Iduna Protects the Brain from Glutamate Excitotoxicity and Stroke by Interfering with Parthanatos (Poly (ADP-ribose) Dependent Cell Death)

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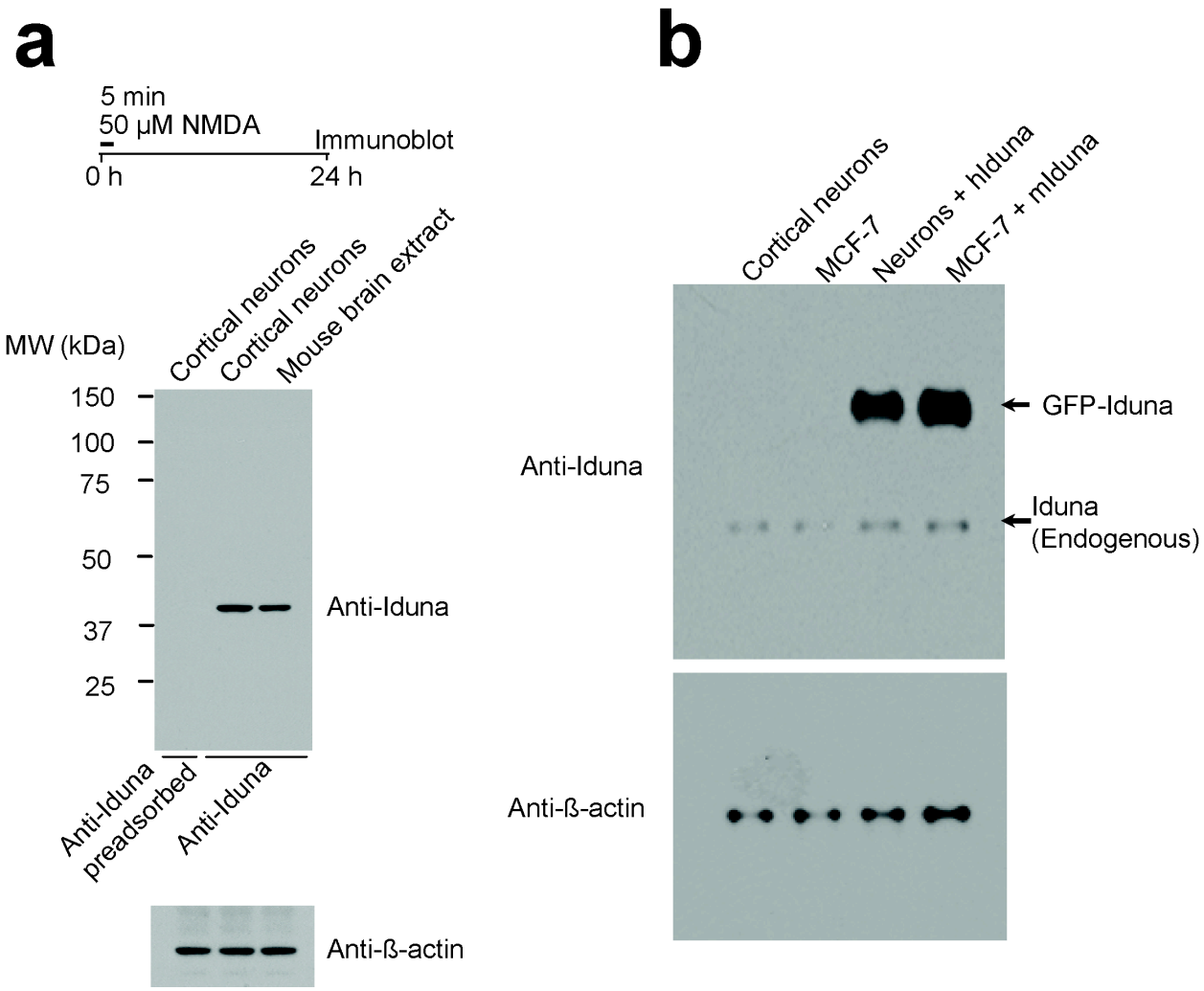
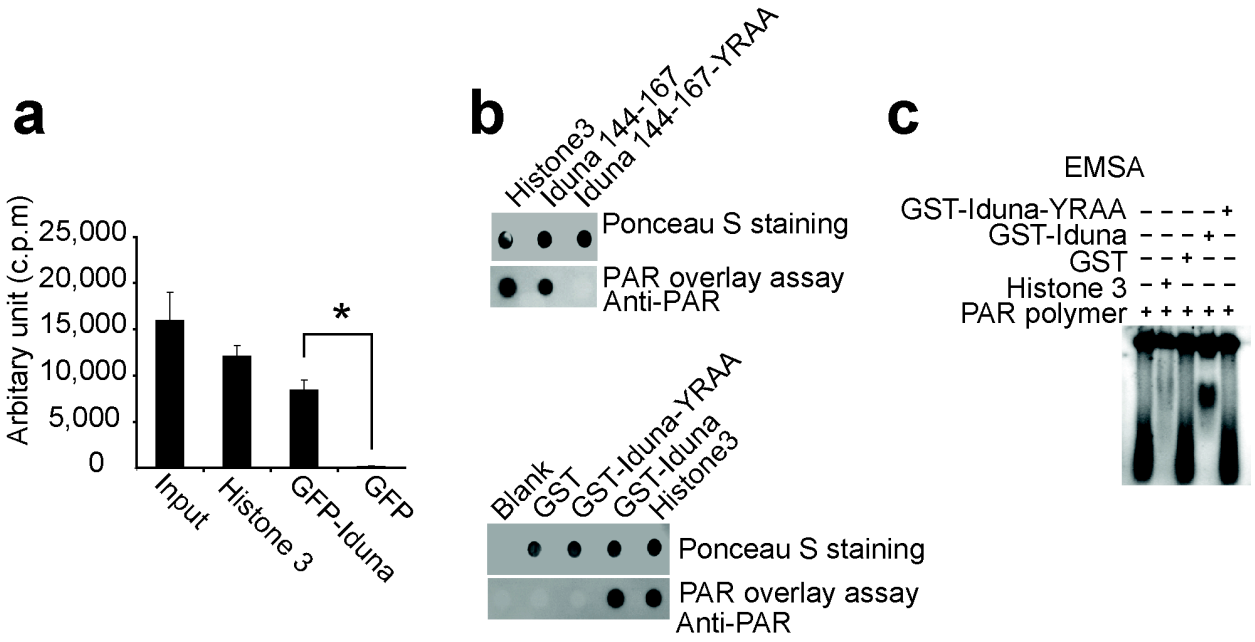
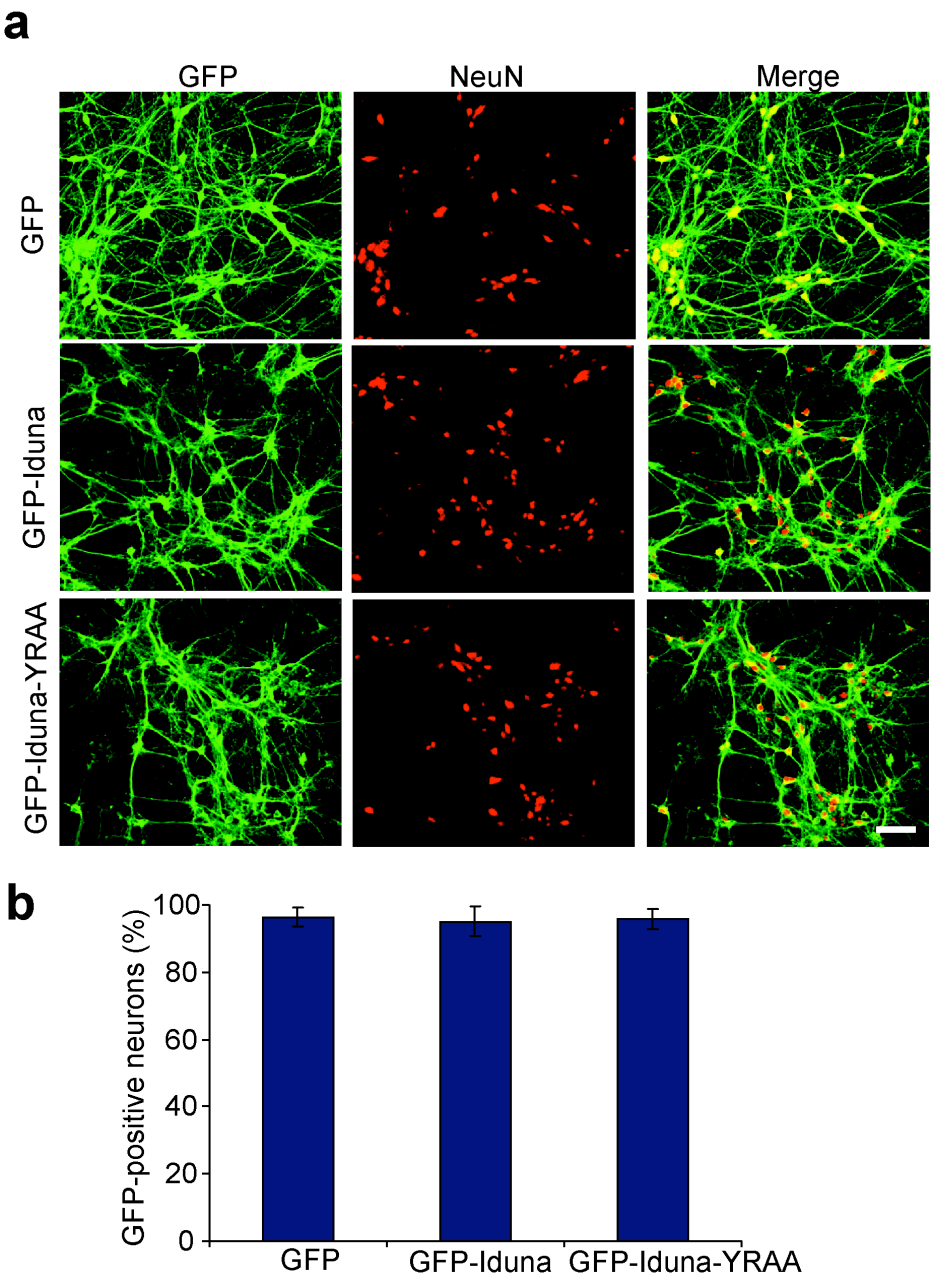


