Synaptic NMDA receptor activity boosts intrinsic antioxidant defences

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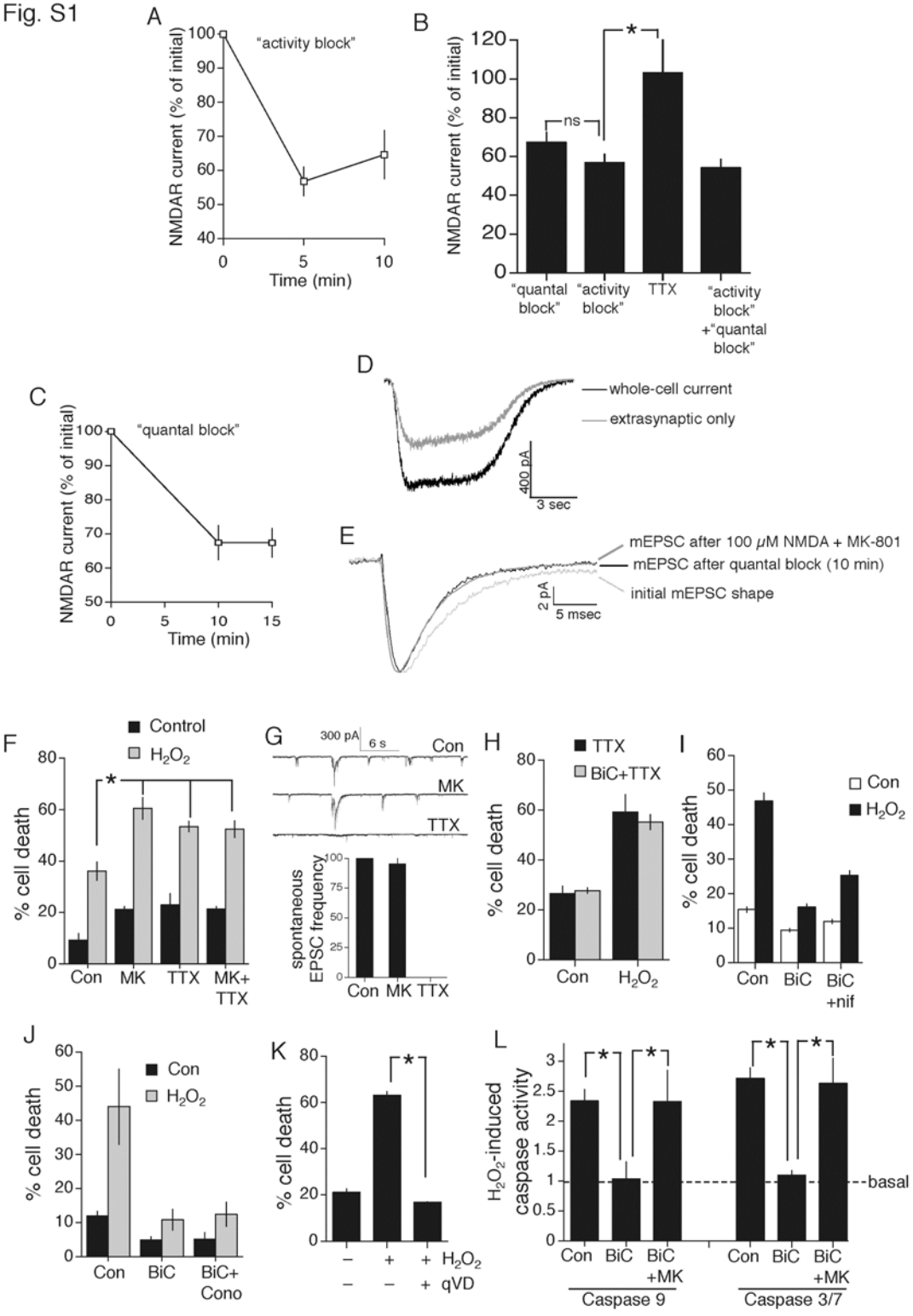
Supplemental Figures 1-7 and Tables 1-2

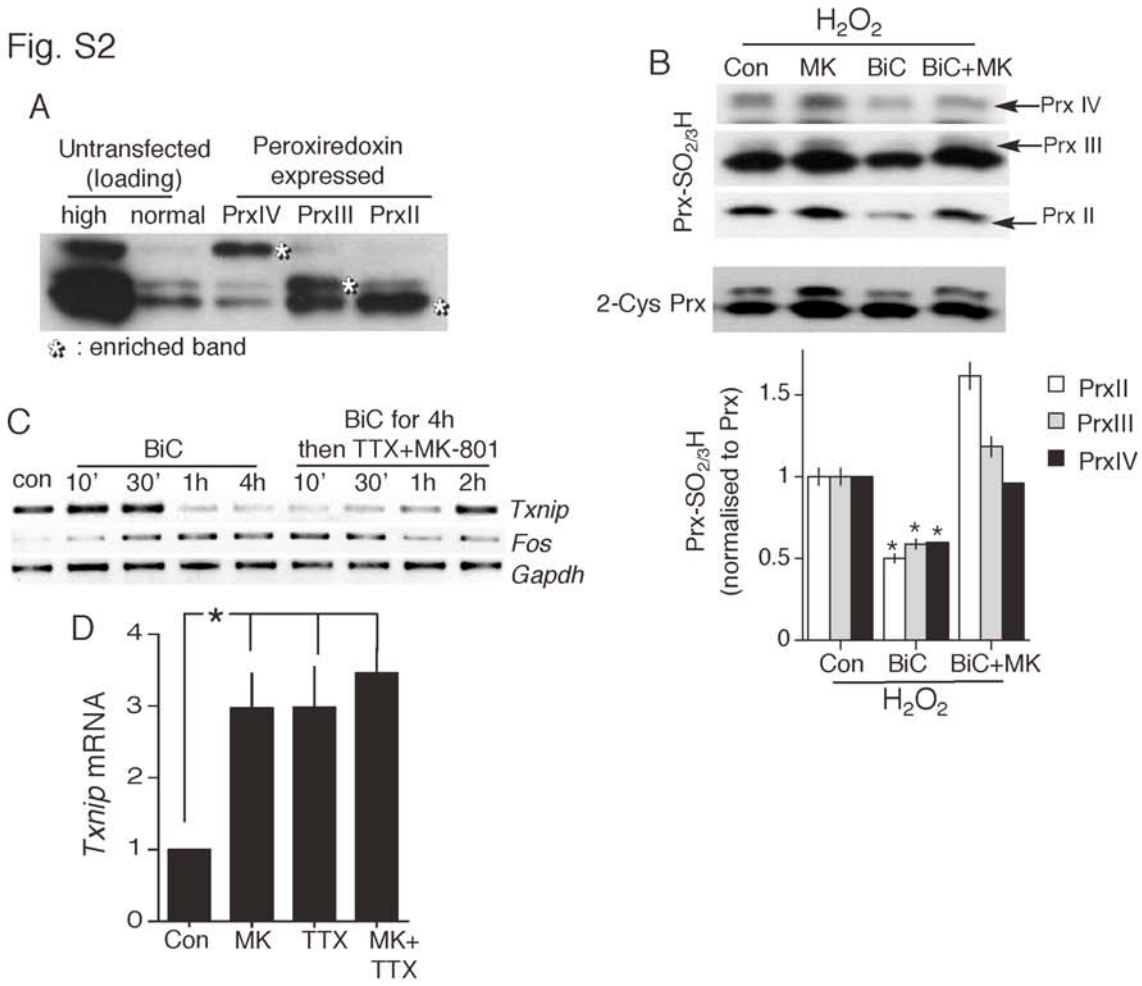
Fig. S1



Supplementary Fig. 1. Supplemental data associated with main Fig. 1. A) Loss of whole-cell current due to MK-801 exposure of neurons experiencing normal spontaneous synaptic activity. After recording of whole-cell NMDAR currents, neurons were switched to current-clamp mode in normal Mg^{2+}-containing, TTX-free medium in the presence of MK-801 for the indicated times, n=3-6 per timepoint. This protocol is referred to as "activity block". B) Comparison of the blockade of NMDARs by MK-801 under different conditions. "Quantal block" refers to blockade by MK-801 in zero Mg^{2+} + TTX, as described in (C). "Activity block" refers to blockade by MK-801 in normal Mg^{2+}-containing, TTX-free medium. "TTX" refers to blockade (or absence thereof) by MK-801 in normal Mg^{2+}-containing medium containing TTX. "Activity block+quantal block" refers to the application of the protocol described in (A) followed by the protocol described in (C). *$p<0.05$ (unpaired t-Test), n=7 (activity block), n=3 (TTX treatment), n=8 (quantal block), n=5 (activity block+quantal block)**.** C) Loss of whole-cell current due to MK-801 exposure under quantal transmission plateaus after 10 min (n=3). Neurons were placed under voltage clamp and whole-cell NMDAR-mediated currents were measured. Neurons were then placed in Mg^{2+}-free external recording solution containing TTX and MK-801 for the indicated times, to allow open-channel blockade of synaptic NMDARs following their activation by quantal release of glutamate. D) Example of a whole-cell current trace before and after treatment described in (A). E) Confirmation that all synaptic NMDARs are blocked by the procedure described in (C). Waveforms representing an average of >200 mEPSCs before and after 10 min of MK-801 block of NMDARs activated by quantal transmitter release. Also for comparison is a waveform average of >200 mEPSCs recorded at the end of the experiment where all NMDARs were blocked by addition of a high concentration of agonist in the presence of MK-801. Note that the mEPSC timecourse in this instance is similar to that of mEPSCs recorded after 10 min "quantal block" protocol. F) Cell death due to 24 h H_2O_2 (100 μM) insult in the face of the indicated treatments, applied 12 h before insult. *$p<0.05$ compared to control (n=3). G) Spontaneous EPSC frequency measured in the

