPEL overexpression in the cerebral cortex reduces NMDA neurotoxicity. This neuroprotection reflects GOSPEL's binding to GAPDH, as mutant GOSPEL that cannot bind GAPDH displays greater neurotoxicity. The competition between GOSPEL and Siah for GAPDH binding may reflect a regulatory system that maintains cellular homeostasis in response to stressors.

RESULTS

Identification of GOSPEL

In a yeast two-hybrid analysis with the N-terminal 150 amino acids of GAPDH and a rat hippocampal cDNA library, we identified 27 positive interactors out of 10^6 clones, 25 of which represented various fragments of GAPDH. This presumably reflects the tendency for this protein to form homo-tetramers (Carlile et al., 2000). The two non-GAPDH interactors are a fragment of GOSPEL and an uncharacterized EST clone (Figure 1A). By contrast, yeast two-hybrid studies employing the C-terminal 183 amino acids of GAPDH identify Siah, but not GOSPEL (Hara et al., 2005). After cDNA library screening, we identified the entire coding frame of GOSPEL. GOSPEL contains 406 amino acids with no obvious recognition motifs except for a four amino acid stretch of glutamines (Supplementary Figure 1A). The intron-exon structure of GOSPEL suggests the possibility of alternatively spliced isoforms (Supplementary Figure 1B), though there is high sequence conservation between rodents and humans (Supplementary Figure 1C).

Northern blot analysis reveals GOSPEL expression highest in heart, skeletal muscle, brain, and lung (Figure 1B). Western blot analysis, employing an affinity-purified polyclonal antibody developed against a recombinant GST-fused GOSPEL, reveals a similar tissue distribution to that shown by Northern blotting (Figure 1C). In brain, heart and skeletal muscle, Western blots show an apparent molecular weight for GOSPEL of about 52 kDa, while in lung the apparent size is somewhat greater, about 55 kDa. Interestingly, tissues such as brain, heart, lung and skeletal muscle, that are most enriched in GOSPEL, also contain highest levels of GAPDH. In brain, GOSPEL is widely expressed in neurons, especially abundant in neurons that contain high levels of GAPDH, such as Purkinje cells in the cerebellum as well as pyramidal cells of CA1-3 and granule cells of the dentate gyrus in the hippocampus (Figure 1D).

We examined the intracellular localization of GOSPEL by immunofluorescent cell staining with confocal microscopy (Figure 1E). Overexpressed GOSPEL in HEK293T cells and endogenous GOSPEL in PC12 cells are both predominantly cytosolic.

GOSPEL *S*-nitrosylation is required for binding GAPDH

We mapped binding sites in GAPDH and GOSPEL by performing selective deletions followed by immunoprecipitation from HEK293 cells (Figure 2A, B; Supplementary Figure 2A, B). We identified amino acids 160-200 of GOSPEL as critical for binding to GAPDH. In GAPDH a domain comprising amino acids 80-120 is required for binding to GOSPEL.

To determine the importance of *S*-nitrosylation for the interaction of these proteins, we examined the binding of GOSPEL to GAPDH in extracts of NMDA-treated or no treated cerebral cortical cultures prepared from wild-type or nNOS knockout mice (Figure 2C; Supplementary Figure 2C). It has been demonstrated that NMDA stimulation of cerebral cortical neurons elicits the generation of NO from nNOS, leading to *S*-nitrosylation of various proteins, including GAPDH (Hara et al., 2005; Takahashi et al., 2007). Low level of GAPDH-GOSPEL binding is detected in the absence of NMDA treatment. The NMDA-augmented GAPDH-GOSPEL protein interaction is markedly reduced in nNOS knockout cultures. To ascertain whether the requirement for NO involves GOSPEL, we examined whether GOSPEL can be *S*-nitrosylated. In cerebral cortical cultures stimulated with NMDA, biotin-switch assays reveal robust endogenous *S*-nitrosylation of GOSPEL, which is absent in nNOS knockout cultures (Figure 2D). The *S*-nitrosylation of GOSPEL and GAPDH is time-dependent (Figure 2E). Following NMDA treatment, *S*-nitrosylated GOSPEL (SNO-GOSPEL) is detected as early as 3 h and peaks at 9-16 h, while GAPDH *S*-nitrosylation is not evident till 6 h and continues to increase between 9 and 16 h. We wondered whether the *S*-nitrosylation of GOSPEL might reflect transnitrosylation from GAPDH. This is unlikely, as incubation of *S*-nitrosylated GAPDH with GOSPEL fails to elicit augmented *S*-nitrosylation of GOSPEL (Supplementary Figure 2D).

GOSPEL contains a single cysteine at position 47 (C47), which conforms to an S-nitrosylation motif, as the glutamate immediately following C47 resembles the pattern for numerous *S*-nitrosylated proteins (Kim et al., 1999) including hemoglobin, ras, NMDA-receptor and dynamin. *S*-nitrosylation of GOSPEL is abolished by replacing the cysteine with serine (GOSPEL-C47S), indicating that this single amino acid is the site for *S*-nitrosylation of GOSPEL (Figure 2F). *S*-nitrosylation at C47 is crucial for GOSPEL binding to GAPDH, as their binding is greatly reduced for GOSPEL-C47S in HEK293 cells (Figure 2G). We have confirmed the importance of *S*-nitrosylation at C47 for GOSPEL-GAPDH interaction utilizing *in vitro* binding of the purified proteins (Supplementary Figure 3A). GAPDH *S*-nitrosylation occurs exclusively on C150 (Hara et al., 2005). The GAPDH-C150S mutant, which cannot be *S*-nitrosylated, binds significantly less to GOSPEL than the wild-type protein, in HEK293 cells (Supplementary Figure 3B, C). Therefore, *S*-nitrosylation of both GAPDH and GOSPEL is crucial for their binding (Supplementary Figure 3C). Of note, GOSPEL, SNO-GOSPEL, and Siah do not influence the catalytic activity of wild-type GAPDH (Supplementary Figure 3D, E).

Competition by GOSPEL and Siah for GAPDH binding

Interaction of GAPDH and Siah occurs without *S*-nitrosylation of Siah, but is augmented by *S*-nitrosylation of GAPDH. As both Siah and SNO-GOSPEL can bind to *S*-nitrosylated GAPDH (SNO-GAPDH), we compared the potencies of Siah and SNO-GOSPEL in competing for binding to SNO-GAPDH (Figure 3A, B). SNO-GOSPEL potently inhibits binding of SNO-GAPDH and Siah with an IC_{50} of about 10 nM. The inhibition curve is steep with a decrease of maximal to minimal binding occurring over a 3-fold increase in SNO-GOSPEL concentration. Siah is less potent in inhibiting the binding of SNO-GAPDH and SNO-GOSPEL with an IC_{50} of about 100 nM. The inhibition curve is also steep with maximal to minimal binding evident over a 4-fold increase in Siah concentration. Different portions of GAPDH respectively bind to GOSPEL and Siah (Figures 1A and 2B). To determine whether GOSPEL

and Siah nonetheless compete for binding to GAPDH, we overexpressed GOSPEL, Siah and GAPDH in HEK293 cells (Figure 3C). Overexpression of GOSPEL markedly diminishes the binding of GAPDH to Siah in the presence of the NO-donor GSNO. Moreover, purified GST-GOSPEL blocks protein interactions of GAPDH and His-Siah in the presence of GSNO (Supplementary Figure 4).

NMDA also enhances the GAPDH-GOSPEL interactions more rapidly than GAPDH-Siah binding (Figure 3D). GAPDH-GOSPEL binding is detectable under basal conditions with increases evident by 3 h after NMDA. By contrast, GAPDH-Siah binding is absent basally and is not detected until 9 h after NMDA. The differential kinetics of GAPDH-GOSPEL binding from GAPDH-Siah binding may reflect more rapid *S*-nitrosylation of GOSPEL (required for GAPDH-GOSPEL binding) than of GAPDH (required for GAPDH-Siah binding) (Figure 2E).