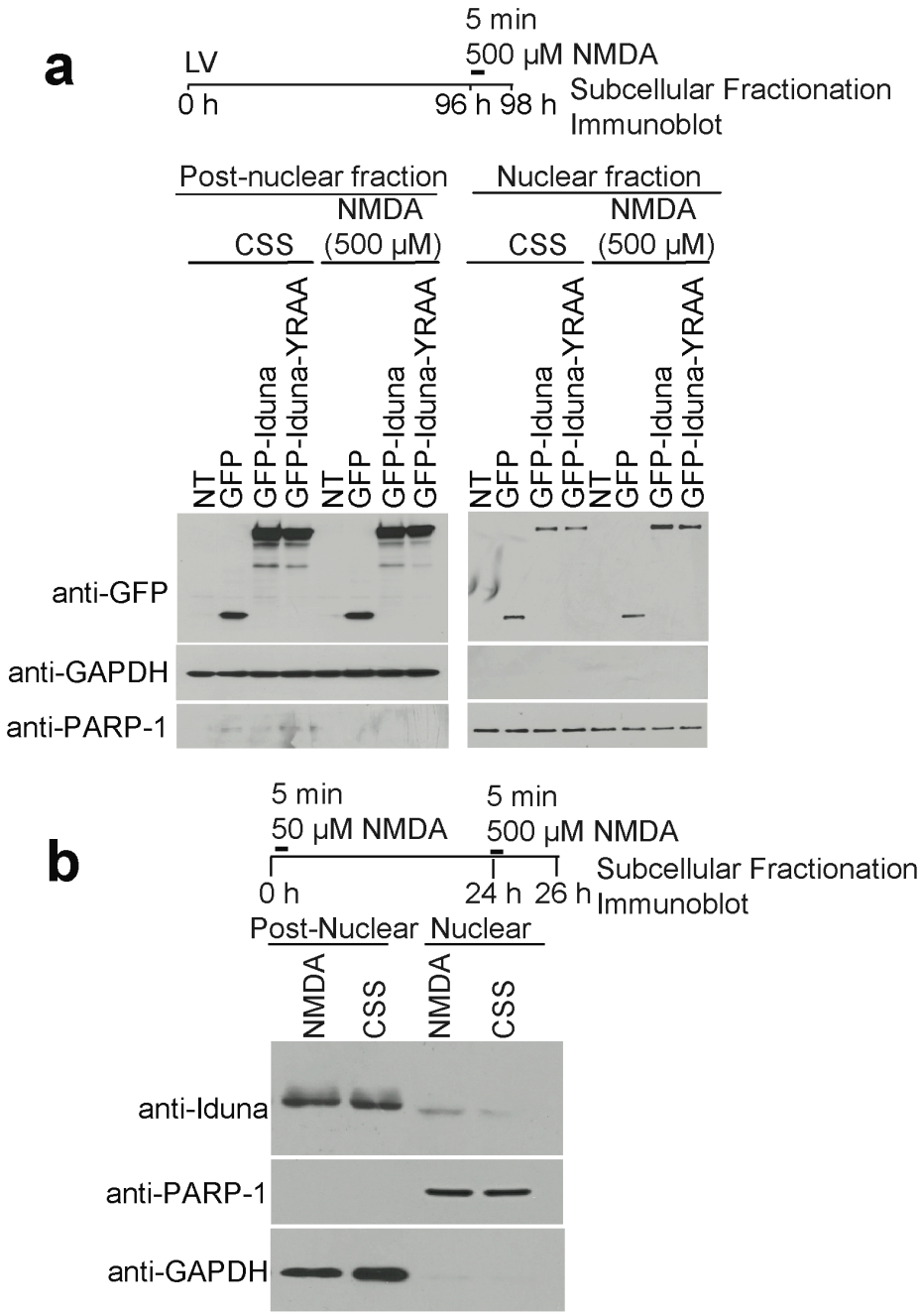
Supplementary Figure 1. Characterization of Iduna Antibody.
(a) Characterization of Iduna polyclonal antibody. Primary cortical cultures were treated with a neuroprotective dose of NMDA (50 μM for 5 min) and harvested 24 h after treatment. To test the specificity of the antibody, endogenous Iduna was pre-adsorbed from the lysate by immunoprecipitation with anti-Iduna antibody. Samples were analyzed by 8-16% SDS-PAGE. Following immunoblotting, the membranes were probed with anti-Iduna and anti-β-actin antibodies. Mouse brain lysate was used as a control. Time course of this experiment is indicated at top of the figure.
(b) The Iduna antibody recognizes both human Iduna (hIduna) and mouse Iduna (mIduna) equally. Mouse cortical neurons were transduced with a lentivirus expressing hIduna, and a plasmid expressing mIduna was transfected to human MCF7 cells. Following SDS-PAGE of the lysates and immunoblotting, nitrocellulose membranes were probed with anti-Iduna antibody. Cell lysates from both mock infected cortical neurons and mock transfected MCF7 cells were used as control to detect endogenous Iduna.



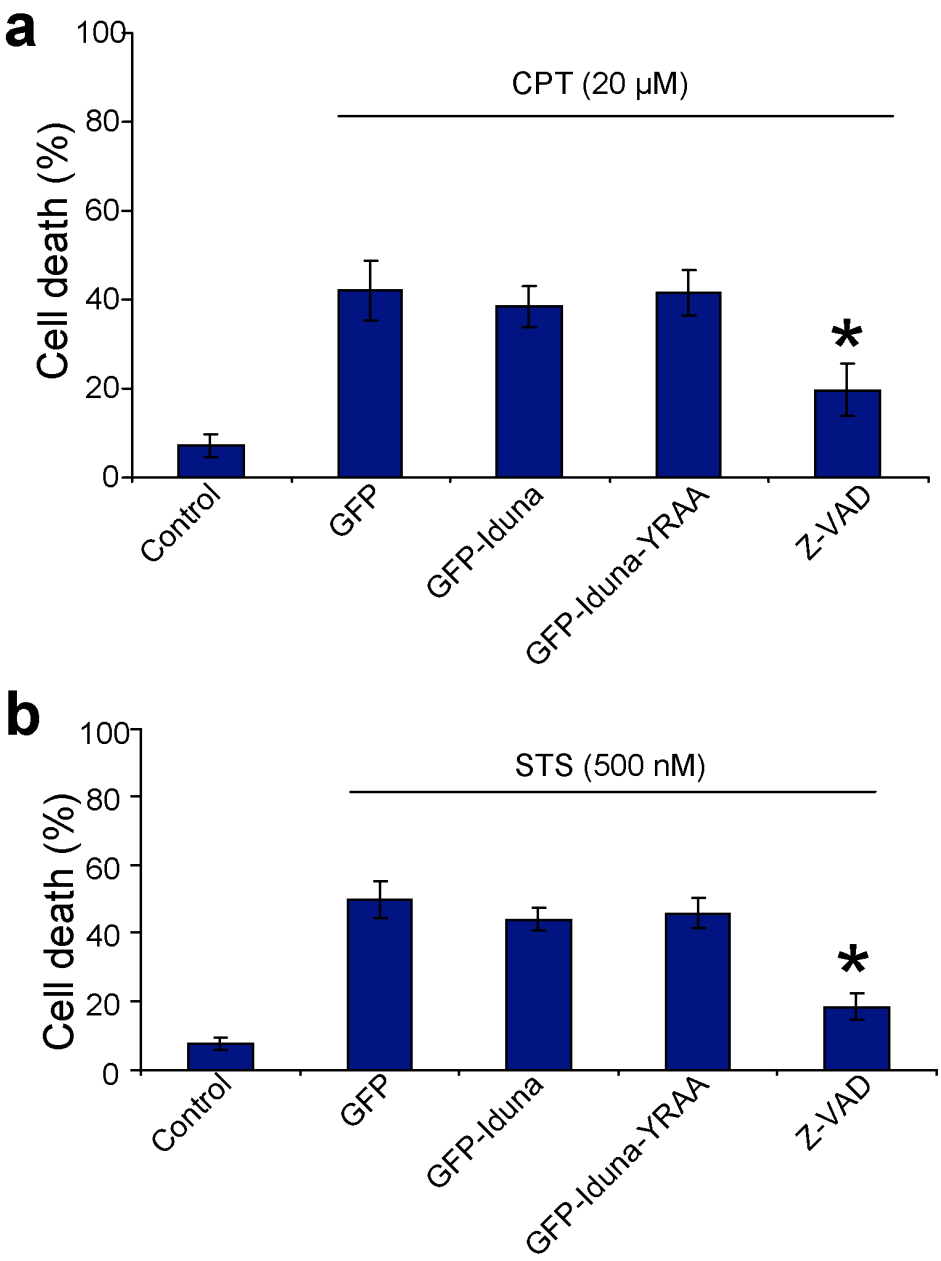
Supplementary Figure 2. PAR binding activity of Iduna.
(**a**) Immunoprecipitated GFP-Iduna, GFP or recombinant Histone 3 (H3) were incubated with synthesized [^{32}P]-PAR polymer and the amount of bound PAR polymer was measured by scintillation counting. Data are the mean ± S.E.M. from two experiments. *$p < 0.05$ by ANOVA with Tukey-Kramer's posthoc test.
(**b**) Histone 3, Iduna 144-167 wild type peptide and Iduna 144-167 YRAA (YRAA) mutant peptide (top panel), and PAR binding activity of recombinant GST, GST-Iduna-YRAA, GST-Iduna and Histone 3 (bottom panel) were analyzed for PAR binding activity by a biotin-labeled-PAR probe and subjected to immunoblot analysis with antibodies to PAR using a dot blot. Ponceau S stain of blotted proteins and peptides served to confirm that an equal amount was blotted onto nitrocellulose.
(**c**) PAR binding activity of GST-Iduna-YRAA, GST-Iduna, GST and Histone 3 were analyzed by electrophoretic gel mobility shift assay (EMSA) of [^{32}P]PAR polymer.