presence or absence of MK-801 (10 μM) to verify that it has no inherent effect on spontaneous electrical activity, emphasising that NMDAR activity and not electrical activity *per se*, is the key regulator of vulnerability to oxidative stress. H) Cell death due to 24 h H_2O_2 (100 μM) insult in the face of the indicated treatments, applied 12 h before insult (n=3). BiC/4-AP stimulation is labelled as "BiC" in this and subsequent figures. I) Cell death due to 24 h H_2O_2 (100 μM) insult in the face of the indicated treatments, applied 12 h before insult. Nifedipine was used at 5 μM. J) Cell death due to 24 h H_2O_2 (100 μM) insult in the face of the indicated treatments, applied 12 h before insult. ω-conotoxin GVIA was used at 1 μM. K) Cell death due to 24 h H_2O_2 (100 μM) treatment, in the presence ± Q-VD-Oph (50 μM) applied 1 h before H_2O_2 treatment (n=4). L) Neurons treated with BiC/4-AP ± MK for 12 h prior to overnight treatment with H_2O_2 (100 μM) . Cell extracts were then assayed for caspase 9 and 3/7 activity (see methods) which were normalized to protein levels assayed by BCA assay *$p<0.05$ (n=3).

Fig. S2


A
Untransfected (loading)
Peroxiredoxin expressed
high
normal
PrxIV
PrxIII
PrxII
: enriched band
B
H_2O_2
Con
MK
BiC
BiC+MK
Prx IV
Prx III
Prx II
Prx-$SO_{2/3}$H
2-Cys Prx
Prx-$SO_{2/3}$H (normalised to Prx)
PrxII
PrxIII
PrxIV
C
BiC
BiC for 4h then TTX+MK-801
con
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30'
1h
4h
10'
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2h
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Fos
Gapdh
D
Txnip mRNA
Con
MK
TTX
MK+ TTX
E
NeuN
DAPI
Merged
GFAP
DAPI
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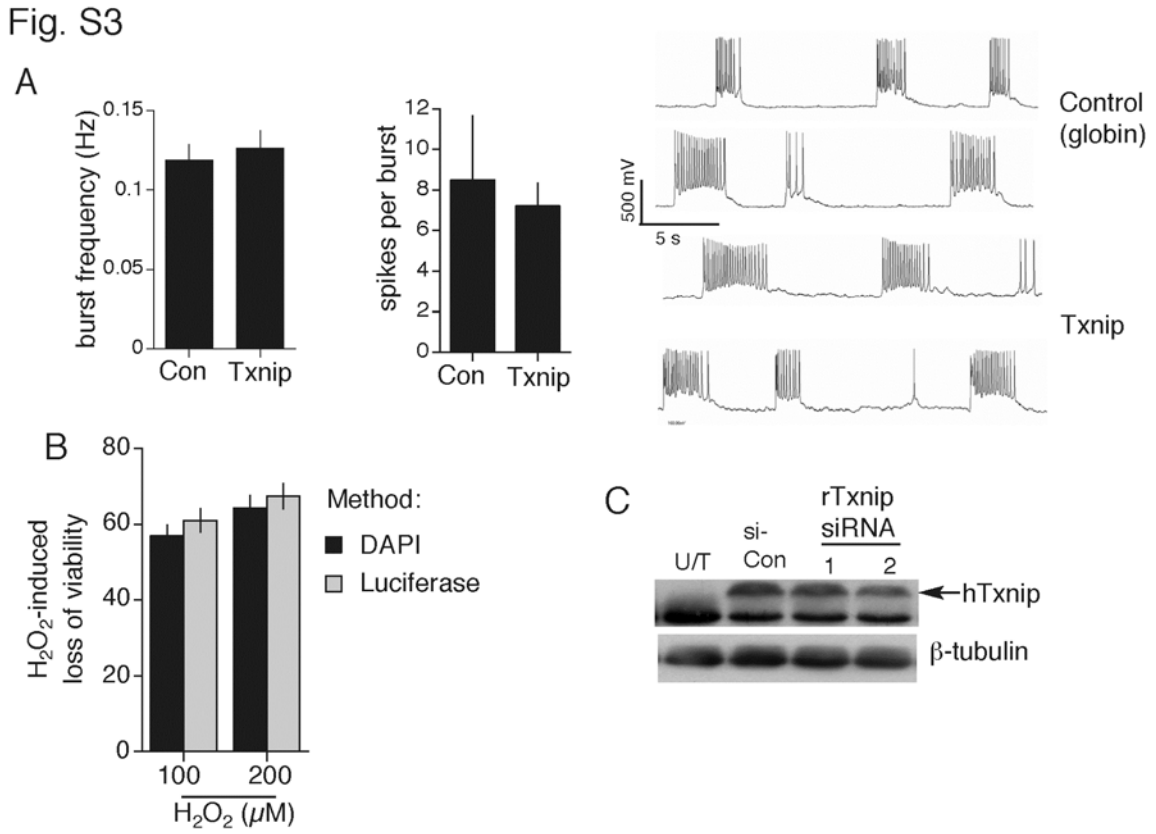
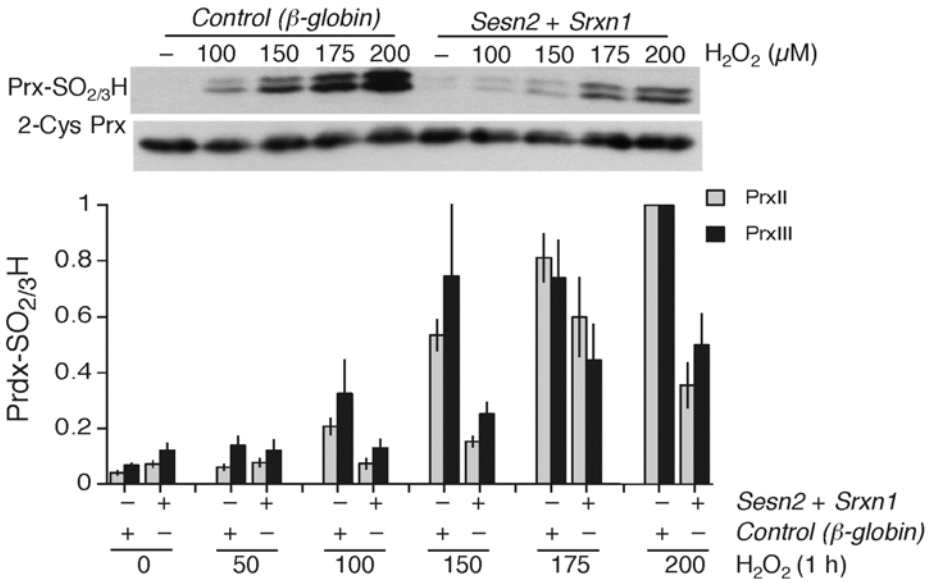
Supplementary Fig. 2. Supplemental data associated with main Fig. 2. A) PrxII, III and IV were ectopically expressed in cortical neurons, and protein extracts subjected to western analysis with a pan-2-Cys Prx antibody. A * indicates which of the endogenous bands is enriched in each case. PrxIV expression enriches upper band by 10-fold, PrxIII enriches middle band by 2-fold, PrxII enriches bottom band by 2-fold. B) Western analysis of Prx overoxidation using an anti-Prx$SO_{2/3}$H specific antibody. Analysis involved normalisation to appropriate Prx band intensity. *$p<0.05$ compared to control, H_2O_2-treated neurons (n=4). C) Conventional RT-PCR illustrating time course of activity-dependent suppression, and activity blockade-dependent elevation, of *Txnip*, in comparison to *c-fos*. D) qPCR-based analysis of Txnip expression after 24 h treatment with the indicated drugs (MK-801 at 10 μM, TTX at 1 μM, n=4). E) Example pictures of immunofluorescent staining of cortical cultures with anti-NeuN (upper, to identify neurons), -GFAP (middle, to identify glial cells) and NeuN/GFAP-double-staining (lower). F) Example pictures of immunofluorescent staining of glial cultures with an anti-GFAP antibody.