GOSPEL prevents the nuclear translocation of GAPDH

To ascertain whether endogenous GOSPEL modulates GAPDH-Siah interactions, we depleted endogenous GOSPEL from primary neuronal cultures of cerebral cortex utilizing RNA interference (RNAi) conveyed by a lentivirus. To rule out the possibility of "off-target" effects of RNAi, we carried out quantitative real-time PCR for 2′5′-oligoadenylate synthetase (OAS1), which monitors such off-target effects (Supplementary Figure 5A). The control and GOSPEL RNAi treatment has no influence on the levels of OAS1 mRNA. Furthermore, such RNAi treatment does not affect the levels of nNOS and NO generated in response to NMDA treatment in neurons (Supplementary Figure 5B, C, D). The RNAi to GOSPEL reduces the endogenous protein level by 90% (Supplementary Figure 6A, B). NMDA-elicited GAPDH-Siah binding is markedly augmented in GOSPEL depleted cultures, establishing that GOSPEL inhibits this interaction (Figure 4A).

Since Siah mediates nuclear translocation of GAPDH, we wondered whether GOSPEL prevents such translocation and retains GAPDH in the cytosol. Treatment of HEK293 cells with GSNO, as previously reported, leads to substantial accumulation of GAPDH in the nucleus (Hara et al., 2005). Overexpression of GOSPEL diminishes the nuclear localization of GAPDH (Figure 4B). Endogenous GOSPEL also regulates the NMDA-elicited nuclear translocation of GAPDH, as depletion of GOSPEL by RNAi markedly enhances nuclear GAPDH levels in neuronal cultures (Figure 4C; Supplementary Figure 6C).

GOSPEL diminishes NMDA neurotoxicity by preventing GAPDH-Siah binding

We previously demonstrated that NMDA stimulation of cerebellar granule neurons elicits NO generation from nNOS and *S*-nitrosylation of GAPDH, which translocates to the nucleus together with Siah and elicits neurotoxicity (Hara et al., 2005; Hara et al., 2006b). We wondered whether the prevention of GAPDH-Siah binding by GOSPEL impacts such neurotoxicity. Accordingly, we examined neurotoxicity in primary cerebellar granule neurons following NMDA treatment (Figure 5A). Overexpression of GOSPEL almost completely prevents the 75% decrease in neuronal viability elicited by NMDA treatment. Deletion of the N-terminal 200 amino acids of GOSPEL (GOSPEL-ΔN200), which prevents binding to GAPDH, abrogates the protective effect of GOSPEL. By contrast, deleting the N-terminal 160 amino acids (GOSPEL-ΔN160), which does not impair GOSPEL-GAPDH binding, preserves the protective actions of GOSPEL. *S*-nitrosylation of GOSPEL, which is crucial for binding to GAPDH, is also required for the neuroprotective actions of GOSPEL. Thus, GOSPEL-C47S, which cannot be *S*-nitrosylated and which does not bind to GAPDH robustly, fails to exert a strong neuroprotective action.

To determine whether endogenous GOSPEL influences neurotoxicity, we employed lentivirus-mediated RNAi to deplete endogenous GOSPEL in primary cerebellar granular cells and

cortical neurons. In both cell types we examined NMDA-nNOS-triggered excitotoxicity, a widely employed model of neurotoxicity (Bonfoco et al., 1995; Dawson et al., 1996), in which GAPDH-Siah is a mediator (Hara et al., 2005; Hara et al., 2006b). Both at 12 and 24 h following NMDA treatment, cell survival is substantially reduced in GOSPEL depleted cultures (Figure 5B, C; Supplementary Figure 7A, 7B)

GOSPEL is neuroprotective in intact mice

Earlier we demonstrated that treatment with the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) augments the binding of GAPDH to Siah in mouse corpus striatum (Hara et al., 2006b; Sen et al., 2008). Utilizing a similar paradigm, we show that MPTP treatment stimulates striatal *S*-nitrosylation of both GAPDH and GOSPEL (Supplementary Figure 8A). MPTP treatment also stimulates the binding of GAPDH-GOSPEL as well as GAPDH-Siah, with the increased interaction of GAPDH-GOSPEL occurring earlier than that of GAPDH-Siah (Supplementary Figure 8B).

To determine whether neuroprotective actions of GOSPEL are evident in intact mice, we utilized a viral vector system that delivers into the cerebral cortex wild-type GOSPEL and GOSPEL lacking the N-terminal 200 amino acids (GOSPEL-ΔN200, which does not bind GAPDH). The two GOSPEL constructs display similar levels of expression in the cerebral cortex monitored by Western blotting (Figure 6A) and immunohistochemical staining (Figure 6B). Neurotoxicity was elicited by direct injection of NMDA into the cerebral cortex (Figure 6C, D). NMDA-induced lesions in brains of GOSPEL-overexpressed mice are about 30% smaller than in those injected with GOSPEL-ΔN200. Thus, GOSPEL overexpression in the cerebral cortex leads to selective diminution of NMDA neurotoxicity *in vivo*. The neuroprotective influence of GOSPEL is attributable to its binding to GAPDH, as a mutant GOSPEL which does not bind GAPDH displays diminished neuroprotective action.

DISCUSSION

In this study we report a novel protein, GOSPEL, which physiologically binds GAPDH. *S*-nitrosylation of GOSPEL augments for GAPDH-GOSPEL binding, which competes with the interaction of GAPDH with Siah. This competition prevents the nuclear translocation of GAPDH and associated neurotoxicity (Supplementary Figure 9).

GOSPEL is a hydrophilic protein that contains 406 amino acids and appears to be exclusively cytosolic. It lacks obvious functional motifs such as nuclear export and import signals. GOSPEL mRNA and protein levels are highest levels in organs most enriched in GAPDH, such as brain, heart, lung and skeletal muscle. This is consistent with the notion that GOSPEL physiologically modulates GAPDH functions. By contrast Siah levels are similar in all organs examined.

GOSPEL retains GAPDH in cytosol, as its depletion augments the nuclear translocation of GAPDH that is associated with cell stress. GOSPEL's neuroprotective actions are robust, as the 75% reduction in viability of cortical neurons elicited by NMDA is reversed by overexpression of GOSPEL in a fashion indicating that its neuroprotective actions derive from its binding GAPDH. In turn, depletion of GOSPEL increases NMDA neurotoxicity.

S-nitrosylation in response to NMDA occurs more rapidly for GOSPEL than GAPDH. Conceivably, this is relevant to the dynamics of cellular responses to nitrosative stress. Thus, perhaps a more rapid initiation of cytoprotective (GOSPEL *S*-nitrosylation) than cytotoxic (GAPDH *S*-nitrosylation) mechanisms reflects the cell's approach to coping with nitrosative stress. The more rapid response of GOSPEL to NO suggests that it may have physiologic as well as pathophysiologic functions.

Deprenyl and related neuroprotective drugs very potently prevent GAPDH *S*-nitrosylation and its binding to Siah (Hara et al., 2006b). We speculate that GOSPEL and deprenyl act at similar sites on GAPDH. Thus, GOSPEL binds to amino acids 80-120 of GAPDH, which contains the Rossman fold (Nagy and Rigby, 1995). The blockade of GAPDH-Siah binding by deprenyl *in vitro* requires the presence of NAD^+, which physiologically interacts with the Rossman fold (MR Hara, A Sawa, and SH Snyder: unpublished data). These observations raise the possibility that drugs mimicking GOSPEL might exert neuroprotective actions similar to those of deprenyl.