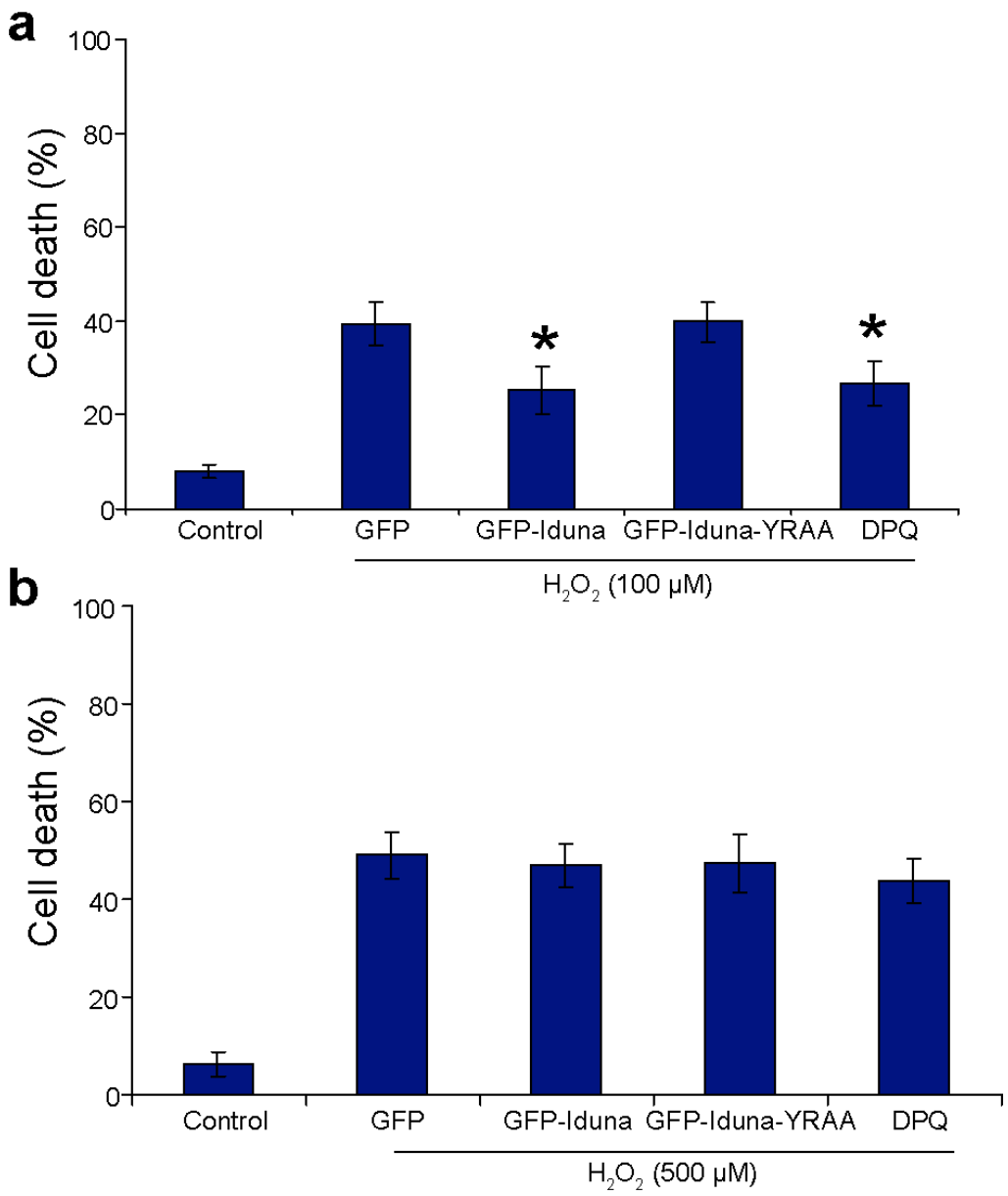
Supplementary Figure 3. Lentiviral Expression of Iduna.
(a) Lentiviral transduction in mouse cortical neurons. Primary cortical neuronal cultures were transduced with GFP, GFP-Iduna or GFP-Iduna-YRAA lentiviruses on DIV 10. On DIV 14, the cultures were fixed in 4% PFA and stained with NeuN and GFP. Following secondary antibody staining (red-NeuN, green-GFP), images were taken on a Zeiss 710 confocal microscope. Scale bar = 50 μm.
(b) Numbers of NeuN-positive and GFP-positive neurons were quantified to evaluate the transduction efficiency of the lentiviral particles. Percent GFP-positive neurons were evaluated by subtracting the number of GFP-positive neurons to the total number of neurons (NeuN-positive cells). All GFP, GFP-Iduna, GFP-Iduna-YRAA viruses have greater than 95% neuronal transduction efficiency. There is no significant difference in expression between GFP, GFP-Iduna, GFP-Iduna-YRAA transduced neurons. Data represent mean ± S.E.M., n = 3.



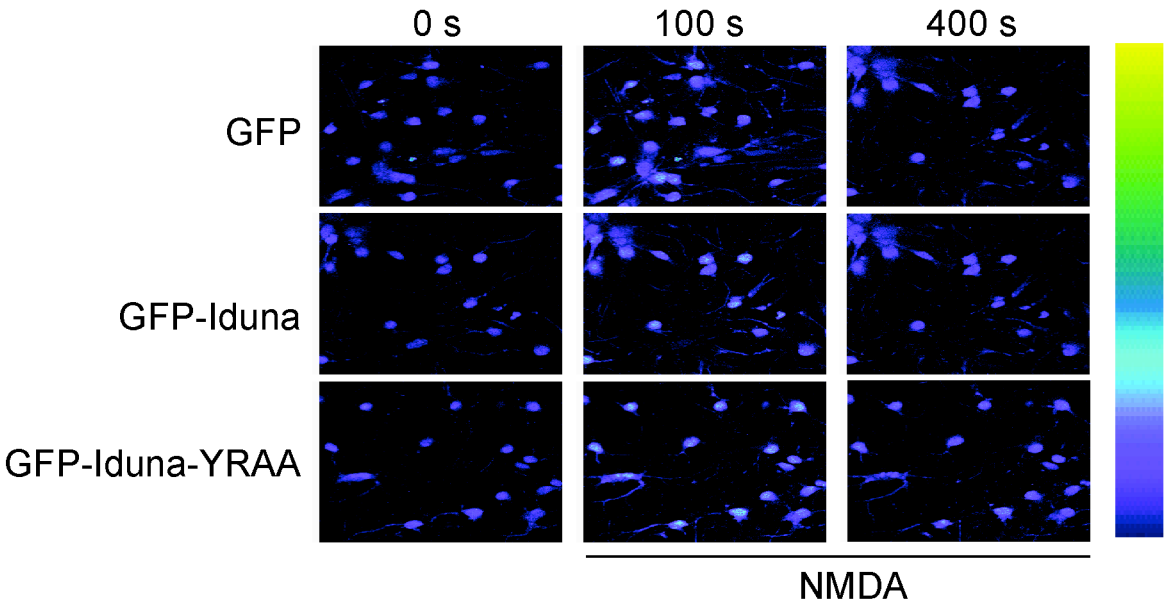
Supplementary Figure 4. Iduna is predominantly a cytosolic protein.
(a) Subcellular fractionation shows cytosolic and nuclear localization of GFP-Iduna or GFP-Iduna-YRAA. Western blots showing lentiviral-mediated expression of GFP-Iduna or GFP-Iduna-YRAA or GFP in cytosolic fractions of mouse cortical neurons 2 h following NMDA (500 μM for 5 min) excitotoxicity. NT is non-transduced cultures. GAPDH and PARP-1 serve as cytosolic and nuclear markers, respectively. CSS treated cultures were treated with CSS alone for 5 min. GFP-Iduna or GFP-YRAA are predominantly localized to the cytosol, whereas only a small amount is in the nuclear fraction. Time course of this experiment is indicated at top of the figure.
(b) Cortical Neurons were treated with NMDA (50 μM for 5 min). 24 hours later, the cells were treated with toxic dose of NMDA (500 μM for 5 min) to assess the localization of endogenous Iduna. Following NMDA toxicity, endogenous Iduna is mainly localized to cytosol (post-nuclear fractions) with a small fraction in the nucleus. These experiments were repeated two times with similar results. The time course of this experiment is indicated at the top of the figure.