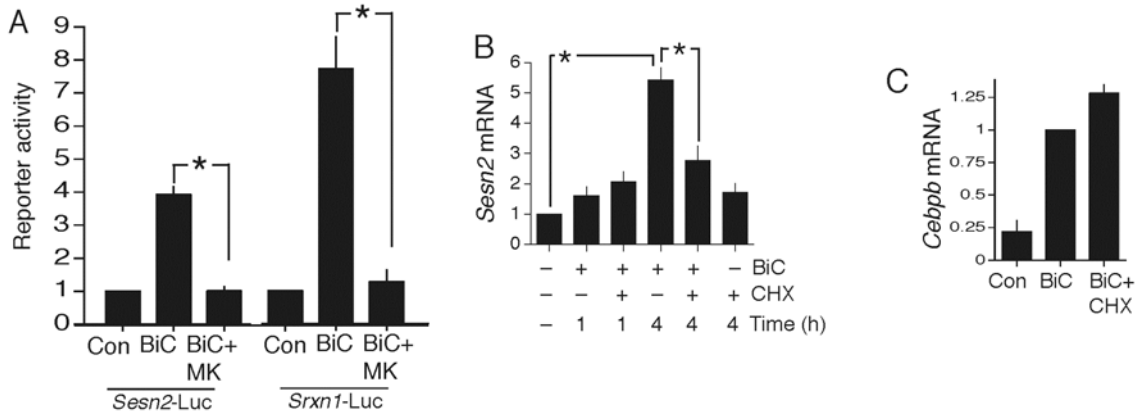
Supplementary Fig. 3. Supplemental data associated with main Fig. 3. A) BiC/4-AP-induced bursting was analyzed in neurons transfected with either Txnip- or globin-expression vectors, plus peGFP used to identify transfected cells. BiC/4-AP-induced bursting was recorded under current clamp and the burst frequency (left) and spikes/burst (middle) measured across 4 control and 4 Txnip expressing neurons. Example traces are shown on the right. B) Comparison of DAPI staining with constitutive luciferase expression as a metric of H_2O_2-induced cell death. Neurons were transfected with a constitutively active vector (SV40-Luc) and treated with H_2O_2, followed by luciferase activity measurement. In a parallel experiment, cell death was assessed conventionally by counting apoptotic nuclei stained with DAPI (n=3). C) Neurons were transfected with an expression vector for human Txnip plus control or one of two rat Txnip-directed siRNAs. Protein was harvested at 24 h and subjected to Western analysis for Txnip protein. Untransfected sample (U/T) shown for comparison.

Fig. S4

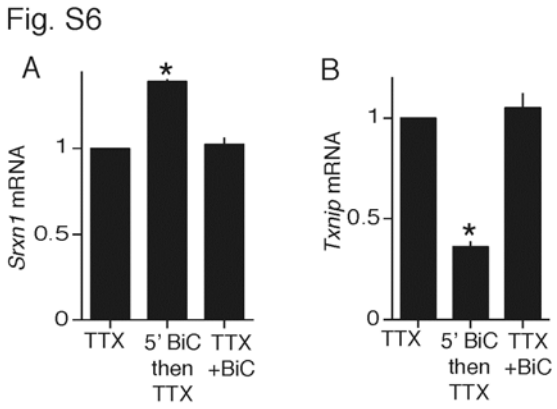


Supplementary Fig. 4. Overexpression of Sesn2 and Srxn1 reduces Prx overoxidation in HEK293 cells. HEK293 cells were transfected with expression vectors for Sesn2 and Srxn1, or control vector (globin). They were then treated with the indicated doses of H_2O_2 and after 1 h harvested and subjected to western analysis of Prx-$SO_{2/3}$H levels. Upper panel shows example western, lower panel shows analysis (n=3-5 westerns per timepoint).

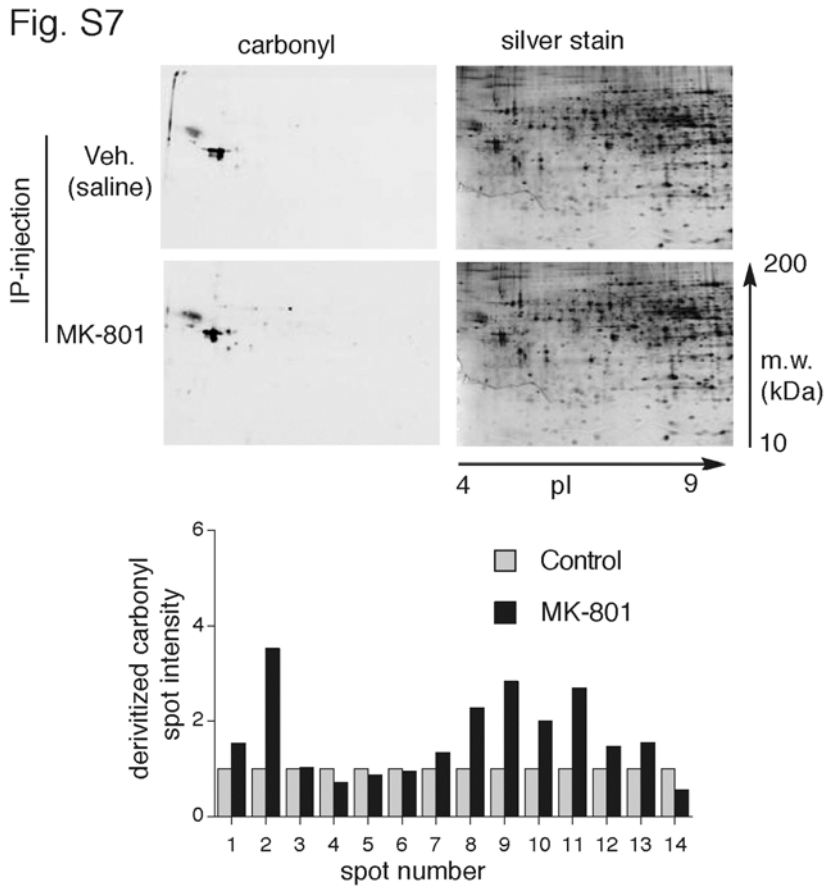
Fig. S5



Supplementary Fig. 5. Supplemental data associate with main Fig 6. A) BiC/4-AP induces luciferase-based reporters of both *Sesn2* and *Srxn1* promoters in an MK-801-dependent manner. *p<0.05 (n=5). B) q-RT-PCR of *Sesn2* induction by synaptic activity at 4 h in the presence or absence of cycloheximide (10 μg/ml, n=3). C) q-RT-PCR of C/EBPβ induction by synaptic activity at 1 h in the presence or absence of cycloheximide (n=3).



Supplementary Fig. 6. Short periods of synaptic activity can promote changes in *Srxn1* and *Txnip* expression. A) Analysis of *Srxn1* expression in neurons stimulated with BiC/4-AP for 5 min, after which activity was blocked by the addition of TTX. Expression was analyzed at 4 h and compared to TTX-treated neurons and to neurons treated with BiC/4-AP 2 min *after* TTX treatment. *$p<0.05$ (n=3). B) As for (A) but *Txnip* levels were analyzed at 1 h. *$p<0.05$ (n=3)



Supplementary Fig. 7. Analysis of protein oxidation following NMDAR blockade in the adult cortex. Analysis of carbonyl content of 2-D separated proteins from the cortices of adult mice subjected to IP-injection of MK-801. Carbonyl content (left) was detected using an antibody specific to the dinitrophenylhydrazone-derivatized residues. Blotted 2-DE gels were stained with silver staining to reveal protein spots (right). Below is a comparison of intensity of representative protein spots *$p<0.05$ ($n=6$). Note that unlike P7 mice, none achieve significance, although a few spots approach significance.

Supplemental Tables T1 and T2

Probe Set ID	Gene Title	Gene Symbol	RefSeq Transcript ID	Fold change (MK-801 *in vitro*)	Fold change (MK-801 *in vivo*)	Fold change (BiC/4-AP *in vitro*)
Genes up-regulated by synaptic NMDAR activity						
1422790_at	natriuretic peptide precursor type C	Nppc	NM_010933	-4.08	-1.75	2.57
1448285_at	regulator of G-protein signaling 4	Rgs4	NM_009062	-3.86	-3.20	2.33
1417065_at	early growth response 1	Egr1	NM_007913	-2.90	-2.15	2.31
1419248_at	regulator of G-protein signaling 2	Rgs2	NM_009061	-2.51	-2.62	2.97
1423427_at	adenylate cyclase activating polypeptide 1	Adcyap1	NM_026249	-2.39	-2.63	4.40
1421340_at	mitogen activated protein kinase kinase kinase 5	Map3k5	NM_009046	-2.13	-1.72	3.71
1415834_at	dual specificity phosphatase 6	Dusp6	NM_009986 /NM_198602	-2.13	-1.63	2.33
Genes down-regulated by synaptic NMDAR activity						
1415996_at	thioredoxin interacting protein	Txnip	NM_023719	5.02	2.62	-3.86

Supplementary Table 1. Gene list obtained by expression analysis. List of genes whose expression is changed by BiC/4-AP treatment (in an MK-801 sensitive manner), and changed in the opposite direction by MK-801, both *in vitro* and *in vivo.*

	age	sex	case number	post-mortem interval	cause of death
young	25	F	1455	7	multiple injuries after vehicle accident
	26	M	a01-78	8	lung transplant, sepsis
	26	F	1489	16	suicide, drowning, history of depression
	35	M	1104	12	vehicle accident
	27	F	1614	18	gunshot wound to abdomen
	30	F	856	7	asthma
	37	F	a03-215	13	unknown
old	81	M	542	03:15	cardiac arrest
	82	M	86756591	04:30	unknown
	89	M	48046480	2	unknown
	82	F	963	8	dissecting aortic aneurysm
	83.7	M	885	2	urinary bladder rupture
	88	F	1037	20:30	lung cancer
	>90	M	911	07:24	Myocardial infarct
	95	M	1158	19	adenocarcinoma

Supplementary Table 2. Information regarding the human samples analysed for Txnip expression.