Heretofore, studies of the NO-GAPDH-Siah/GOSPEL cascade have focused upon cyto/neurotoxicity. However, it is likely that this signaling system possesses physiologic roles. Thus, GAPDH-GOSPEL binding is detectable in untreated cerebral cortical cultures with the binding diminished in nNOS deleted cultures or cultures treated with the NMDA antagonist MK801 (data not shown). Nuclear translocation of GAPDH may convey signals from neuronal activity to epigenetic controls of gene transcription by modulating p300/CBP-mediated histone acetylation (Sen et al., 2008), which may be regulated by GAPDH-GOSPEL interactions.

EXPERIMENTAL PROCEDURES

Unless otherwise noted, all data are representative of 3 individual experiments.

Chemicals, antibodies, plasmids, overexpression and RNAi-virus

Unless otherwise noted, chemicals were purchased from Sigma. Protein G and A agaroses were purchased from Oncogene. Antibodies were obtained from the following companies: anti-GAPDH monoclonal antibody from Biogenesis; anti-histone H2B antibody and anti-actin antibody from Upstate; anti-HA antibody from Babco; anti-Myc antibody from Calbiochem; anti-nNOS antibody from BD Pharmingen anti-HA antibody from Covance and anti-Siah antibody from Santa Cruz; anti-GFP antibody, anti-mouse Ig conjugated to FITC, and anti-rabbit Ig conjugated to rhodamine from Molecular Probes. Polyclonal antibody against GOSPEL was generated with the following procedures: GST-tagged GOSPEL was produced in *E. coli*, purified through glutathione-sepharose beads (Pharmacia Biotech, Piscataway, NJ), and used for antigen. Antisera were produced by a service of Research Genetics, and the sera were immunopurified. Constructs expressing site-directed or deletion mutant proteins were prepared according to the published protocol (Ho et al., 1989).

GOSPEL RNAi mediated by a lentiviral system (target sequence: 5′-AAGGCTCTGATTGAGCAGAAG-3′) was generated as follows: the target sequences were cloned in the pFUGW vector, and this construct was transfected in HEK293FT cells along with two other helper/packaging vectors (Δ8.9 and VSVg constructs) to produce lentiviral particles. For the production of control lentivirus, FUGW vector was transfected into HEK293FT cells along with two other helper/packaging constructs. Viral production was allowed to take place for 48-72 h inside HEK293 FT cells, and the supernatant was collected and ultracentrifuged to bring down viral particles, which were resuspended into OPTI-MEM. About 10^6-10^7 pfu/ml viruses were infected in primary neurons for 24 h. Viral expression was confirmed by observing GFP encoded in pFUGW backbone, 5 days after infection. Wild-type HA-GOSPEL and HA-GOSPEL-ΔN200 expression by a lentiviral system was prepared as follows: both inserts were cloned in pENTR/D-TOPO vector (invitrogen), and then inserted in pLenti4/TO/V5-DEST vector (invitrogen) individually by homologous recombination (according to the manufacturer's protocol). The final constructs were transfected in HEK293T cells along with two other helper/packaging vectors (Δ8.9 and VSVg constructs), and the supernatant was collected and ultracentrifuged to bring down viral particles, which were resuspended into OPTI-MEM. About 10^7-10^8 pfu/ml viruses were infected in primary neurons for 24 h. Viral

expression was confirmed by doing Western blotting with an HA-antibody 5 days after infection.

Yeast two-hybrid and cDNA library screenings

The N-terminal (amino acids 1-150) of rat GAPDH open reading frame were cloned into yeast expression vector pPC97, containing the *Gal4* DNA binding domain. These constructs were used for the screening of a rat hippocampal cDNA library cloned into pPC86, containing the Gal4 transactivation domain. An adult rat brain cDNA library in lambda ZAPII vector (Stratagene) was screened using the yeast two-hybrid fragment for a portion of GOSPEL open reading frame, labeled with [^{32}P]dCTP using a random priming DNA labeling kit (Boehringer-Mannheim). A total of 1×10^6 clones were screened, yielding 61 overlapping clones.

Northern Blotting and in situ hybridization

Rat *GOSPEL* cDNA fragment utilized as a probe was labeled with [^{32}P]dCTP using the High Prime DNA-labeling system (Roche Diagnostics), and Northern hybridization was performed at 68°C for 2 h in QuikHyb solution (Stratagene) with a tissue mRNA blot membrane (CLONTECH) (Burnett et al., 1998).

Biochemical studies

In vitro binding experiments were performed according to the published protocol with glutathione-sepharose (Bae et al., 2005; Kamiya et al., 2005). Co-immunoprecipitation and Western blotting were conducted as published previously (Bae et al., 2005; Kamiya et al., 2005). Subcellular fractionation was carried out with Nuclear/Cytosol Fractionation Kit (BioVision). *S*-nitrosylation biotin switch assay was conducted as published previously (Forrester et al., 2007; Jaffrey and Snyder, 2001). Trans-nitrosylation assay was performed as follows: purified wild-type GAPDH (1.5 μg) purchased from Sigma was incubated with 200 μM GSNO at 37°C for 1 h. The whole reaction mixture was then passed through a desalting column (Pierce) to remove free GSNO. *S*-nitrosylation of GAPDH was confirmed by biotin switch assay. For the trans-nitrosylation reaction, 0.1, 1.0 or 2.5 μg of GST-GOSPEL were incubated with *S*-nitrosylated GAPDH in 4°C for 1 h with mild rotation in 1 ml of buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1 mg/ml BSA, and 0.1% CHAPS). The whole solution was used for the biotin switch assay to detect *S*-nitrosylation of GOSPEL protein.

In cell-free binding assay we have purified GAPDH, GOSPEL, and Siah protein separately. GAPDH and GOSPEL were incubated separately with 200 μM GSNO at 37°C for 1 h. The whole reaction mixture was then passed through a desalting column (Pierce) to remove free GSNO. Then SNO-GAPDH and SNO-GOSPEL were mixed 1:1 molar ratio. On top of this mixture, GST-Siah was added in a concentration dependent manner (0-500 nM) and kept at 37°C for 1 h. In another set of experiments we incubated SNO-GADH and Siah along with different concentration of GST-GOSPEL (0-100 nM) at 37°C for 1 h. Then we conducted GST-pull down assays and western blots for GAPDH. After measuring the optical density of GAPDH, we calculated IC50 values for inhibition of binding between GAPDH and GOSPEL by Siah as well as binding between GAPDH and Siah by GOSPEL.

For quantitative PCR total RNA was isolated from granule neuron with the RNeasy mini kit (Qiagen) and was reversely transcribed with the Omniscript RT kit (Qiagen). Real-time PCR was performed using SYBR green PCR master mix for *OAS1*. The primers used for OAS1 amplification are as follows: forward primer for OAS1 is 5′-CCATCCTCAAGTGGACAAGAACTG-3′; reverse primer for OAS1 is 5′-TTGGGCTTTGGGCACCTTC-3′ (Bridge et al., 2003).

Enzymatic assay for GAPDH

GAPDH (10 nM) or S-nitrosylated GAPDH were preincubated with either GOSPEL (100 nM) or Siah (500 nM) at 37 °C for 30 min. S-nitrosylated GOSPEL (100 nM) was also preincubated separately with 10 nM GAPDH or S-nitrosylated GAPDH in the presence or absence of Siah at 37 °C for 30 min. Then "assay mixture" was added in each tube and the incubation continued at 37 °C for 10 min. The assay mixture contained 10 mM sodium pyrophosphate (pH 8.5), 20 mM sodium arsenite, 2 mM NAD^+, and 2 mM glyceraldehyde 3-phosphate. Optical density was measured at 340 nm. All values are normalized to control and expressed as arbitary units.