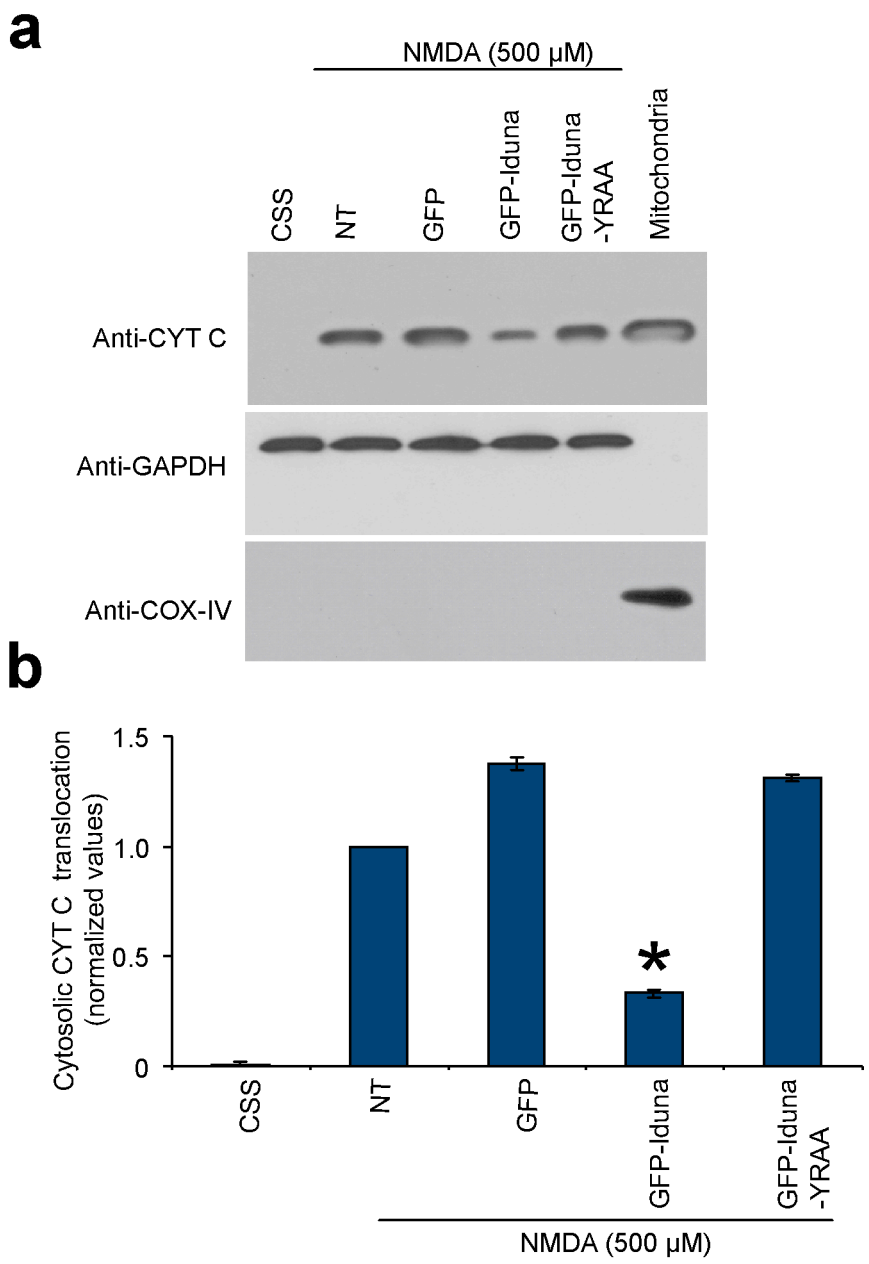
Supplementary Figure 5. Iduna is ineffective against caspase-dependent cell death.
(a) Neuronal cultures expressing lenti-viral mediated GFP, GFP-Iduna or GFP-Iduna-YRAA were treated with camptothecin (CPT, 20 μM) and cell death assessed 24 h later using PI and Hoechst staining. No protection was observed GFP-Iduna expressing neurons. The pan-caspase inhibitor, Z-VAD provides protection confirming the cell death is caspase dependent. Data represent the mean ± SEM, n = 4 of two independent experiments. *p < 0.05 by ANOVA with Tukey-Kramer's posthoc test.
(b) Neuronal cultures expressing lenti-viral mediated GFP, GFP-Iduna or GFP-Iduna-YRAA were treated with staurosporine (STS, 500 nM) and cell death assessed 24 h later using PI and Hoechst staining. No protection was observed in GFP-Iduna expressing neurons. The pan-caspase inhibitor, Z-VAD provides protection confirming the cell death is caspase dependent. Data represent the mean ± SEM, n = 4 of two independent experiments. *p < 0.05 by ANOVA with Tukey-Kramer's posthoc test.



Supplementary Figure 6. Iduna protects against peroxide-induced cell death in primary neurons. Primary neuronal cultures were transduced with lentiviruses expressing GFP, GFP-Iduna or GFP-Iduna-YRAA. 4 days following lentiviral transduction, cultures were treated with H_2O_2 (100 μM or 500 μM) and cell death assessed 24 h later using PI and Hoechst staining.
(a) GFP-Iduna expressing neurons are significantly protected against 100 μM H_2O_2 toxicity in a manner similar to the PARP inhibitor, DPQ.
(b) Neither GFP-Iduna nor DPQ protect against 500 μM H_2O_2.
Data represent the mean ± SEM, n = 4 of two experiments. *p ≤ 0.05 by ANOVA with Tukey-Kramer's posthoc test.



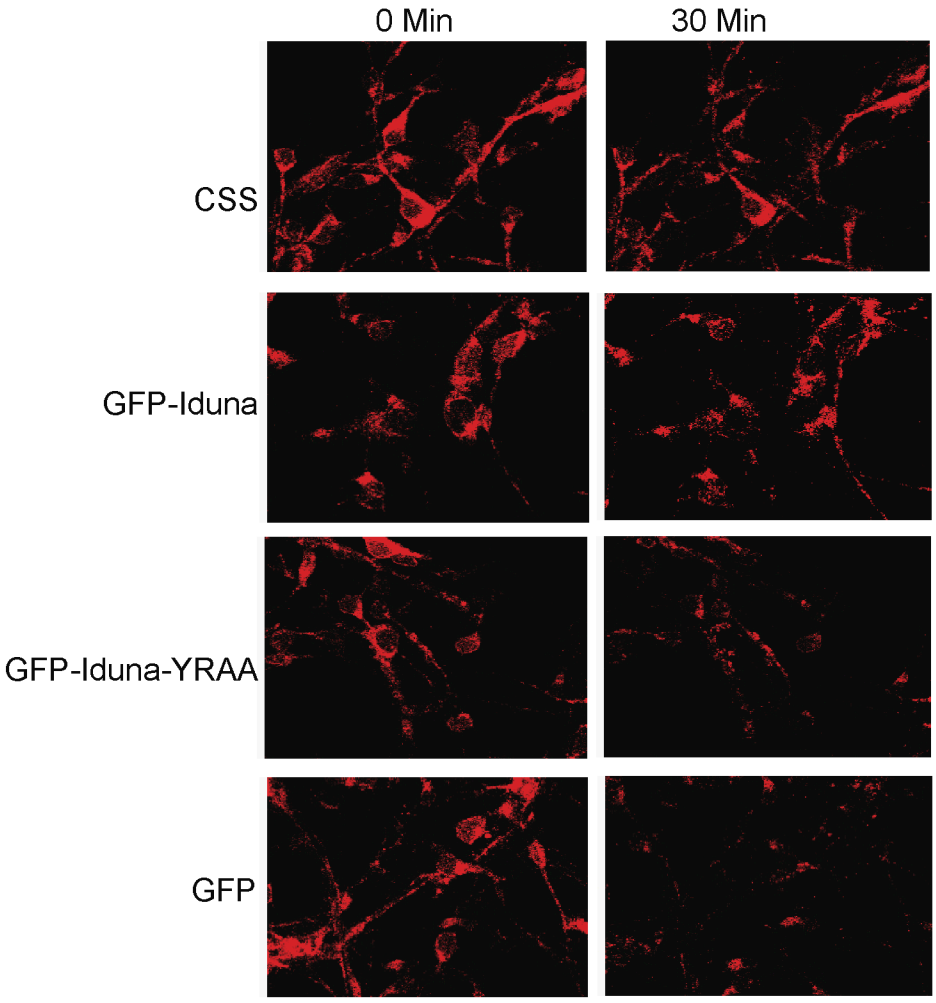
Supplementary Figure 7. Iduna does not interfere with NMDA-induced intracellular Ca^{2+} influx. Primary cortical cultures were transduced with GFP, GFP-Iduna or GFP-Iduna-YRAA lentiviruses on DIV 10. On DIV 14, the cultures were loaded with the Ca^{2+}-sensitive fluorochrome fluo-5f (2.0 µM final concentration) and a time series of confocal imaging was recorded to monitor the fluorescence intensities. NMDA 500 µM was superfused in CSS for 5 min. Representative images of the fluo-5f-loaded cultures show NMDA-mediated Ca^{2+} influx in cortical neurons. The pictures represent 0 s (before NMDA application), 100 s (at the time of NMDA application) and 400 s (after NMDA application) times points of image acquisition.