Supplemental Methods

Plasmids

For construction of Txnip-Luc, approximately 950bp of the m*Txnip* upstream region was amplified from Balb/C genomic DNA using AdvantageGC2 DNA Polymerase with the following amplimers: forward, 5'- cta *gct agc* ctc ttg ttt cct gga gaa aca agg ac -3'; reverse, 5'- ccg *ctc gag* cca aaa ggt gcc tgg aag ttt cag c -3'. The resulting PCR product was digested with NheI and XhoI (sites within primers italicized) and cloned into the corresponding sites upstream of the firefly luciferase construct in the modified pGL3Basic vector, pGL3BAdTATA[1]. A truncated form of this construct leaving only 250bp directly upstream of the putative transcriptional start site was generated by digesting the longer promoter with NheI and SmaI, blunting the NheI site and religating to remove approximately 700bp of sequence. The 5'-upstream region of *Sesn2* was amplified by PCR from rat genomic DNA using the oligonuclotides 5'-tta a*gc tag c*ct ctg ctt tct ggg tat-3' and 5'- cgg c*aa gct t*aa cga gga gaa ctg gcg ata-3' and cloned into NheI and HindIII sites of pGL4.10 reporter vector (Promega; sites within primers italicized), this construct was called -1555/+174-Luc. -1211/+174-Luc was made deleting the -1555/-1211 region of -1555/+174-Luc by EcoICRI digestion. -494/+174-Luc, -107/+174-Luc, +109/+174-Luc were made by digestion with EcoICRI plus XhoI, SmaI and SfiI, respectively, of -1555/+174-Luc, then the ends were blunted using DNA Polymerase I Large (Klenow) Fragment (Promega) enzyme and re-ligated. The 5'-upstream region of *Srxn1* gene was amplified by PCR from rat genomic DNA using the oligonuclotides 5'-tcc tga agc tgg tgc ctt ac-3' and 5'-acg ttg tgc aca gtg tcg atg-3'. The PCR product was digested with PvuI and NotI which cut within the PCR product and cloned in EcoRV/NotI sites of pGL4.10. This plasmid was called -922/-60-Luc. -645/-60-Luc, -234/-60-Luc and -99/-60-Luc were made by digestion with XhoI plus EcoRI, MluI and PflMI, respectively, of -922/-60-Luc, then the ends were blunted using DNA Polymerase I Large (Klenow) Fragment (Promega) enzyme and re-ligated. Site-directed mutagenesis was performed with the QuikChange II XL site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. The following oligonucleotides and their reverse-complementary sequences

were used: FOXO site on *Txnip* 5'- ggt tgg agg cct ggt acc caa ggg cca agt agc -3'; distal C/EBP site on *Sesn2* 5'- ctt act cag aag gga ttt cgg ccc tgg ctg tct ggc-3'; proximal C/EBP site on *Sesn2* 5'- ccg gct tgc aaa agt ttg cgg cct ctg tgc gag gcc aac-3'; distal AP1 site on *Srxn1* 5'- tcg cct ctg agg gcc taa gct tcc acg ctg tgc gtc ac-3' and proximal AP1 site on *Srxn1* 5'- acc tgc aaa ctc acc ctg ggc ccg cga ccc gac gcg tc-3'. All mutations were verified by sequencing. *Sesn2* cDNA (TC128085, OriGene Technologies) was cloned into the NotI site of pEF1/V5-His A expression vector (Invitrogen). To generate Myc-*Txnip*, rat *Txnip* cDNA was amplified (IMAGE ID 7192327, Geneservice Ltd.) using the following primers: 5'-cc*g aat tc*a tgg tga tgt tca aga aga tca ag-3'and 5'-cgc *tct aga t*ca cag atc ttc ttc aga aat aag ttt ttg ttc ctg cac gtt gtt gtt gtt gtt gtt aag g-3' and cloned in XbaI/EcoRI sites of pEF1/V5-His A expression vector (Invitrogen; sites within primers italicized). To generate Myc-*Srxn1*, human cDNA (IMAGE ID 5735551, Geneservice) was amplified with the following primers: 5'- ac*g aat t*ca tgg ggc tgc gtg cag gag-3' and 5'- cgc *tct aga* tca cag atc ttc ttc aga aat aag ttt ttg ttc ctg caa gtc tgg tgt gga tgc tc-3', PCR product was cloned in XbaI/EcoRI sites of pEF1/V5-His A expression vector (Invitrogen). To generate rat Srxn1-Myc, the rat Srxn1 ORF was amplified from neuronal rat cDNA using the primers 5'-acg aat tct gtc cgc caa gga agc aat atg-3' and 5'- cgc tct aga tca cag atc ttc ttc aga aat aag ttt ttg ttc ctg caa gtc tgg tgt gga tgc ac-3' and cloned in XbaI/EcoRI sites of pEF1/V5-His A expression vector (Invitrogen). Expression vectors for Peroxiredoxin 2 (IMAGE ID 3485635), Peroxiredoxin 3 (IMAGE ID 3963113) and Peroxiredoxin 4 (IMAGE ID 2654429) were from Geneservice Ltd. pcDNA-hTxnip was a gift from Richard Lee, pFOXO1myc, pFOXO3a and pFOXOADA [2] were a gift from Domenico Accili. pcDNA3.1TRX-His was a gift from Junji Yodoi. AP1(5X)Luc was a gift from George Wilding. pTK-RL was from Promega.

Electrophysiological recording and analysis

Coverslips containing cortical neurons were transferred to a recording chamber perfused (at a flow rate of approximately 5 ml/min) with an external recording solution composed of (in mM): 150 NaCl, 2.8 KCl, 10 HEPES, 2 $CaCl_2$ and 10 glucose, pH 7.3 (320-330 mOsm). Patch-pipettes were made from thick-

walled borosilicate glass (Harvard Apparatus, Kent, UK) and filled with a K-gluconate-based internal solution containing (in mM): 155 K-gluconate, 2 $MgCl_2$, 10 Na-HEPES, 10 Na-PiCreatine, 2 Mg_2-ATP and 0.3 Na_3-GTP, pH 7.3 (300 mOsm). Electrode tips were fire-polished for a final resistance ranging between 5-10 M . Currents were recorded at room temperature (21 ± 2°C) using an Axopatch-1C amplifier (Molecular Device, Union City, CA) and stored on digital audio tape. Data was subsequently digitized and analyzed using WinEDR v6.1 software (John Dempster, University of Strathclyde, UK). Neurons were voltage-clamped at –70 mV, and recordings were rejected if the holding current was greater than –100 pA or if the series resistance drifted by more than 20% of its initial value (<35 M). ***Analysis of the effect of MK-801 on NMDAR currents of neurons experiencing normal spontaneous synaptic activity: "activity block protocol".*** After measurement of whole-cell NMDAR-mediated currents, the recording solution was switched to the standard Mg^{2+}-containing external solution for 2 min. At that point, spontaneous spike activity could be detected, indicative of TTX-washout and the return of axonal transmission and synaptic activity. The whole-cell recording configuration was then changed to current-clamp to allow the neuron to depolarize freely (measured resting potential of –60 ± 5 mV). The irreversible NMDAR blocker MK-801 was then introduced for a defined period of time, after which the drug was washed-out for 2 min in Mg^{2+}-containing solution, 2 min in Mg^{2+}-free solution (+TTX/picrotoxin (PTX)) and the patch-clamp configuration returned to voltage-clamp at –70 mV. Finally, the residual whole-cell currents were measured again and normalized to pre-MK-801 currents. In several cases, neurons were further treated to the "quantal block" protocol (see below) to verify that both techniques were targeting the same pool of (synaptic) NMDARs. ***Measurement of extrasynaptic NMDAR currents: "quantal block protocol".*** To block synaptically located NMDARs, we used an established method [3]. In Mg^{2+}-free external recording solution containing TTX, release of glutamate into the synaptic cleft can occur only via action potential independent (i.e. spontaneous) release of synaptic vesicles (quanta) of glutamate. In the added presence of the open channel blocker MK-801 (acting irreversibly in our experimental time-frame) only the NMDARs activated by spontaneously released quanta and by definition, "synaptic", were blocked. Following an MK-801 application that allowed

sufficient block of synaptic NMDARs and a 3 min washout of MK-801, only extrasynaptic NMDAR contributed to subsequent whole-cell NMDAR-mediated currents. The amplitude of these extrasynaptic NMDAR-mediated currents were normalized to the amplitude of pre-MK-801 NMDAR-mediated currents. To ensure that all synaptic NMDARs were blocked by this protocol we analysed the shape of mEPSCs before and after blockade by MK-801. There is a clear change in the decay kinetics, indicative of a loss of the NMDAR component of the current (Fig. S1e). To confirm that this loss is complete, we compared the kinetics of these mEPSCs with those where all NMDARs are blocked by a high agonist application (100 μM glutamate) in the presence of MK-801. Under these conditions, the mEPSC kinetics are identical to those subjected to the "quantal block" protocol (Fig. S1e). ***Effect of MK-801 on spontaneous events:*** Spontaneous EPSCs were recorded in voltage-clamp for 4 min. The external solution was then switched to one containing MK-801 (10 μM) for 2 min and events were recorded for a further 4 min. To quantify the intrinsic activity in the neuronal network, EPSCs greater than 50 pA (to exclude mEPSCs) and separated in time by more than 1 sec were manually selected for analysis. ***Determination of NMDAR sensitivity to NR2B-specific antagonist:*** Whole-cell NMDAR-mediated currents were measured in external recording solution in which the $MgCl_2$ was replaced by equimolar NaCl and supplemented with 100 μM glycine, 0.3 μM tetrodotoxin (TTX) and 50 M PTX. NMDAR-mediated currents were elicited by applying NMDA (150 μM), 5-10 sec (or until a steady-state current was achieved). Agonist applications were separated by at least 1 min washout and were repeated 2-3 times. `To examine the ifenprodil sensitivity of NMDAR-mediated currents, ifenprodil (3 μM) was applied to the recording chamber 3 min prior to agonist application. Currents recorded in the presence of ifenprodil were normalized to those recorded prior to antagonist application. ***Effects of various blockers on BiC/4AP-induced bursting:*** We assessed the involvement of different receptors and channels in BiC + 4AP-induced bursting by recording from cortical neurons in current-clamp configuration (mean resting potential = -60 ± 5 mV). Antagonists used: CNQX (AMPA receptors, 10 μM, Tocris), nifedipine (L-type VGCC, 5 μM, Sigma), ω-conotoxin MVIIC (N/P/Q-type VGCC, 5 μM, Alomone labs), ω-conotoxin GVIA (N-type VGCC, 1 μM, Alomone labs) and ω-Agatoxin IVA (P/Q-type VGCC, 100

nM, Alomone labs) were added in the recording solution and perfused for at least 5 min. When drugs were dissolved in DMSO, the final concentration of DMSO in the external recording solution was lower than 0.2%.