Cell culture and staining

HEK293 cells and HEK293FT cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 2 mM L-glutamine at 37°C with a 5% CO_2 atmosphere in a humidified incubator. PC12 cells were maintained in DMEM with 10% FBS, 5% horse serum, 2 mM L-glutamine, and 100 U/ml PS in the same environment. Primary cortical neurons were prepared from fetal mice (14-day gestation) derived from wild-type and nNOS knockout mice, and cultured in Neurobasal media supplemented with B27, 2 mM L-glutamine, and 100 U/ml PS at 37°C with 5% CO_2 atmosphere in a humidified incubator. Cerebellar granule neurons were prepared from neonatal mice and cultured in Basal Eagle Medium (BEM) containing 10% FBS, 25 mM KCl, and 2 mM L-glutamine. Immunocytochemistry using confocal microscopy was performed as described (Sawa et al., 1999). Confocal microscropy images were obtained using a Zeiss Meta Confocal System. For neuron cultures anti-GFP antibody was used to enhance the signal from transfected cells.

Measurement of NMDA-induced neurotoxicity in primary neurons

To activate NMDA receptors in neurons, 8-10-day-old cultures (DIC 8-10) were treated with Mg^{2+} free Earle's Balanced Salt Solution (EBSS) containing 300 μM NMDA and 5 μM glycine for 10 min.

Three days prior to NMDA receptor activation, cerebellar granule neurons were transfected with plasmids as follows: after pre-incubation of cells with Neurobasal (Gibco BRL) including B27 for 24 h from DIV 5, neurons were transfected with 1 μg of GFP construct and a total of 3 μg of varied combinations of plasmids using lipofectamine 2000. The molar ratio of expression constructs to GFP plasmid was 3:1. Using fluorescent microscopy with a digital camera, we captured images of more than 20 fields per preparation, which were randomly chosen in a blind manner. GFP positive neurons were tallied in each field and added together to determine the percentage of viable cells compared to control.

Three days prior to NMDA receptor activation, RNAi-lentivirus was infected into granule or cortical neurons. Viability of neurons was assayed 12 h or 24 h after exposure to NMDA as follows: cells were washed with PBS and incubated in 1 μg/ml propidium iodide for 10 min (dead cells are stained in red). After extensive washing to remove non-specifically attached propidium iodide to cell debris by PBS, neurons were then fixed in 4% paraformaldehyde in PBS and stained with DAPI to visualize the total cell population. As the majority of neurons were infected, the ratio of propidium iodide-stained cells to DAPI-stained cells was employed to reflect toxicity.

Measurement of nitric oxide species

To measure reactive nitric oxide level inside cells, we used 4,5 diamino fluorescein diacetate (DAF-FM, Molecular probes), a cell permeable derivative of the indicator DAF-2 whose fluorescence reflects the level of endogenous nitric oxide (Choi et al., 2000; Chvanov et al., 2006). Briefly, neurons were washed once with Hanks Balanced Salt Solution (HBSS) and

then were loaded with 5 μM DAF-FM in HBSS and incubated at 37°C with 5% CO_2 atmosphere in a humidified incubator for 60 min. NMDA (300 μM) and glycine (5 μM) were added to the cells and incubated for another 10 min. Cells were washed, harvested in HBSS and fluorescence was measured in a fluorimeter utilizing excitation 485 nm and emission 530 nm. DAF-2 fluorescence was also detected using a laser scanning confocal microscope (Zeiss Meta).

Animals and MPTP Treatment

All experiments were approved and conformed to the guidelines set by the Institutional Animal Care Committee. Eight-week-old male CD1 mice (The Jackson Laboratory) were used. Mice received four i.p. injections of MPTP-HCl (20 mg/kg free base; Sigma) in saline at 2-h intervals and were sacrificed after 16 h and 24 h after the first injection (n = 5). Control mice received saline only. In each case the striatum was isolated and was used for imunoprecipitation and *S*-nitrosylation experiments (Hara et al., 2006b).

Stereotaxic injection into intact mice

Adult male C57BL/6 mice (20-25 g; Charles River, Wilmington, MA) were used for NMDA mediated cytotoxicity measurement. Animal protocols, approved by the Institutional Animal Care and Use Committee of Johns Hopkins University, were used in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animal well being was determined by monitoring the weight and rectal temperature before the surgical procedure. Mice were anesthetized with 3.0% isoflurane, maintained with 1.0% isoflurane and mounted on a stereotaxic frame (Stoelting Co., Wood Dale, IL). The skull was exposed and 5.0 μl (10^7-10^8 pfu/ml) of viral particles of either full length GOSPEL (n=8) or delta-N200 GOSPEL (n=9) protein was slowly injected into the cortex (anterior 0.5 mm, lateral 3.5 mm from bregma, and ventral 1.0 mm relative to dura) over a period of 20 min with the help of a 5.0 μl Hamilton® syringe, and the needle was left in place for additional 5 min. Seven days after viral infection, overexpressed full length GOSPEL or delta-N200 GOSPEL in coronal sections were detected by western blot and immunohistochemistry with anti-HA antibody (1:200). On day 8, 15 nmol NMDA was slowly injected at the same location with 1.0 μl Hamilton® syringe. After each injection, mice were placed in a thermoregulated chamber maintained at 31 ±0.5°C and returned to their cages after full recovery from anesthesia. The rectal temperatures were monitored and maintained at 37.0 ± 0.5 °C during the experimental procedure.

NMDA-induced lesion quantification

At 24 h post-NMDA injection, mice were trancardially perfused with 0.1 M PBS and fixed with 4% PFA. Brains were harvested and soaked in 4% PFA overnight, then equilibrated with 30% sucrose. Sequential brain sections of 25 μm obtained on cryostat were stained with cresyl violet to estimate the lesion volume as described previously (Ahmad et al., 2006; Ahmad et al., 2007). One-way ANOVA followed by Tukey's post-hoc analysis was used to calculate the difference between the groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

Ahmad AS, Zhuang H, Echeverria V, Dore S. Stimulation of prostaglandin EP2 receptors prevents NMDA-induced excitotoxicity. J Neurotrauma 2006;23:1895–1903. [PubMed: 17184197]

Ahmad M, Ahmad AS, Zhuang H, Maruyama T, Narumiya S, Dore S. Stimulation of prostaglandin E2-EP3 receptors exacerbates stroke and excitotoxic injury. J Neuroimmunol 2007;184:172–179. [PubMed: 17275922]

Bae BI, Hara MR, Cascio MB, Wellington CL, Hayden MR, Ross CA, Ha HC, Li XJ, Snyder SH, Sawa A. Mutant huntingtin: nuclear translocation and cytotoxicity mediated by GAPDH. Proc Natl Acad Sci U S A 2006;103:3405–3409. [PubMed: 16492755]

Bae BI, Xu H, Igarashi S, Fujimuro M, Agrawal N, Taya Y, Hayward SD, Moran TH, Montell C, Ross CA, et al. p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. Neuron 2005;47:29–41. [PubMed: 15996546]

Benhar M, Stamler JS. A central role for S-nitrosylation in apoptosis. Nat Cell Biol 2005;7:645–646. [PubMed: 15990893]

Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 1995;92:7162–7166. [PubMed: 7638161]

Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet 2003;34:263–264. [PubMed: 12796781]

Burnett PE, Blackshaw S, Lai MM, Qureshi IA, Burnett AF, Sabatini DM, Snyder SH. Neurabin is a synaptic protein linking p70 S6 kinase and the neuronal cytoskeleton. Proc Natl Acad Sci U S A 1998;95:8351–8356. [PubMed: 9653190]

Carlile GW, Chalmers-Redman RM, Tatton NA, Pong A, Borden KE, Tatton WG. Reduced apoptosis after nerve growth factor and serum withdrawal: conversion of tetrameric glyceraldehyde-3-phosphate dehydrogenase to a dimer. Molecular pharmacology 2000;57:2–12. [PubMed: 10617673]