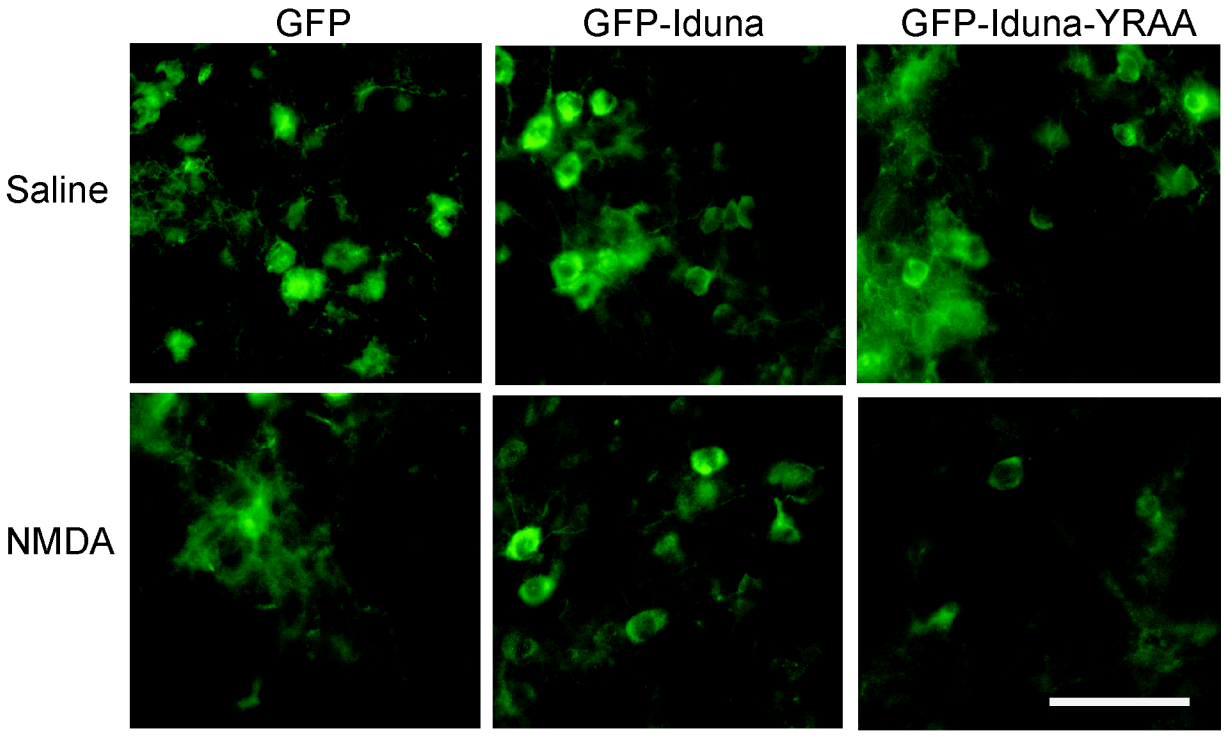
Supplementary Figure 8. Iduna reduces cytochrome c (CYT C) release after NMDA excitotoxicity.

(a) Immunoblot showing release of CYT C. Mouse cortical neurons over-expressing GFP, GFP-Iduna or GFP-Iduna-YRAA were subjected to NMDA-excitotoxicity (500 μM for 5 min) on DIV 14. Following subcellular fractionation into mitochondrial and cytosolic (post-mitochondrial) fractions, CYT C release was monitored 4 hrs following NMDA stimulation. COX IV was used as a marker for mitochondria and GAPDH was used a marker for cytosol. Data were repeated three times with similar results.

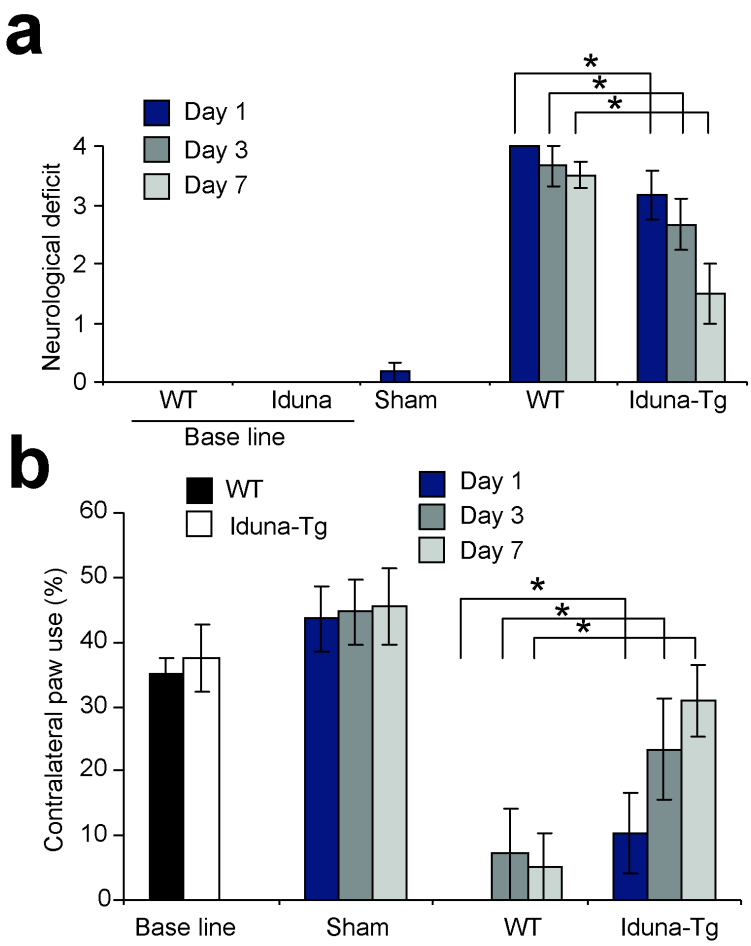
(b) Quantification of cytochrome c release from mitochondria following NMDA-toxicity. Iduna significantly (*$p < 0.05$) reduces CYT C release monitored 4 hrs following NMDA-stimulation. Values of optical density were quantified using Image J software and the data were normalized to the values of GAPDH in the cytosol.



Supplementary Figure 9. Iduna protects against NMDA-induced loss of $\Delta\psi_m$ in mouse cortical neurons. Representative images of TMRM fluorescence before and after NMDA-application. Primary cortical neurons expressing GFP, GFP-Iduna or GFP-Iduna-YRAA were loaded with TMRM (100 nM) for 20 min and then live imaging was conducted for 20 min, using an LSM 5 Live confocal microscope (Carl Zeiss, Germany). NMDA-stimulation (500 µM for 5 min) leads to a substantial loss of $\Delta\psi_m$ (TMRM fluorescence) in mitochondria. Overexpression of Iduna in mouse cortical neurons protects against NMDA-induced loss of $\Delta\psi_m$, where as no protection was observed in Iduna-YRAA or GFP-expressing neurons.



Supplementary Figure 10. Iduna protects against NMDA-induced neuronal loss *in vivo*. Representative pictures taken from striatum of 6 week old C57BL/6 mice showing expression of GFP, GFP-Iduna or GFP-Iduna-YRAA following lentivirus transduction. GFP positive cells are lost following injection of NMDA (20 nmoles) but not normal saline 5 days after lentiviral transduction. Magnification bar = 50 μm.



Supplementary Figure 11. Behavioral Assessment following MCAO.
(a) Neurological deficit was evaluated on a scale of 0-4 (0 no neurological deficit, 4 severe neurological deficit) by the following criteria: 0= mice appeared normal, explored the cage environment and moved around in the cage freely; 1= mice hesitantly moved in cage and didn't approach all sides of the cage, 2= mice showed postural and movement abnormalities and had difficulty to approach the walls of the cage, 3= mice with postural abnormalities tried to move in the cage but did not approach to the wall of the cage, 4= mice were unable to move in the cage and stayed at the center. Recordings were scored by an observer blind to the treatment and genotype. Data represent the mean ± SEM, n = 5. *p ≤ 0.05 by ANOVA with Tukey-Kramer's posthoc test.
(b) Forelimb use of the mouse was recorded for 10 minutes in a glass cylinder and analyzed according to the following criteria: Ipsilateral (right) forelimb use (number of touches to the cylinder wall) independent of the left limb, contralateral (left) forelimb use (number of touches to the cylinder wall) independent of the right limb and simultaneous use of both limbs. The percent use of the contralateral (left) limb was quantified by subtracting contralateral forepaw touches from the total number of touches made by the mouse during the period of observation. Recordings were scored by an observer blind to the treatment and genotype. Data represent mean ± SEM, n = 5. *p ≤ 0.05 by ANOVA with Tukey-Kramer's posthoc test.