Neuronal cultures, stimulation, and the induction of oxidative stress

Cortical rat neurons were cultured as described[4] from E21 rats except that growth medium was supplemented with B27 (Invitrogen). Mouse cortical neurons were cultured in an identical manner from E17 pups. Stimulations were done in both cases after a culturing period of 8-10 days during which cortical neurons develop a network of processes, express functional NMDA-type and AMPA/kainate-type glutamate receptors, and form synaptic contacts. Stimulations were performed after transferring neurons into defined medium lacking trophic support "TMo" [5]: 10% MEM (Invitrogen), 90% Salt-Glucose-Glycine (SGG) medium ([6]; SGG: 114 mM NaCl, 0.219 % $NaHCO_3$, 5.292 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 1 mM Glycine, 30 mM Glucose, 0.5 mM sodium pyruvate, 0.1 % Phenol Red; osmolarity 325 mosm/l, hereafter TMo). Bursts of action potential firing were induced by treatment of neurons with 50 μM bicuculline, and burst frequency was enhanced by addition of 250 μM 4-amino pyridine[7]. MK-801 (used at 10 μM) was from Tocris, TTX (at 2 μM) and 4-amino pyridine from Calbiochem.

Stimulations were initiated approximately 12 h prior to the application of an oxidative insult in the form of H_20_2 (stabilized solution: Sigma). For assessing the long-lasting effects of synaptic activity (Fig. 1c), activity was terminated by the addition of TTX +MK-801 at 12 h, prior to the addition of H_20_2 to the culture medium. Neurons were fixed after a further 24 h and subjected to DAPI staining and cell death quantified by counting (blind) the number of apoptotic nuclei as a percentage of the total. Approximately 1500 cells were counted per treatment, across 4 independent experiments. Morphologically, peroxide-treated neurons show typical signs of apoptotic-like cell death (shrunken cell body and large round chromatin clumps). Furthermore, death was blocked by the pan-caspase inhibitor Q-VD-Oph (50 μM,

Fig. S1k). In addition to counting pyknotic nuclei, cell viability was measured using the Cell Titer-Glo assay kit (Promega).

In vivo MK-801 and memantine administration

Animal experiments were performed in accordance with the guidelines of the Humboldt University in Berlin, Germany. Six-day-old (P6) male CD1 mice (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin BgVV, Berlin, Germany) received two (at 0 and 8 h) consecutive intraperitoneal injections of saline vehicle or the noncompetitive NMDA receptor antagonist dizocilpine (MK801, Tocris, Bristol, UK) in a dose of 0.5 mg/kg per injection (10 ml/kg). Pups were exposed to MK801 or vehicle for up to 12 h after the first dose. Memantine was injected at a dose of 20 mg/kg, a dose consistently shown to be therapeutically active in protecting against ischemic insults in vivo [8-14]. The cingulate, retrosplenial, frontal and parietal cortices were rapidly collected, pooled and snap-frozen in liquid nitrogen. Samples were kept at -80°C until further processing. Total cellular RNA was isolated by acidic phenol/chloroform extraction (Peqlab, Erlangen, Germany). To determine the effect of blocking NMDA receptor function in the neonatal brain (P0 MK-801, n=4 mice), dams were treated with MK-801 (0.5 mg/kg; Sigma-Aldrich, St. Louis MO) via subcutaneous injection starting 24 h before birth (E18.5) at t=0, 8, and 16 h. For controls (P0 vehicle, n = 4 mice) dams were treated with vehicle (saline). Plug date is E0.5, with a gestation period of 19.5 days. Processing of P0 brains for nissl and TUNEL staining was as described [15].

TUNEL Histology

For terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL)-based histological analysis, the mouse pups received an overdose of intraperitoneal chloral hydrate and were transcardially perfused with heparinized 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde in cacodylate buffer (pH 7.4). Brains were post-fixed for 5 days at 4ºC and paraffin-embedded. TUNEL staining was performed on 12 μm-thick paraffin sections using the ApopTag Peroxidase kit (Oncor Appligene,

Heidelberg, Germany) according to the manufacturer's instructions. Briefly, after pretreatment with proteinase K and quenching of endogenous peroxidase, sections were incubated in equilibration buffer followed by working-strength TdT enzyme (incorporating digoxigenin-labeled dUTP nucleotides to free 3'-OH DNA termini) (1 h, 37ºC). Sections were incubated in stop/wash buffer (30 min, 37ºC), then with anti-digoxigenin-peroxidase conjugate (30 min) followed by DAB substrate (Sigma, Deisenhofen, Germany) and lightly counterstained with methylgreen. TUNEL-positive cells were determined in the cortex (frontal, parietal, cingulate and retrosplenial), caudate nucleus (mediodorsal part), thalamus and hippocampus (CA1, subiculum) by means of stereological dissector [16], estimating the mean numerical cell densities (Nv) of degenerating cells (cells/mm^3). An unbiased counting frame (0.05 x 0.05 mm, dissector height 0.07 or 0.012 mm) and a high aperture objective were used for the sampling. The Nv for each brain region was determined with eight dissectors. To assess overall severity of damage and enable comparisons among treatment groups, a scoring system was based on the cumulative numerical densities of degenerated cells in all brain regions examined. Highest levels of death were observed in the cortex.

Protein Extraction and Protein Carbonyl Assay

Total protein extracts were prepared from cortices by an extraction procedure described previously, with some modifications [17-19]. In short, frozen brain tissue, 1.6 parts v/w of buffer 1 (0,11 M CHAPS, 50 mM TRIZMA♦ Base (Sigma-Aldrich, Steinheim, Germany), 50 mM KCl and 20% w/v glycerol at pH 7.5), 0.08 parts of protease inhibitor solution I (1 Complete™ tablet (Roche Applied Science, Mannheim, Germany) dissolved in 2 ml of buffer 1) and 0.02 parts of protease inhibitor solution II (1.4 μM pepstatin A and 1 mM phenylmethylsulfonyl fluoride in ethanol) were ground to fine powder in a mortar pre-cooled in liquid nitrogen. The tissue powder was transferred into a 2 ml tube, quickly thawed and supplied with an average number of 11 glass beads (0.034 units of glass beads per combined weight of tissue, buffers and inhibitors in mg). Each sample was sonicated 6 times in an ice-cold water bath for 15 s each, with cooling intervals of 1 min 45 s in between. The homogenate was stirred for 30 min in the presence of 0.025 parts v/w of benzonase (Merck, Darmstadt, Germany) and 0.021 parts v/w 5 mM

magnesium chloride in buffer 1 without CHAPS at 4 °C. Subsequently, 6.5 M urea and 2 M thiourea were added, and stirring was continued for 30 min at room temperature until urea and thiourea were completely dissolved. The protein extract was supplied with 0.1 v/w parts dithiothreitol (Biorad, Munich, Germany), 0.1 parts v/w of ampholyte mixture Servalyte pH 2-4 (Serva, Heidelberg, Germany), corrected by the amount of urea added (correction factor = sample weight prior to addition of urea/ sample weight after addition of urea) and stored at –80 °C. Protein concentrations were determined using Biorad DC Protein Assay according to the protocol supplied by the manufacturer (Biorad).

We separated brain proteins by two-dimensional electrophoresis (2-DE) as described previously [19, 20]. The gel format was 16 cm (isoelectric focussing; IEF) x 14 cm (SDS-PAGE) x 0.75 mm. Brain proteins were extracted as described above, and 8 µl (~160 µg) of protein was assayed for protein carbonyls using OxyBlot™ Protein Oxidation Detection Kit (Chemicon, Planegg-Munich, Germany) according to the manufacturer's instructions with some modifications described previously [19]. Prior to derivatization, brain proteins were separated in 16 cm IEF gels according to their isoelectric point using the mobile ampholyte technique with a carrier ampholyte mixture pH 3-10. Following IEF, gels were incubated in DNP, neutralized after 10 min and then prepared for molecular weight separation in SDS-PAGE polyacrylamide gels. 2-DE gels were Western-blotted, and oxidatively damaged proteins were identified using an antibody specific to the dinitrophenylhydrazone-derivatized residues. Blotted 2-DE gels were stained with high sensitivity silver staining.

Carbonyl assay blots were evaluated with Proteomeweaver™ imaging software version 2.1 (Definiens, Munich, Germany). Following automatic spot detection, spot matching between all gels was viewed and edited where appropriate. The intensity of individual spots in blots of MK801-treated and control mice was quantified using spot volumes of 16 bit grayscale images, and the difference between pairs determined. Densitometric measurements of spot intensities were statistically analyzed for matched spots by Student's t-test to reveal the significance of differences between groups.