Choi YB, Tenneti L, Le DA, Ortiz J, Bai G, Chen HS, Lipton SA. Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. Nat Neurosci 2000;3:15–21. [PubMed: 10607390]

Chuang DM, Hough C, Senatorov VV. Glyceraldehyde-3-phosphate dehydrogenase, apoptosis, and neurodegenerative diseases. Annu Rev Pharmacol Toxicol 2005;45:269–290. [PubMed: 15822178]

Chvanov M, Gerasimenko OV, Petersen OH, Tepikin AV. Calcium-dependent release of NO from intracellular S-nitrosothiols. EMBO J 2006;25:3024–3032. [PubMed: 16810320]

Dawson VL, Kizushi VM, Huang PL, Snyder SH, Dawson TM. Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. J Neurosci 1996;16:2479–2487. [PubMed: 8786424]

Forrester MT, Foster MW, Stamler JS. Assessment and application of the biotin switch technique for examining protein S-nitrosylation under conditions of pharmacologically induced oxidative stress. J Biol Chem 2007;282:13977–13983. [PubMed: 17376775]

Hara MR, Agrawal N, Kim SF, Cascio MB, Fujimuro M, Ozeki Y, Takahashi M, Cheah JH, Tankou SK, Hester LD, et al. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. Nat Cell Biol 2005;7:665–674. [PubMed: 15951807]

Hara MR, Cascio MB, Sawa A. GAPDH as a sensor of NO stress. Biochim Biophys Acta 2006a;1762:502–509. [PubMed: 16574384]

Hara MR, Thomas B, Cascio MB, Bae BI, Hester LD, Dawson VL, Dawson TM, Sawa A, Snyder SH. Neuroprotection by pharmacologic blockade of the GAPDH death cascade. Proc Natl Acad Sci U S A 2006b;103:3887–3889. [PubMed: 16505364]

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 1989;77:51–59. [PubMed: 2744487]

Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. Sci STKE 2001;2001:PL1. [PubMed: 11752655]

Kamiya A, Kubo K, Tomoda T, Takaki M, Youn R, Ozeki Y, Sawamura N, Park U, Kudo C, Okawa M, et al. A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. Nat Cell Biol 2005;7:1167–1178. [PubMed: 16299498]

Kim WK, Choi YB, Rayudu PV, Das P, Asaad W, Arnelle DR, Stamler JS, Lipton SA. Attenuation of NMDA receptor activity and neurotoxicity by nitroxyl anion, NO. Neuron 1999;24:461–469. [PubMed: 10571239]

Nagy E, Rigby WF. Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD(+)-binding region (Rossmann fold). J Biol Chem 1995;270:2755–2763. [PubMed: 7531693]

Sawa A, Wiegand GW, Cooper J, Margolis RL, Sharp AH, Lawler JF Jr. Greenamyre JT, Snyder SH, Ross CA. Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. Nat Med 1999;5:1194–1198. [PubMed: 10502825]

Sen N, Hara MR, Kornberg MD, Cascio MB, Bae BI, Shahani N, Thomas B, Dawson TM, Dawson VL, Snyder SH, Sawa A. Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. Nat Cell Biol 2008;10:866–873. [PubMed: 18552833]

Sirover MA. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochimica et biophysica acta 1999;1432:159–184. [PubMed: 10407139]

Sirover MA. New nuclear functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells. Journal of cellular biochemistry 2005;95:45–52. [PubMed: 15770658]

Takahashi H, Shin Y, Cho SJ, Zago WM, Nakamura T, Gu Z, Ma Y, Furukawa H, Liddington R, Zhang D, et al. Hypoxia enhances S-nitrosylation-mediated NMDA receptor inhibition via a thiol oxygen sensor motif. Neuron 2007;53:53–64. [PubMed: 17196530]

Tatton W, Chalmers-Redman R, Tatton N. Neuroprotection by deprenyl and other propargylamines: glyceraldehyde-3-phosphate dehydrogenase rather than monoamine oxidase B. J Neural Transm 2003;110:509–515. [PubMed: 12721812]

Waldmeier PC, Boulton AA, Cools AR, Kato AC, Tatton WG. Neurorescuing effects of the GAPDH ligand CGP 3466B. J Neural Transm Suppl 2000:197–214. [PubMed: 11205140]

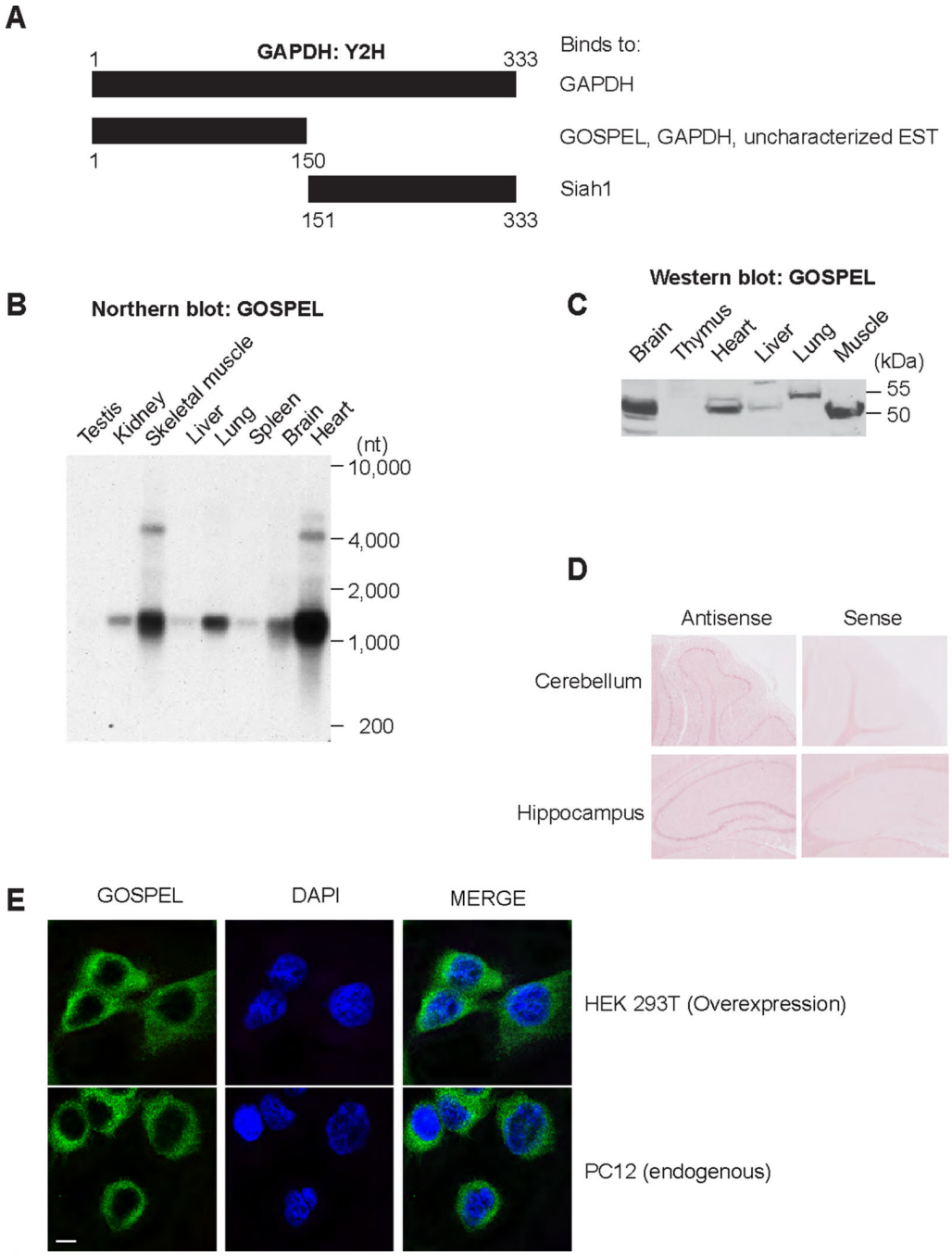

Figure 1. Identification and characterization of GOSPEL

(A) Yeast two-hybrid analysis with two GAPDH constructs (amino acids 1-150 or 151-333) and a rat hippocampal cDNA library. The 1-150 amino acid fragment binds with GOSPEL, but not with Siah.