Supplemental Methods

Primary Neuronal Culture Preparation: Primary cortical cell cultures were prepared from gestational day 15 mouse embryos as previously described [1]. Experiments were performed at DIV (day *in vitro*) 14. Under these conditions, neurons represent 90% of the cells in the culture. Mature neurons were washed with control salt solution (CSS) containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM Tris-HCl pH 7.4 and 15 mM D-glucose. To induce NMDA-mediated protection, 50 μM NMDA and 10 μM glycine in CSS solution was applied to the cells for 5 min, then the cells were washed and re-supplemented with minimum essential medium containing 10% horse serum. Sham treatment control was performed as above except for a 5 min treatment with CSS alone. NMDA excitotoxicity was induced by treating cultures with 100 or 500 μM NMDA and 10 μM glycine in CSS for 5 min. MNNG, 50 μM was applied to neurons for 15 min, cells were washed and re-supplemented with the normal growth media. All experiments were performed in accordance with the National Institutes of Health Guidelines and were approved by the institutional animal care and use committee.

Oxygen-Glucose Deprivation (OGD): For oxygen-glucose deprivation the culture medium was removed and the cells were washed with glucose free media to remove the entire medium containing glucose. OGD was initiated by addition of glucose-free medium that was pre-bubbled for 20 min with a mixture of OGD gas (5% CO_2, 9.8% hydrogen and the rest N2, (Airgas Ltd. USA) to remove the dissolved O_2 from the media. The cultures were then immediately transferred into a hypoxia chamber connected to an O_2 sensor/monitor (Biospherix Ltd. USA) and maintained at 37°C. OGD is terminated by resupplying the normal growth media and transferring back the incubator containing 5% CO_2 in room air.

Cell Death Assessment: Following exposure of neuronal cultures to the various treatments neuronal survival was quantified and presented as percent of cell death. Percent cell death was determined as the ratio of live-to-dead cells compared with the percent cell death in control wells to account for cell death attributable to mechanical stimulation of the cultures. Quantification of neuronal survival was determined by staining treated cultures with 5 μM Hoechst 33342 and 2 μM propidium iodide (PI) (Invitrogen, Carlsbad, CA) [1]. Culture plates were placed on a mechanized stage of a Zeiss microscope and photomicrographs were collected by a blinded observer. The numbers of total and dead (PI positive) cells were counted by automated computer assisted software (Axiovision 4.6, Zeiss, Germany) [1]. The raw counts are presented in an Excel file for generation of percent cell death and statistical analysis. Glial nuclei fluoresce at a lower intensity than neuronal nuclei and were gated out by the software program. At least two separate experiments using four separate wells were performed for all

data points.

Cell Death Assessment in GFP-Transfected Mouse Neuronal Cultures: GFP, GFP-Iduna or GFP-Iduna-YRAA constructs were transfected into mouse cortical neurons on DIV 11, using lipofectamine 2000 (Invitrogen). On DIV 13, the cultures were treated with NMDA (500 μM for 5 min) to induced excitotoxicity. 24 h later, images were taken from the transfected neurons using Axiovert M 200 Zeiss microscope and Axiovision 6 software. GFP expressing neurons with fragmented processes were considered as dead cells. % cell death was assessed by subtracting the number of fragmented (dead) cells to the total number of transfected (GFP-positive) cells in the cultures.

Northern Analysis: FirstChoice Mouse blot was purchased from Ambion. Each probe from Iduna cDNA, ß-tubulin, and ß-actin was labeled with γ-^{32}P dATP using Strip-EZ DNA kit (Ambion). The membrane was prehybridized in hybridization buffer and then hybridized with each probe at 55°C overnight. The membrane was washed with 1 X SSC at 37°C and 0.5 X SSC at 65°C, respectively. The membrane was exposed on storage phosphor screen (Packard) for 24 h. Signals were detected using Cyclone Storage Phosphor System (Packard).

Cloning of Iduna Genes: Iduna complementary DNAs were cloned from mouse cDNA and sequenced. Iduna PCR products were cloned into the phCMV1-Xi vector (Gene Therapy Systems), pEGFP-C2 vector (Clontech), pCMV-Tag5 vector (Stratagene) and pGEX-6p vector (GE Health Care). Deletion mutants and YRAA mutants were constructed by PCR and were verified by sequencing.

Antibody Preparation and Immunoprecipitation: The peptide NH2-GCDAPVVVAQHSLTQQRPLVPN-OH was synthesized from the amino acids 298 - 317 of Iduna (Gly and Cys were added to the sequence). The purified peptide was injected as an antigen to raise rabbit polyclonal anti-Iduna anti-sera. Iduna antibody was purified from anti-sera using Sulfolink (Pierce) with the purified Iduna protein. GST-Iduna fusion protein was cloned into pGEX-6p vector, expressed in E. coli, and Iduna protein was purified with glutathione-Sepharose 4B beads after cleavage of GST by Precision protease according to the manufacturer's instruction (Amersham Biosciences).

For immunoprecipitation, neuronal cell extracts were collected in 0.5 ml IP buffer (Phosphate buffered saline containing 1 mM EDTA, 1 mM EGTA, 0.5 % NP-40, 1 % Triton X-100, 0.25 mM sodium orthovanadate, 0.25 mM PMSF, 2.5 μg/ml leupeptin, 2.5 μg/ml aprotinin) and incubated for 30 min at 4°C with constant agitation. After centrifugation (16,000 X g, 4°C for 15 min), the resulting supernatants were subjected to immunoprecipitation by incubation for 1 h

at 4°C with anti-PARP-1 (BD Biosciences), anti-GFP (Abcam), anti-PAR (96-10) or anti-Iduna antibodies. Following the additions of protein G-agarose beads, the mixtures were incubated for 1 h at 4°C. After washing with IP buffer, bound proteins were subjected to immunoblot analysis.

Immunoblot Analysis: Neuronal cultures were exposed to NMDA for 5 min. Cell lysates were subjected to centrifugation at 12,000 X g for 10 min at 4°C. The resulting supernatant was subjected to SDS-PAGE, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was incubated with a Tris-buffered saline solution containing 5% nonfat milk and 0.1% Tween 20. The membrane was then incubated for 1 h at room temperature with the indicated antibodies in a Tris-buffered saline solution containing 0.1% Tween 20 and subsequently with appropriate secondary antibodies conjugated with horseradish peroxidase (Amersham Biosciences). The immunoblots were visualized in X-ray films by an enhanced chemiluminescence method (Pierce, USA). Antibodies used include: anti-c-myc (Roche Applied Sciences USA), anti-PAR [2], anti-PARP-1 (BD Pharmigen USA), anti-GFP, anti-COXIV (Abcam Inc Cambridge, MA), HRP conjugated anti-β-actin, anti-β-tubulin, and anti-biotin (Sigma, USA), anti-AIF (Epitomics, Burlingame, CA) [3].

*FAR Western, PAR Overlay and EMS*A: Synthetic peptides, purified proteins or immunoprecipiated samples were diluted in TBS-T buffer (1 µg/µl) and loaded onto a nitrocellulose membrane (0.05 µm) using a dot blot manifold system (Life Technologies) for far western analysis. For the PAR overlay assay, immunoprecipiated samples were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were washed once with TBS-T buffer and air-dried followed by incubation with indicated concentrations of biotin-labeled PAR polymer for 1 h at room temperature with constant shaking. After washing in TBS-T buffer at 4°C, the membranes were probed with anti-PAR or anti-biotin antibodies. Immunoblots were visualized in X-ray films by an enhanced chemiluminescence method (Pierce). For EMSA analysis, 100 ng of purified proteins (0.1 µg/µl) were incubated with [^{32}P]-labeled PAR polymer for 2 min at RT thereafter samples were resolved in 5% PAGE-gel. The gel was heat dried and developed using a Typhoon 9400 Imager (GE Health Care).

Biotin and [^{32}P]-labeled Automodified PARP-1 Synthesis and PARP-free PAR Preparation: Biotin and [^{32}P]-labeled automodified PARP-1 were synthesized according to Shah *et al.* [4] and modified as described in Gagné *et al.* [5]. Briefly PARP-1 purified up to the DNA-cellulose step (600 U/mg) was incubated with biotin labeled NAD^+ and [^{32}P]-NAD^+ for 2 min at 30°C, thereafter nonlabelled/nonisotopic NAD^+ was added to the reaction mixture and incubated for further 28 min at 30°C. The high specific activity biotin labeled NAD^+ and [^{32}P]-labeled automodified PARP-1 (80 cpm/nmol) were precipitated as described [6]. Biotin-labeled, non-radioactive and

[^{32}P]-labeled free PAR was prepared and purified on a DHBB column as described [7]. Polymer size was assessed by 20% TBE-PAGE (90 mM Tris-borate pH 8.0, 2 mM EDTA) 45 and HPLC chromatography using a DEAE-NPR column [8].