Measuring ROS accumulation

Following treatment with H_2O_2 for the indicated times, neurons were loaded with H_2DCFDA (50 μM) or Dihydrorhodamine 123 (10 μM) for 45', in the presence of TTX (to prevent any activity-dependent differences in probe uptake). These indicators are oxidized by a variety of ROS including peroxyl radicals, peroxides, and peroxynitrites (formed when superoxide reacts with nitric oxide). After incubation, neurons were washed twice in fresh medium and then a cell lysate was prepared (by addition of 400 μl of Cell titer-Glo lysis buffer-Promega) and sample fluorescence measured to indicate ROS levels (Exc 475-495 nM, Em. 510-550). After subtracting basal (i.e. 'no probe added' sample fluorescence), sample fluorescence was normalized to cell number as measured using the Cell Titer-Glo assay kit (Promega). Note that imaging experiments performed to test the possibility of an increase in ROS probe oxidation at the point of lysis revealed no increase. However, we cannot completely rule out an extra increase in ROS signal caused by the lysis procedure, although this would only cause an additional signal to each sample, and would be unlikely to alter any treatment-to-treatment differences.

Caspase assays and inhibition

Cortical neurons plated in a 24-wells plate were incubated for 5 min in 150 μl PBS supplemented with Triton X-100 0.5 %. Lysates were further homogenized by pipetting. 50 μl of the lysates were transferred in a 96-wells plate containing 50 μl of either Caspase-Glo® 3/7 or Caspase-Glo® 9 reagents (Promega Corporation, Southampton, UK) previously equilibrated at room temperature. As described in the manufacturer's instructions, MG-132 was added in the Caspase-Glo® 9 reagent to reduce non-specific background activity. Measurements were performed on FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK). Caspase activity was then normalized to protein concentration measured by bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, USA) and expressed in fold induction compared to the corresponding control. Where used, Q-VD-Oph (Merck Biosciences) was used at 50 μM and incubated for 1 h prior to H_2O_2 treatment.

Thioredoxin Assay

The thioredoxin "insulin-reducing assay" [21] was performed with some modifications. Briefly, cells were treated for 24 h or 48 h with MK-801 or BiC/4-AP, then lysed by scraping on ice in 150 µl lysis buffer/35 mm dish (20mM HEPES pH 7.9, 100 mM KCl, 300mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 1:100 Protease Inhibitor Cocktail III- Calbiochem) after being washed twice in pre-warmed TMo medium. Cell lysates were collected in microcentrifuge tubes and cell debris discarded by centrifugation for 3 min at 10000rpm at 4°C. The appropriate volume of lysate containing 30µg protein was diluted in lysis buffer to a final volume of 34 µl. 1 µl DTT activation buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 1 mg/ml BSA, 2 mM DTT) was added to all tubes. After mixing, the tubes were incubated at 37°C in a waterbath for 15 min, for reduction of endogenous thioredoxin. 20 µl reaction mixture (200 µl 1 M HEPES pH 7.6, 40 µl 0.2 M EDTA, 40 µl NADPH 40 mg/ml and 500 µl insulin 10mg/ml) was then added to each tube, as well as 0.5 units of TRX reductase or H_2O for negative controls, and the samples were incubated at 37 °C for 20 min. The reaction was stopped by addition of 250 µl Stop Buffer (6 M guanidine HCl, 1 mM DTNB (Sigma) in 0.2M Tris-HCl pH 8.0), 200 µl of sample were transferred to a 96-well plate and absorbance was measured at 405 nm. Thioredoxin-specific reducing activity was calculated by subtracting the value obtained for the sample in the absence of thioredoxin reductase. Thioredoxin activity in MK-801 treated cortical cultures was then calculated as a % of that observed in BiC/4-AP treated neurons.

Since our cortical cultures also contained glial cells, we wanted to determine the extent to which they contributed to thioredoxin activity. Immunofluorescence experiments revealed that our cortical cultures were 90-95 % NeuN-positive neurons, 4-9% GFAP-positive glial cells (Fig. S2f). Comparison of thioredoxin activity in cortical cultures with that in pure glial cultures (>98% GFAP-positive, Fig. S2e), combined with cell counts of parallel dishes revealed that a neuron has 3.8 ± 0.86 fold more thioredoxin activity than a glial cell. Thus, the glial cells that represent 4-9% by number in cortical cultures only represent 1-2.5% of thioredoxin activity. Furthermore, neither BiC/4-AP nor MK-801 altered the thioredoxin activity of glial cultures (data not shown). Thus, the changes in thioredoxin activity we observe (Fig. 2g) are due to changes in neuronal thioredoxin.

Western blotting and antibodies

Total cell lysates were boiled at 100 °C for 5 min in 1.5x sample buffer (1.5 M Tris pH 6.8; Glycerol 15 %; SDS 3 %; -mercaptoethanol 7.5 %; bromophenol blue 0.0375 %). Gel electrophoresis and western blotting were performed using Xcell Surelock system (Invitrogen) using precast gradient gels (4-20%) according to the manufacturer's instructions. The gels were blotted onto PVDF membranes, which were then blocked for 1 hour at room temperature with 5 % (w/v) non-fat dried milk in TBS with 0.1% Tween 20. The membranes were then incubated at 4° C overnight with the primary antibodies diluted in blocking solution: Anti- phospho-FKHR (Thr24)(1:1000, Cell signalling), β-tubulin isotype III (1: 125000, Sigma), Thioredoxin (1:500, Santa Cruz), Txnip (1:500, MBL), 2-Cys Prx (1:500, Abcam) Prx-$SO_{2/3}$H (1:1000, Abcam), Sulfiredoxin (P16, 1:250, Santa Cruz), Sestrin 2 (1:750, Proteintech Group Inc), Thioredoxin Reductase (1:500, Abcam), Phospho-(Ser 473) Akt (1:500, Cell Signalling), Akt (1:500, Cell Signalling). For visualisation of Western blots, HRP-based secondary antibodies were used followed by chemiluminescent detection on Kodak X-Omat film. Western blots were analysed by digitally scanning the blots, followed by densitometric analysis (ImageJ). All analysis involved normalizing to a loading control, often β-tubulin, or where appropriate, to the unmodified version of the modification under study (e.g. phospho-Akt normalized to Akt, Prx-$SO_{2/3}$H normalized to Prx).

RNA isolation, RT-PCR and qPCR

RNA was isolated using the Qiagen RNeasy isolation reagents (including the optional Dnase treatment) following passage of the cells through a QiaShredder column. Conventional RT-PCR was performed using the Pro-star one step RT-PCR kit from Stratagene, following the manufacturer's cycling conditions. For each gene, cycle time was optimised such that reactions were terminated within the linear amplification range. The sequences of the primers used were: forward 5' TGTCGTGGAGTCTACTGG 3', reverse 5' CAGCATCAAAGGTGGAGG 3' for GAPDH; forward 5'

GGAAACAAATATGAGTACAAGTTCG 3', reverse 5' CCATTGGCAAGGTAAGTGTG 3' for Txnip; forward 5' CATCCACATCTGGACTCC 3' , reverse 5' CTACAGTACGTGGATATAGC 3' for rat c-fos. Annealing temperatures for the primers were 57°C for Txnip and 51 °C for c-fos and GAPDH; Txnip PCR had 28 cycles and c-fos 30 cycles of amplification, with GAPDH PCR cycles adjusted accordingly.

For qPCR, cDNA was synthesized from 1-3 μg RNA using the Stratascript QPCR cDNA Synthesis kit (Stratagene, Amsterdam, Netherlands) according to the manufacturer's instructions. Briefly, the required amount of RNA (up to 3 g) was diluted in RNase-free water (up to 7 l final volume) and mixed on ice with 1x cDNA Synthesis master mix (10 l), random primers: oligo-dT primers 3:1 (total 2 l- 200 ng) and either 1 l RT/RNase block enzyme mixture (for RT reactions) or 1 l water (for No RT control reactions). Reaction mixtures were mixed and span down and incubated for 2 min. at 25°C, 40 min. at 42 °C and 5 min. at 95°C. cDNA was stored at -20°C.