(B) Tissue distribution of GOSPEL mRNA.

(C) Tissue distribution of GOSPEL protein detected with an immunopurified polyclonal antibody against GOSPEL.

(D) GOSPEL in brain tissues. *In situ* hybridization reveals high expression of GOSPEL in Purkinje cells of the cerebellum as well as CA1-3 pyramidal and dentate granule neurons in the hippocampus.

(E) Cellular distribution of GOSPEL. Exogenous GOSPEL in HEK293 cells (GOSPEL-HA detected by anti-HA antibody) and endogenous GOSPEL in PC12 cells (detected by anti-GOSPEL antibody) are exclusively cytosolic. Scale bar, 40 μm.

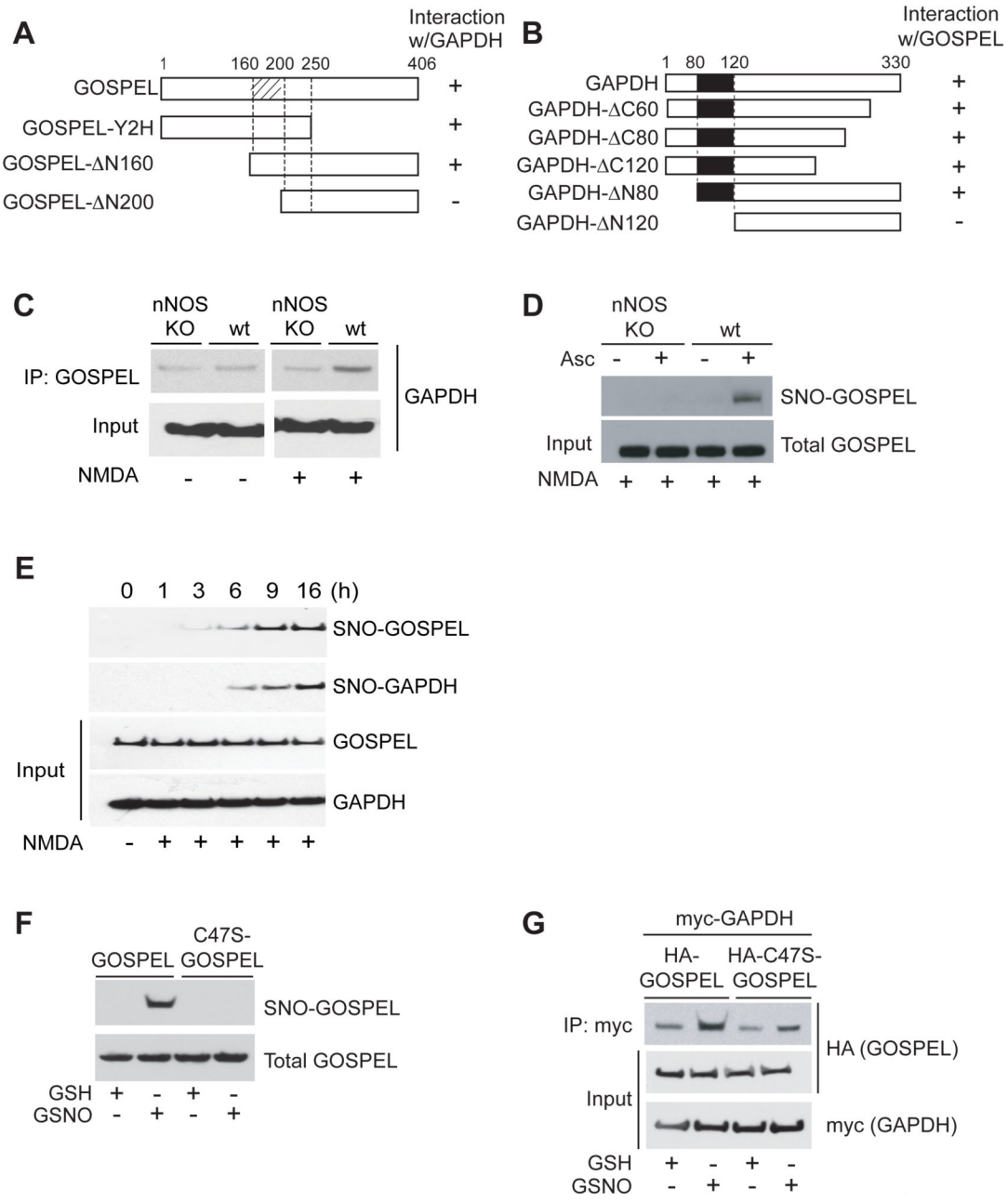

Figure 2. GOSPEL-GAPDH protein interaction

(A) Schematic representation of the binding domain of GOSPEL for GAPDH. Amino acids 160-200 GOSPEL are responsible for binding to GAPDH.

(B) Schematic representation of the interaction domain of GAPDH for GOSPEL. Amino acids 80-120 GAPDH are responsible for binding with GOSPEL.

(C) GAPDH-GOSPEL interaction with or without NMDA exposure is diminished in primary cortical neurons from nNOS deleted mice.

(D) *S*-nitrosylation of GOSPEL (SNO-GOSPEL) in primary cortical neurons elicited by NMDA exposure. SNO-GOSPEL was detected by biotin switch assay as an ascorbate-sensitive signal. Asc, ascorbate.

(E) Time course for *S*-nitrosylation of GOSPEL and GAPDH following NMDA treatment in primary granule neuron over 16 h. GOSPEL is *S*-nitrosylated earlier than GAPDH. *S*-nitrosylation of GAPDH and GOSPEL were detected by biotin switch assay.
(F) Requirement of cysteine-47 (C47) for *S*-nitrosylation of GOSPEL. HEK293 cells were transfected with either HA-GOSPEL or HA-GOSPEL-C47S mutant. Cells were then treated with GSH or GSNO for 16 h and subjected to biotin switch assay. The C47S mutant of GOSPEL is not *S*-nitrosylated.
(G) Requirement of cysteine-47 (C47) for efficient binding of GOSPEL and GAPDH. HEK293 cells were transfected with myc-GAPDH along with HA-GOSPEL or HA-GOSPEL-C47S, and treated with either GSH or GSNO for 16 h. The protein interaction was examined by co-immunoprecipitation.