Nitrocellulose PAR-binding Assay: Synthetic peptides or purified proteins were diluted in TBS-T buffer (1 µg/µl) and loaded onto a nitrocellulose membrane (0.05 µm) using a dot blot manifold system (Life Technologies). The membranes were washed once with TBS-T buffer, removed from the manifold and air dried. The membranes were then incubated in 10 ml of TBS-T buffer containing an indicated concentration of both [^{32}P]-labeled automodified PARP-1 and [^{32}P]-labeled PAR polymer for 3 h at room temperature with constant shaking. The membranes were washed with TBS-T buffer at 4°C until no radioactivity could be detected in the waste. Finally, the membrane was air dried and subjected to autoradiography on Bio-Max MR (Kodak) films or analyzed by Cerenkov counting using an Instant Imager system (Perkin Elmer).

Chemiluminescent PARP assay for poly(ADP-ribosyl)ation of Histone H1: Activity of PARP-1 was determined by Trevigen Universal chemiluminescent PARP assay kit (Trevigen, Gaithersburg, MD, USA) in the presence of Iduna or the PARP-1 inhibitor 3-aminobenzamide (3-AB). According to manufacturer's instructions, the incorporation of biotinylated NAD+ (Trevigen) into the poly(ADP-ribose) polymer primed at solid phase immobilized histone H1 (Trevigen) was detected by the chemiluminescent assay, either in the presence or absence of recombinant Iduna. Reactions were performed with 34 µM total biotinylated NAD+ for 60 min at 25°C in triplicates. Chemiluminescence was measured using a fluorescence multi-well plate reader (SOSTmax, Sunnyvale, CA) with an excitation at 544 nm excitation/ 590 nm emission. In the parallel wells, PARP1 activity was measured after the 3-aminobenzamide treatment at the same concentration of biotinylated NAD+ (34 µM).

PARP Inhibition Assay: To evaluate the ability of Iduna to act as a PARP inhibitor, [^{32}P]-labeled automodified PARP-1 was synthesized in presence of GST, GST-Iduna or PARG, respectively. To purify the [^{32}P]-labeled automodified PARP-1, the reaction mixture was incubated with anti-PAR antibody for 4 h at 4°C and then sequentially incubated with protein-G slurry for 1 h at 4°C. Samples were washed two times with PBS and the amount of [^{32}P]-labeled automodified PARP-1 was measured by LS 6500 Liquid Scintillation Counting System.

Lentiviral Preparations for Overexpression and RNAi: We used Invitrogen ViraPower lentiviral packaging system and obtained high-titer viral preparations for effective transduction in primary neuronal cultures and for intra-striatal injections. For developing efficient shRNA lentiviruses, we subcloned a siRNA oligo directed to the coding region +556 – 576 of Iduna into a lentiviral

expression vector, cFUGw. The oligo was PCR amplified with primers flanked by PacI restriction sites. Following digestion and ligation, clones were selected and verified for the inserted sequence. The lentiviral construct co-expresses EGFP driven by the Ubiquitin C promoter, in addition to the mouse U6 Pol II promoter driving the shRNA. To control for off-target and non-specific effects of shRNA, a shRNA against dsRed was used. The over expression lentiviral system was developed by removing the EGFP open reading frame from the cFUGw construct by a BamHI/XbaI digestion and replacing it with the cDNA of GFP-Iduna or GFP-Iduna YRAA. Near 100% neuron specific expression is observed, using either our over expression or RNAi lentiviral system. The cDNA of human Iduna was cloned from human MCF-7 cells mRNA by reverse transcription-PCR (RT-PCR) and then it was subcloned to pEGFP-C2 to create EGFP human-Iduna. The construction of cFUGW-EGFP human-Iduna, was performed by digesting the pEGFP- human-Iduna by BamHI/XbaI followed by subcloning into the same enzyme restriction sites of cFUGW. DNA sequences were verified by sequencing.

Real Time PCR: RNA was isolated from primary cortical neurons. Reverse transcription by PCR was performed using an oligo-dT primer. Following cDNA generation, quantitative PCR was performed using Iduna primers: sense- 5'-tgg gtg gtg gca gta tga tga gc-3', and antisense- 5'-ctt cac ctc tgt gac tcc gtt cag c-3'. Actin primers: sense- 5'- gct cgt cgt cga caa cgg ctc-3', and antisense- 5'-caa aca tga tct ggg tca tct tct c-3' were used for normalization. 50 cycles were used for quantitative PCR, using conditions: 94˚C (30 s), 58˚C (30 s) and 72˚C (30 s).

Calcium Imaging: Neuronal cells were plated on 17 mm glass cover slips, pre-coated with poly-L-ornithine (0.1 mg/ml). On DIV 14, the cultures were loaded with calcium sensitive dye Fluo-5f (2.5 µM, dissolved in pleuronic acid/DMSO solution) for 45 min at 37˚C and thereafter placed on a thermostatically confocal microscopic stage (Carl Zeiss). HEPES balanced salt solution (HBSS: NaCl 137 mM, KCl 5 mM, HEPES 20 mM, glucose 10 mM, $CaCl_2$ 1.4 mM, $NaHCO_3$ 3 mM, Na_2HPO_4 0.6 mM, KH_2PO_4 0.4 mM, pH 7.4) was superfused to the cultures for 100 s to obtain a steady base-line. NMDA (500 µM for 5 min) was used to induce intracellular calcium influx. Florescence values were monitored at every 10 s and calculated using LSM 510 Meta software and represent the changes in cellular calcium.

Mitochondrial Membrane Potential ($\Delta\psi_m$): TMRM was used to determine $\Delta\psi_m$. Mouse neuronal cultures were loaded with TMRM (100 nM) for 20 min. Thereafter live-cell imaging was captured using LSM 510 Live Confocal microscope (Carl Zeiss, Germany) for 20 minutes and fluorescence values for TMRM were calculated using LSM 510 confocal software (Carl Zeiss). Following 50-100 seconds base-line stabilization, NMDA (500 µM for 5 min) was directly perfused during image acquisition on the confocal microscopic stage using a peristaltic pump

(Gilson). Live images were acquired at an interval of 20 s using a low laser power to avoid excessive bleaching. Due to spectral overlap with GFP, plasma membrane potential could not be determined

Mitochondrial Isolation: Mitochondria were isolated from C57B6 mice by percoll gradient [9]. Mice were sacrificed and forebrains were rapidly removed, minced and homogenized in isolation buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES / KOH (pH 7.4), 1 mM EGTA and 1 mg/ml fatty acid free bovine serum albumin (BSA). Two pooled mouse forebrain homogenates were centrifuged at 1,800 g for 5 min and the resulting the supernatant was again centrifuged at 12,000 g for 10 min. The pellet containing mitochondria was resuspended in 15% percoll solution and layered over a 23%-40% percoll gradient. Following centrifugation at 30,000 g for 10 min, the synaptosomal-free mitochondrial fraction was collected between the interface of 23% and 40% percoll layers. The mitochondrial fraction was carefully collected with minimum contamination from the lower 40% percoll layer and washed twice with isolation buffer at 12,000 g for 10 min. The resulting pellet was resuspended in 100 µl of isolation buffer without EGTA and BSA. The samples were kept on ice until use. All mitochondrial preparations were used within 3-4 h of isolation.

Measurements of Mitochondrial Ca^{2+}*-uptake* Capacity: Extramitochondrial free Ca^{2+} was monitored in the presence of isolated mitochondria or digitonin-permeabilized cells, using an indicator of extramitochondrial free Ca^{2+} (Calcium green-5N, Invitrogen). Isolated mitochondria (100 µg protein) were suspended in potassium chloride (KCl) media containing 125 mM KCl, 2 mM K_2HPO_4, 1 mM $MgCl_2$, 20 mM HEPES (pH 7.0) and 0.1 µM Calcium green-5N. Mitochondrial substrates 5 mM glutamate, 5 mM malate and 1mM ADP were added to media at the time of assay. Fluorescence was continuously monitored at an excitation/emission at 488/532 nm respectively. All the assays were performed at 37°C using an attached circulating water bath. For measurement of mitochondrial Ca^{2+} uptake in digitonin-permeabilized cells, the cultures were harvested in growth media by trypsinization. Following centrifugation in growth medium at 2000 x g for 3 min, the cells were re-suspended in KCl medium (1×10^7 cells/ml) containing mitochondrial substrates 5 mM glutamate, 5 mM malate and 1mM ADP in presence of 0.1 µM Calcium green-5N. The plasma membranes were then selectively permeabilized with digitonin (50 µg/ml, Sigma USA). Ca^{2+} uptake was monitored by addition of 50 µM $CaCl_2$ to the assay medium using a Hamilton syringe.