Dilutions of this cDNA were subsequently used for real-time PCR (cDNA equivalent to 6 ng of initial RNA per 15 l qPCR reaction for all genes except GAPDH; cDNA equivalent to 2 ng initial RNA per 15 l reaction for GAPDH). qPCR was performed in an Mx3000P QPCR System (Stratagene) using Brilliant SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer's instructions. Briefly, the required amount of template was mixed on ice with 1x Brilliant SYBR Green Master Mix, the required concentration of forward and reverse primers, 30 nM ROX passive reference dye and water to the required reaction volume. Technical replicates as well as no template and no RT negative controls were included and at least 3 biological replicates were studied in each case. The sequence and concentration of the primers used are as follows: Rat Txnip –F: 5'-CTACAGGTGAGAACGAGATGG-3' 200 nM, -R: 5'- GAGAAAAGCCTTCACCCAG-3' 100nM; mouse Txnip -F: 5'- GGAAACAAATATGAGTACAAGTTCG-3' 200 nM, -R: 5'-CCATTGGCAAGGTAAGTGTG -3' 200 nM; Sesn2 F: 5'- GGATTATACCTGGGAAGACC -3' 200 nM, -R: 5'- CGCAGTGGATGTAGTTCC-3' 200 nM; Srxn1 –F: 5'-GACGTCCTCTGGATCAAAG-3' 200 nM, -R: 5'- GCAGGAATGGTCTCTCTCTG-3' 200 nM;

C/EBPbeta – F: 5'-AGGCGGTGGACAAGC-3' 200 nM, – R: 5'- TCTTCTGCAGCCGCTC 200 nM; rat GAPDH –F: 5'- AGAAGGCTGGGGCTCACC-3' 200 nM, -R: 5'- AGTTGGTGGTGCAGGATGC-3' 100nM; mouse GAPDH –F: 5'- GGGTGTGAACCACGAGAAAT-3' 200nM, -R: 5'- CCTTCCACAATGCCAAAGTT-3' 200 nM; 18s rRNA – F: 5'- GTGGAGCGATTTGTCTGGTT -3', -R: 5' – CAAGCTTATGACCCGCACTT – 3'. For Sesn2, Srxn1, C/EBPbeta the primers were common for rat and mouse. The cycling programme was 10 min. at 95 °C; 40 cycles of 30 sec. at 95 °C, 40 sec. at 60°C with detection of fluorescence and 30 sec. at 72 °C; 1 cycle (for dissociation curve) of 1 min. at 95 °C and 30 sec. at 55 °C with a ramp up to 30 sec. at 95 °C (ramp rate: 0.2°C/sec) with continuous detection of fluorescence on the 55-95 °C ramp. The data were analysed using the MxPro QPCR analysis software (Stratagene). Expression of the gene of interest was normalized to GAPDH, a commonly used control. To confirm that normalizing to GAPDH is not introducing any systematic errors to our studies on activity-dependent gene expression we measured levels of another commonly used control gene (18S rRNA) compared to GAPDH under our different stimulation paradigms and found no significant differences. Compared to unstimulated neurons, GAPDH levels in MK-801 treated neurons (normalised to 18S rRNA) levels were 86 ± 21 % (p=0.55, T-test). In neurons stimulated for 4 h with BiC/4-AP, GAPDH levels (normalised to 18S rRNA) were 109 ± 17 % (p=0.72, T-test).

The human postmortem brain tissue samples used in this study were neuropathologically normal for age and were derived from non-demented individuals (for details see supplemental table T2). RNA was extracted from cortical samples dissected from the frontal pole as described [22]. mRNA abundance was assessed by quantitative real time RT-PCR on an iCycler iQ system (BioRad) using SYBR Green one step RT-PCR kits (Qiagen). All reactions were performed in 24 μl mixture containing 0.5 M primers (forward and backward) and 1 ng total RNA. Primers specific for *TXNIP* and internal controls (*GAPDH* and *28s*) (Integrated DNA Technologies, Inc.) were designed to cross intron-exon boundaries so as to exclude the amplification of genomic DNA. The specificity of the primers was confirmed by melting curve analysis and by assessing product size by electrophoresis. As a negative control, reactions were

carried out in the absence of RT enzyme. Each sample was measured in quadruplicate. The fluorescence threshold was set within the linear range of amplification using the iCycle iQ system software. A standard curve derived from 10-fold serial dilutions on purified PCR products was used to determine the absolute concentrations. The concentration of *TXNIP* mRNA was normalized to 28s and *GAPDH* because the expression of each standard in the human frontal cortex does not change significantly as a function of age [22].

Microarray analysis

For microarray-based expression analysis, total RNA was quality confirmed on RNA 6000 Nanochips in the Agilent 2100 Bioanalyzer. All samples were of very high quality RNA (RIN>7.5) and thus were suitable for micro-array screening. Double-stranded DNA was synthesized using One-cycle cDNA Synthesis Kit (Affymetrix) followed by purification with GeneChip Sample Cleanup Module (Affymetrix). The double-stranded DNA was used as template for the *in vitro* transcription using GeneChip IVT Labeling Kit (Affymetrix) yielding biotin-labeled cRNA. Following cleanup and quantifying spectrophotometrically, the purified biotinylated target cRNA was then fragmented into short sequences. The hybridisation cocktail consisted of 15 g fragmented biotin-labeled cRNA spiked with eukaryotic hybridisation control. Eighty microliters of the hybridisation cocktail was first hybridized to the test-chips to check the cRNA integrity and assess the system veracity. After that, the Mouse Genome 430A plus 2.0 microarrays (Affymetrix) were directly loaded with 130 l of hybridisation cocktail solution and then placed in Genechip Hybridisation Oven 640 (Affymetrix) rotating at 60 rpm at 45 °C for 16 h. After hybridisation, the arrays were washed on Genechip Fluidics Station 450 (Affymetrix) and scanned using Genechip Scanner 3000 (Affymetrix) according to the manufacturer's procedure. Expression was calculated using the robust multiarray average algorithm [23] implemented in the Bioconductor (http://www.bioconductor.org) extensions to the R statistical programming environment[24]. Robust multiarray average generates a background-corrected and quantile-normalized measure of expression[25] on the log 2 scale of measurement.

Co-immunoprecipitation

For each treatment, 2x35 mm dishes (approx $1.5x10^6$ neurons) were lysed in 400 l of Lysis buffer (0.05 M Tris Base, 0.9 % NaCl, pH 7.6, 0.5 % Triton X-100 plus Protease Inhibitors Cocktail Set III (1:100; Promega)). Lysate was clarified at 16000 g for 15 min at 4 ºC. The supernatant was then precleared with 25 l of 50% Protein A Sepharose (Sigma) for 1 hour at 4 ºC, and, after a brief spin, the pellet was discarded. 10 % of the supernatant was retained as input for normalization purposes. The remainder was incubated o/n with 7.5 g anti-Txnip antibody (Zymed) at 4 ºC. The immuno-complex was precipitated with 25 l of 50% Protein A Sepharose for 1 h at 4ºC. The pellet was washed 3 times in lysis buffer, then boiled for 10 min in 25 l 1.5x sample loading buffer (1.5 M Tris, pH 6.8, 15 % glycerol, 3 % SDS, 7.5 % β-mercaptoethanol, 0.0375 % bromophenol blue) prior to gel electrophoresis and Western blotting.

Transfection and following the fate of transfected cells

Neurons were transfected at DIV8 using Lipofectamine 2000 (Invitrogen). Transfection efficiency was approximately 5 %. >99% of eGFP-expressing transfected neurons were NeuN-positive, and <1% were GFAP positive (data not shown) confirming their neuronal identity. For the first set of neuronal viability assays, peGFP was used to track the fate of transfected neurons expressing the plasmid of interest (Txnip vs. globin control). To ensure that GFP-positive neurons were also expressing the plasmid of interest, a favorable ratio was used (peGFP: plasmid of interest, 1:2). Coexpression at this ratio was confirmed in the case of pRFP (data not shown). Where used, siRNA (Txnip, Sesn2, Srxn1 or Dharmacon's control non-targetting siRNA #2 siRNA) was at 100 nM. Pictures of GFP-expressing neurons were initially taken on a Nikon D70 digital camera mounted on a fluorescence microscope. Later experiments utilized a Leica AF6000 LX imaging system, with a DFC350 FX digital camera. Neurons were then treated with 100 μM H_20_2 and images of the same cells were taken 24 h after H_20_2 exposure. Cell death was assessed by counting the number of surviving GFP-positive neurons pre- and post- exposure to H_20_2. In the vast majority of cases, death was easily spotted as an absence of a healthy GFP-expressing cell where one

once was. In place of the cell, there was in most cases (>90%) evidence of death in the form of fragmented neurites, fluorescent cell debris, and an apoptotic nucleus. This confirmed that the cells were genuinely dying as opposed to more unlikely scenario such as peroxide-induced quenching of eGFP fluorescence in a sub-population of neurons. This is also underlined by the fact that death measured by this technique is blocked by caspase inhibitors (data not shown). For each combination of plasmid(s) and/or siRNA, the fate of approx. 130-250 neurons was monitored over 4-7 independent experiments. For the second type of neuronal viability assay, neurons were transfected with pSV40-Luc (Promega) plus the plasmid of interest (Txnip vs. control) at a ratio of 1:2. After transfection, neurons were stimulated with BiC/4-AP overnight, prior to 24 h ± H_20_2 (100 μM). After this period, neurons were washed twice in PBS and neuronal viability was assayed by measuring the amount of luciferase expression in the surviving cells (using Promega Steady-Glo assay system), normalizing to the control value (cells not exposed to H_20_2). Mean ± s.e.m given of 5-7 independent experiments. siRNA sequences used were as follows: Txnip (1): aaacagaccttggactacttt; Txnip (2): agggaaatatggctgtgtatt; Sesn2 (1): ggatgagaagtttcaggtatt; Sesn2 (2): gctcgaggctctgatgtcttt; Srxn1 (3): gtgcagagcctcgtggacatt; Srxn1 (3): tcgacgtcctctggatcaatt

Luciferase assays

Viability assays: Neurons were transfected with a luciferase vector drive by a constitutively active promoter (SV40). After 24 h H_20_2 exposure treatment, luciferase was assayed using the Steady Glo kit (Promega). To validate this technique as a metric of cell death, we compared peroxide-induced cell death as assayed by loss of transfected luciferase signal, with cell death as assayed by counting % apoptotic nuclei. As shown in Fig. S3b, they correlate well, indicating that the luciferase-based assay is appropriate for measuring cell death of transfected cells.