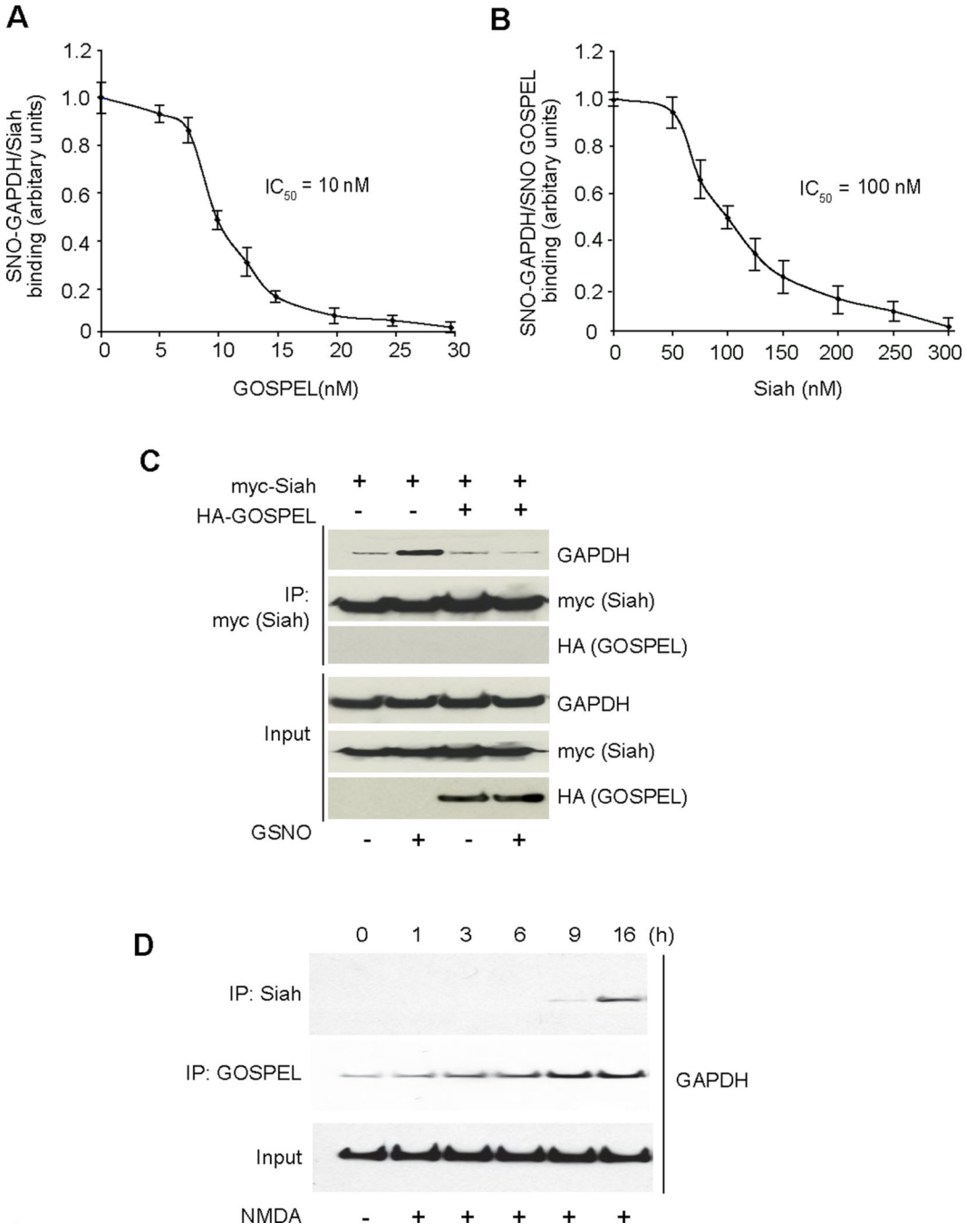

Figure 3. GOSPEL-GAPDH binding prevents GAPDH-Siah interaction

(A) Inhibition of binding between *S*-nitrosylated GAPDH (SNOGAPDH) and Siah by the addition of purified GOSPEL in a concentration-dependent manner.

(B) Inhibition of binding between *S*-nitrosylated GAPDH (SNOGAPDH) and *S*-nitrosylated GOSPEL (SNO-GOSPEL) by the addition of purified Siah in a concentration-dependent manner.

(C) Decreased GAPDH-Siah interaction in the presence of GOSPEL. HEK293 cells were transfected with HA-GOSPEL and/or myc-Siah constructs and then treated with GSH or GSNO for 16 h. Cell lysates were co-immunoprecipitated by an anti-myc antibody, and endogenous GAPDH was detected by anti-GAPDH antibody.

(D) Contemporaneous measurement of binding between GAPDH and GOSPEL as well as GAPDH and Siah in primary neurons 16 h following NMDA treatment.

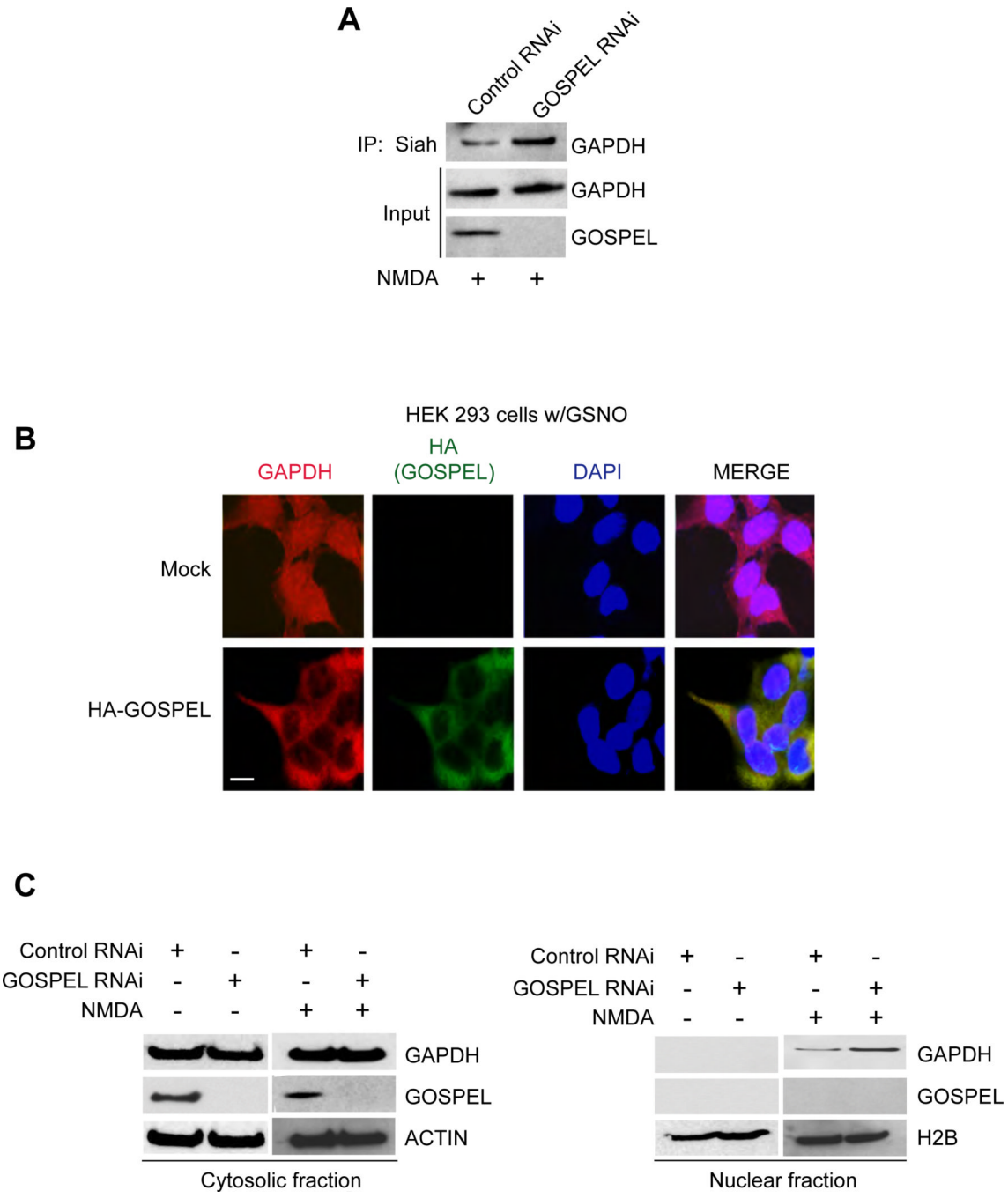

Figure 4. GOSPEL-GAPDH binding prevents GAPDH-Siah interaction

(A) GOSPEL depletion augments GAPDH-Siah binding in primary neuronal cultures after NMDA exposure. Neuron extracts were immunoprecipitated with anti-Siah antibody, and immunoprecipitates were analyzed with SDS-PAGE followed by Western blotting with anti-GAPDH antibody.