Construction of the ROSA26-Iduna Targeting Vector: The plasmid pBigT, which has adenovirus splice acceptor (SA), followed by a *loxp* site, phosphoglycerine kinase (PGK)-neo cassette, transcriptional stop sequences (tpA), another *loxp* site, a multiple cloning site (MCS), and the

bovine growth hormone polyadenylation sequence (bpA). Both PacI and AscI sites were 5' to the SA, and an AscI site 3' to the bpA, respectively. To generate the pBigT-Iduna construct, the *Iduna* cDNA was excised from pEGFP-Iduna with XhoI and NotI and inserted into the same sites in a MCS of pBigT as indicated in Figure 6A. The resulting plasmids were digested with Pac1 and Asc1 to release the SA-*loxp*-(PGK)Neo-tpA-*loxp*-iduna-bpA cassette, which was then inserted into a ROSA26 targeting vector, pROSA26PA. pROSA26PA was kindly provided by Dr. Soriano (University of Washington, Seattle, Washington, USA)

Transgenic Mice: ES cells were targeted and screened as described in Soriano et al. [10,11]. Briefly, the pROSA26PA-iduna plasmid was linearized with KpnI and electroporated into ES cells derived from mouse strain 129SvEv. Twelve of 277 G418-resistant colonies had undergone correct homologous recombination, as determined by Southern blot and PCR of the ROSA26-Iduna allele. Two of these ROSA26-Iduna ES clones were injected into C57BL/6 blastocysts. Resulting chimeras were bred to C57BL/6 mice and offspring were tested for germline transmission. Heterozygous mice for the Rosa26PA-Iduna allele were crossed to Nestin-Cre$^{(+/+)}$ mice (on C57BL/6 background), which were obtained from the Jackson Laboratory, to obtain ROSA26PA-Iduna$^{(+/loxP)/}$Nestin-Cre$^{(-/+)}$ mice. Nestin-cre/ROSA26PA-Iduna transgenic mice, in which Cre recombinase expression is under the control of the nestin promoter, specifically expresses Iduna in brain. Mice were genotyped by PCR analysis using primers (5′-AAAGTCGCTCTGAGTTGTTAT-3′, 5′-GCGAAGAGTTTG TCCTCAACC-3′ and 5′-GGAGCGGGAGAAATGGATATG-3′) to select the ROSA26PA-Iduna locus. Nestin-Cre mice were genotyped by PCR using following primers; CreA, 5'-CCCGGCAAAACAGG TAGTTA-3'; CreS, 5'-CATTTGGG CCAGCTAAA CAT-3' (93°C for 30 s, 51°C for 30 s, 65°C for 40 s).

*Bilateral Common Carotid Artery Occlusion (*BCCAO*):* 8 week old C57BL/6 mice were anesthetized with 3% isoflurane and kept under anesthesia with 1% isoflurane in air for the entire period of surgery. Body temperature was maintained at 37˚C with a heating pad and a rectal probe (Harvard Apparatus, USA). Skin along the midline on the throat was cleaned and swabbed 3 times with 70% ethanol and betadine. A midline incision was given to expose the common carotid arteries (CCA) on either sides of the trachea. Sterile normal saline was used on the incision and CCAs to keep the tissues and arteries moist, and avoid any tissue dehydration. Micro-vascular clamps (Fine Science Tools, USA) were used to occlude both right and left CCA (bilateral CCA occlusion, BCCAO) for 5 minutes. Blockade of blood flow to the brain through CCAs could be seen visually. After 5 minutes the micro-vascular clamps were slowly released to allow CCA reperfusion to the brain. The incision was sutured using a silk 5-0 suture (Ethicon, USA). Anesthesia was withdrawn and animals were transferred to a warm chamber to allow recovery from anesthesia. Following complete recovery from anesthesia, the animals were

returned to the cages and housed individually. At 48 h following BCCAO, animals were sacrificed under decapitation and brain tissue was collected for biochemical analysis. The entire surgical procedure was performed except BCCAO in sham operated mice.

Middle Cerebral Artery Occlusion (MCAO): To occlude the middle cerebral artery, mice were anesthetized with 1.5–2% isoflurane and maintained at normothermic temperature. A 7-0 monofilament with an enlarged silicone tip was passed through the right internal carotid artery to the base of the middle cerebral artery. Occlusion was confirmed by laser-Doppler flowmetry with a probe placed on thinned skull over the lateral parietal cortex. After 60 min of occlusion, the filament was removed and reperfusion was verified. At 24 h of reperfusion, the brain was harvested, sectioned into five coronal slabs, and stained with the vital dye, triphenyltetrazolium chloride. Infarct area was measured on the anterior and posterior surfaces of each slab and integrated to obtain infarct volume with correction for tissue swelling. The investigator performing the surgery and analyzing infarct size was unaware of the genotype of the mouse.

Neurobehavioural Activity: Spontaneous motor activity was evaluated for 5 min by placing the animals in a mouse cage for 5 minutes. A video camera was fitted on top of the cage to record the activity of a mouse in the cage. Neurological deficits were evaluated by an observer blinded to the treatment and genotype of the animals with a scale of 0-4 (0 no neurological deficit, 4 severe neurological deficit). The following criteria were used to score deficits: 0= mice appeared normal, explored the cage environment and moved around in the cage freely; 1 = mice hesitantly moved in cage and didn't approach all sides of the cage, 2 = mice showed postural and movement abnormalities and had difficulty approaching the walls of the cage, 3 = mice with postural abnormalities tried to move in the cage but did not approach the wall of the cage, 4 = mice were unable to move in the cage and stayed at the center. The cylinder test was performed to assess the forelimb performance in mice. For this test, a transparent glass cylinder (9 cm in diameter and 15 cm in height) was placed in the center of a chamber containing two video cameras on opposite sides. A mouse was placed in the cylinder and the cameras on opposite sides were aligned at a straight axis with the cylinder to allow recordings of mouse forelimb movements on all sides of the cylinder. Recordings were evaluated by an observer blinded to the treatment and genotype of the animals. Forelimb use of the mouse was recorded for 10 minutes and analyzed according to the following criteria: (1) Ipsilateral (right) forelimb use (number of touches to the cylinder wall) independent of the left limb (2) Contralateral (left) forelimb use (number of touches to the cylinder wall) independent of the right limb (3) Simultaneous use of both limbs. The percent use of the contralateral (left) limb was quantified by subtracting contralateral fore paw touches from the total number of touches made by the mouse during the period of observation.

Stereotaxic Injections: Mice were anesthetized by intra-peritoneal injection of sodium pentobarbital (45 mg/kg body weight) and the head was fixed in a stereotactic frame (Kopf, Tujunga, CA) for the intrastriatal injection. Following a midline incision on the scalp, a small hole was drilled using coordinates rostral, 0.5 mm; lateral, 1.7 mm; ventral, 3.5 mm from bregma. 2 µl of high titer virus was injected using a Digital Stereotaxic Apparatus and a Nanomite Injector Syringe Pump (Harvard Apparatus, USA). over a period of 10 min followed by 3 min needle pause to permit proper diffusion. 5 days following viral injections, NMDA (20 nmoles) was injected using the same coordinates. After the surgery the animals were put in separate cages and the body temperature was maintained with a heating pad and rectal probe. Following full recovery from the anesthesia, the animals were placed back on the animal cage racks in the JHMI animal facility

Stereology: Mice were anesthetized and perfused with ice-cold PBS and ice-cold 4% paraformaldehyde in PBS (pH 7.4). Brains were removed and post-fixed overnight in 4% PFA. Following cryoprotection in 30% sucrose/PBS (pH 7.4), brains were frozen and 40 µM thick coronal sections were cut with a microtome. Free-floating sections were blocked in blocking solution (10% donkey serum plus 0.3% Triton-X-100 in PBS) for 1 h at room temperature. A primary antibody against GFP (rabbit polyclonal; Abcam, USA) was incubated overnight at 4^{o}C, followed by incubation with anti-rabbit alexa488 conjugated antibody (Invitrogen, USA). The sections were mounted on glass slides and cover glasses were mounted on the sections, using Immu-Mount (Thermo, USA). To evaluate protection offered by Iduna against NMDA-toxicity, an unbiased stereological methodology was employed to count GFP-positive neurons. For the stereological counts, a computer assisted optical fractionator probe, Stereo Investigator (MicroBrightField, Williston, VT, USA) software was used to count the green cells in every fourth section throughout the entire striatal region of the brain.

Animals: The Johns Hopkins Medical Institutions are fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). All research procedures performed in this study were approved the Johns Hopkins Medical Institutions Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act regulations and Public Health Service (PHS) Policy.

Data Analysis: In homologous competitive binding experiments, the ligand binding program of SigmaPlot 9.0 software (Systat) was used to determine the EC_{50} and B_{max} of Iduna and Iduna mutants against PAR polymer, whereas the K_d was calculated using the Cheng-Prusoff equation. The curve fitting was established by a standard dose-response curve fitting equation

with ≤ 95% confidence. EC_{50} and B_{max} were directly determined from plots of specifically-bound [^{32}P]-labeled PAR polymers and calculated as a function of [^{32}P]-labeled PAR polymer concentration (mean size of 40 ADP-ribose units). Alignment of Iduna PAR-binding sequences with the consensus PAR-binding motif was performed with ClustalW and PATTINPROT tool on the NPS@server (Network Protein Sequence Analysis).

Statistical Analysis: One-way analysis of variance (ANOVA) was used followed by Tukey's post hoc test. Data represents mean ± SD or SEM, $p \leq 0.05$ was considered statistically significant.

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