Reporter assays: Firefly luciferase-based reporter gene constructs (*Txnip*-Luc, *Sesn2*-Luc, *Srxn1*-Luc and mutated variants) were transfected along with a renilla expression vector (pTK-RL), and also, where relevant, other expression vectors. Neurons were stimulated (where appropriate) 24 h after transfection (for 6 h or 24 h). Luciferase assays were performed using the Dual Glo assay kit (Promega) with Firefly luciferase-based reporter gene activity normalized to the Renilla control (pTK-RL plasmid) in all cases.

Immunofluorescence

Immunofluorescence was performed as described [7]. Pictures of FOXO1-myc expressing neurons were taken on a CCD camera driven by Openlab software, and the subcellular distribution scored as either exclusively nuclear, nuclear + cytoplasmic (even distribution in nucleus and cytoplasm), or cytoplasmic (higher level in cytoplasm than nucleus). 9E10 Anti-myc antibody (Santa Cruz) was used (1:200) and visualized using biotinylated secondary antibody/cy3-conjugated streptavidin. Nuclei were counter-stained with DAPI. For each treatment/timepoint combination, ca. 150 cells were analysed within 3 independent experiments. Other immunofluorescence antibodies used: Sulfiredoxin (S17, 1:500, Santa Cruz), Sestrin 2 (1:500, Proteintech Group Inc), NeuN (1:15 Chemicon), GFAP (Sigma, 1:1000).

Chromatin ImmunoPrecipitation (ChIP)

ChIP assays were performed following the Upstate Chromatin Immunoprecipitation Protocol (www.upstate.com) with modifications. For each treatment, 2x 60 mm dishes were used (approx 4.5 x 10^6 cells). Medium was removed and treated cells were washed with pre-warmed medium and incubated for 12 min at RT with 1% para-formaldehyde in pre-warmed medium, to crosslink proteins to DNA. The reaction was stopped by addition of glycine to a final concentration of 125 mM for 5 min. Cells were washed twice with ice-cold PBS containing protease and phosphatase inhibitors (1:100 Protease Inhibitor Cocktail III (Calbiochem); 1:500 each Phosphatase Inhibitor Cocktail I and II (Sigma)) and harvested on ice in lysis buffer (1 % SDS, 10 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.1) including inhibitors as above, using plastic scrapers [600 µl lysis buffer per 60 mm dish]. Cell lysates were transferred to 1.75

ml microcentrifuge tubes and sonicated on ice with five 20 s pulses at 10 % power on a Branson 450 Sonifier, with 15 second intervals between pulses. This gives chromatin fragments of ca. 600 bp average size, as demonstrated in test experiments for optimizing sonication conditions.

Cell lysates were centrifuged for 10 min at 13000rpm, 4°C to remove cell debris and the supernatants were transferred to 15ml tubes. ChIP dilution buffer (0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA pH 8.0, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl) including protease and phosphatase inhibitors was added to cell lysates for a 10-fold dilution. A fraction of each sample was kept at this point as input. Diluted cell lysates were pre-cleared by incubation with protein-A agarose beads/salmon sperm DNA (Upstate) for 1 h at 4 °C with agitation, beads were collected by centrifugation at 7600 rpm for 1 min, 4° C and supernatants were collected and subjected to immunoprecipitation. The appropriate antibody (anti-FKHR, anti-PiFKHR (Cell Signaling) at 1.75 μl/ 100 μg protein) was added for overnight incubation at 4 °C with agitation. Immune complexes were collected by 1 h incubation with protein-A agarose beads/salmon sperm DNA at 4 °C. The beads were collected by centrifugation at 7600 rpm for 1 min, 4°C, transferred to a microcentrifuge tube, and subjected to a series of washes, for 3-5 min at 4°C with agitation. There were one wash each in Low Salt Buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), High Salt Buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl Buffer (0.25 M LiCl, 1 % NP40, 1 % deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1) and two washes in 1xTE (10 mM Tris-HCl pH 8.1, 1 mM EDTA pH 8.0). The immune complexes were eluted from the beads by addition of elution buffer (1 % SDS, 0.1 M $NaHCO_3$), vortexing for 30 seconds and mixing at RT for 15 min and centrifugation to collect the eluates. Elution was performed twice, the eluates were combined and NaCl was added (0.33 M final concentration) to reverse crosslinking by incubation overnight at 65 °C. DNA was isolated and purified by phenol:chloroform extraction and isopropanol precipitation and subsequently used for PCR with primers designed to be flanking the putative FOXO-binding site of the Txnip promoter. The primer sequences were: forward 5’ AGCACACACCCAAACAACC 3’, reverse 5’

TCTCCCATTGGCTACTGG 3'. The cycling conditions were: 95 °C for 2 min, [95 °C for 40 seconds, 58 °C for 30 seconds, 72 °C for 1 minute] for 40 cycles and 72 °C for 10 min.

In vivo focal cerebral ischemia and analysis of peroxiredoxin overoxidation

All experiments were carried out in adult male C57Bl/6J mice (Charles River, UK) under an appropriate Home Office Licence and adhered to regulations as specified in the Animals (Scientific Procedures) Act (1986). Transient focal ischemia (60 min) was induced by intraluminal filament occlusion of the right middle cerebral artery (MCA). Animals were anaesthetized and maintained with halothane (2 %) in a mixture of 30 % O_2 and 70 % N_2O by face mask. Focal cerebral ischemia was induced by occlusion of the right MCA with an 8-0 nylon monofilament (Ethicon) coated with a mixture of silicone resin (Xantoprene, Bayer Dental, Osaka, Japan) and hardener (Elastomer Activator, Bayer Dental). Briefly, the right common carotid (CCA), external carotid (ECA) and internal carotid (ICA) arteries and their branches were exposed through a midline cervical incision. A 6-0 silk suture was tied around the CCA proximal to the bifurcation of the ECA and ICA and then a second suture tied around the ECA distal to the superior thyroid artery (STA). The STA and occipital artery (OA) were closed by electrocoagulation. The silicone-coated monofilament (diameter 220 μm) was introduced into the CCA via a small incision and advanced 10 mm distal to the carotid bifurcation so as to occlude the MCA. Mice were then recovered from anaesthesia and placed in an incubator (30 C) for the duration of occlusion. The monofilament was completely withdrawn after 1 hour to allow reperfusion. Sham operated mice underwent the same surgical procedure except the MCAO was not occluded. Wounds were sutured closed and anaesthesia discontinued. Mice were briefly re-anaesthetised and the filament withdrawn to induce reperfusion 60 min after occlusion onset. After reperfusion of 3 hr (n=6) or corresponding times for sham-operated mice (n=5), the mice were reanesthetized with 5 % halothane and decapitated. The brains were rapidly removed and subsequent dissections performed on ice. Brain tissue slices were sectioned in 1mm thick coronal sections using a mouse brain mould. The regions supplied by the MCA (striatum and cortex) were dissected within the ipsilateral and contralateral MCAO territory, frozen in

liquid nitrogen and kept at -80 ^{0}C for subsequent analyses. Frozen tissue samples (ipsi- and contra-lateral) from ischemia and sham models were first weighed. Each sample was placed in a 1ml glass Dounce homogeniser and 5 volumes (5 l/mg tissue) of ice-cold lysis buffer (0.5 M Tris base; 0.9 % NaCl; 0.5 % Triton X-100) containing 1:100 v/v Protease Inhibitor Cocktail III (Calbiochem) were added. The tissue was homogenised, transferred to a cold microcentrifuge tube and left on ice for 15 min. The samples were then centrifuged at 10000 rpm for 10 min at 4 °C and the supernatant was collected in new pre-cooled tubes. A small volume (3- 7 l diluted 1:10-1:20 in lysis buffer) was kept for a BCA assay to calculate protein concentration in the samples, according to the manufacturer's instructions (Pierce; Perbio Science, Northumberland, UK). The appropriate amount of lysis buffer was added to the remainder of the samples so that the concentration of the homogenised stocks was 10 g protein/ l. For Western blots, aliquots of the homogenates were taken and mixed with 4x Protein Sample Buffer (0.24 M Tris pH 6.8; 40 % glycerol; 8 % SDS; 20 % -mercaptoethanol; 0.1 % bromophenol blue, in H_2O) to a final concentration of 7.5 g protein/ l. The samples were boiled for 2-3 min to denature the proteins, cooled down and spun briefly before loading.

Statistical analysis

Most statistical testing involved a 2-tailed paired student T-test. For studies employing multiple testing (e.g. the use of two pairs of siRNA, or comparisons between multiple deletion/mutant luciferase reporters constructs) we used a one-way ANOVA followed by Fisher's LSD post-hoc test.

Equipment and settings

For western blots we used chemiluminescent detection on Kodak X-Omat film. Appropriate exposures were taken such that bands were not saturated. Western blots were analysed by digitally scanning the blots, followed by densitometric analysis of the raw scans (ImageJ). For figure preparation of example western blots, linear adjustment of brightness/contrast was applied (Photoshop) equally across the entire image, taking care to maintain some background intensity. Pictures of GFP-expressing neurons were

initially taken on a Nikon D70 digital camera mounted on a fluorescence microscope. Later experiments utilized a Leica AF6000 LX imaging system, with a DFC350 FX digital camera. The DFC350 FX digital camera is a monochrome camera, and so coloured images (e.g. of green fluorescent protein) essentially involve taking a black and white image (using the appropriate filter set) and applying a colour to the image after capture. All luminescent assays were performed on a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK). Light collection time and gain were set such that counts were substantially lower than the maximum level collectable.

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