(B) Blockade of GAPDH nuclear translocation by GOSPEL. HEK293 cells were transfected with mock or HA-GOSPEL constructs followed by treatment with 200 μM GSNO for 16 h, and immunostained with anti-HA (GOSPEL) and anti-GAPDH antibodies. Representative confocal images are shown. Red, GAPDH; green, HA (GOSPEL); blue, DAPI for nucleus. Scale bar, 40 μm.

(C) GOSPEL depletion enhances nuclear GAPDH levels in primary neuronal cultures after NMDA exposure. Neurons were separated into cytosolic and nuclear fractions. Nuclear GAPDH levels were increased in GOSPEL RNAi treated cells. Anti-histone H2B and anti-actin antibodies were used as nuclear and cytosolic markers, respectively.

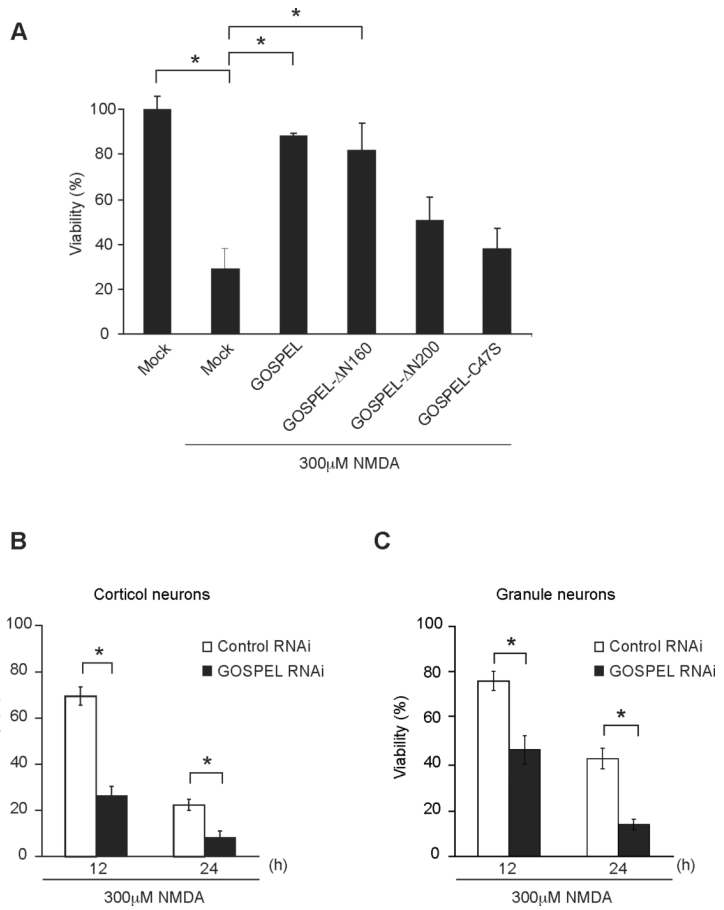

Figure 5. Modulation of NMDA-mediated neurotoxicity by GOSPEL

(A) Overexpression of GOSPEL protects neurons from excitotoxicity in a GAPDH-GOSPEL interaction dependent fashion. Primary cerebellar granule neurons were transfected with or without various GOSPEL constructs (wild-type, ΔN160, ΔN200, and C47S). Cell viability was measured 24 h after transient exposure to 300 μM of NMDA. *p<0.01, n=3, one-way ANOVA, mean ± S.E.M.

(B) Depletion of GOSPEL enhances NMDA-mediated cytotoxicity of cortical neurons. Transfection of GOSPEL RNAi in primary cortical neurons elicits a high level of neurotoxicity 12-24 h after treatment with NMDA. *p <0.01, n=3, one-way ANOVA, mean ± S.E.M. Scale bar, 20 μm.

(C) Depletion of GOSPEL enhances NMDA-mediated cytotoxicity of cerebellar granule neurons. Transfection of GOSPEL RNAi in primary granule neurons elicits a high level of neurotoxicity 12-24 h after treatment with NMDA. *p <0.01, n=3, one-way ANOVA, mean ± S.E.M.

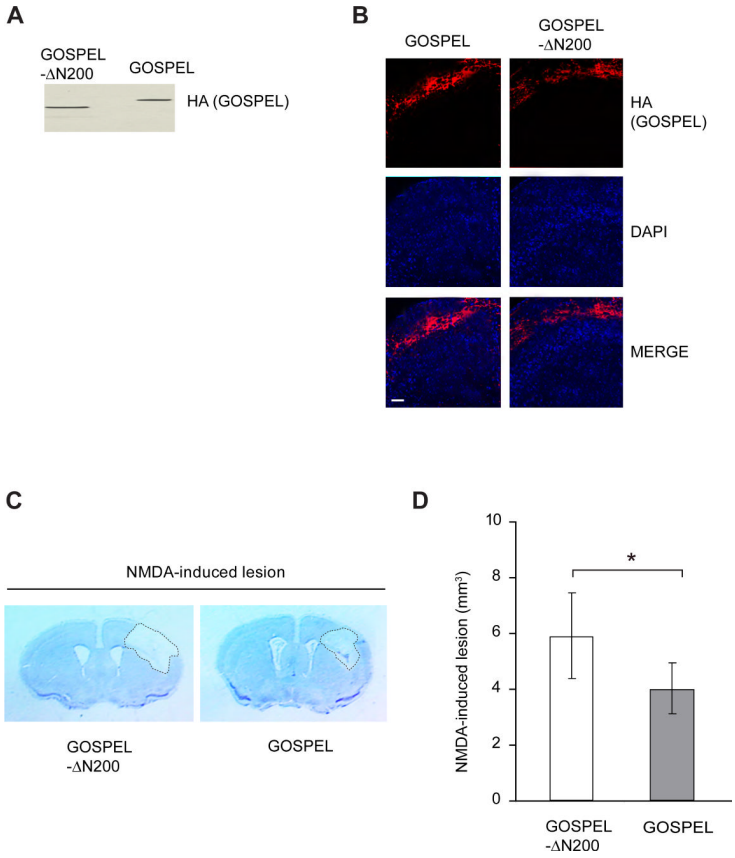

Figure 6. Prevention of NMDA induced toxicity in brains expressing wild-type GOSPEL but not GOSPEL-ΔN200 deficient in GAPDH binding

(A) Lentivirus-mediated overexpression of GOSPEL and GOSPEL-ΔN200 in the right cortex of C57BL/6 mouse brain. Proteins are expressed at similar levels for the same amount of viral particles.

(B) Lentiviral vectors encoding either for HA-GOSPEL or HA-GOSPEL-ΔN200 were stereotaxically injected in the right cortex of mice. Sections were immunostained with a polyclonal anti-HA antibody, revealing a significant overexpression of GOSPEL and GOSPEL-ΔN200 in the injected hemisphere. Scale bar, 100 μm.

(C) Representative micrographs of mouse brain sections injected with NMDA into the right cortex at the site of viral particle injection. Brains were stained with cresyl violet. Lesions are indicated by dotted lines.

(D) Quantitative analysis of NMDA-elicited brain damage. Lesion volume was 31 ± 4% smaller in mice treated with GOSPEL than GOSPEL-ΔN200. Lesion volumes are reported as mean ± S.E.M.