Synaptic NMDA receptor activity boosts intrinsic antioxidant defences

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Supplemental Figures S1-S7 and Tables T1-T2

Fig. S1

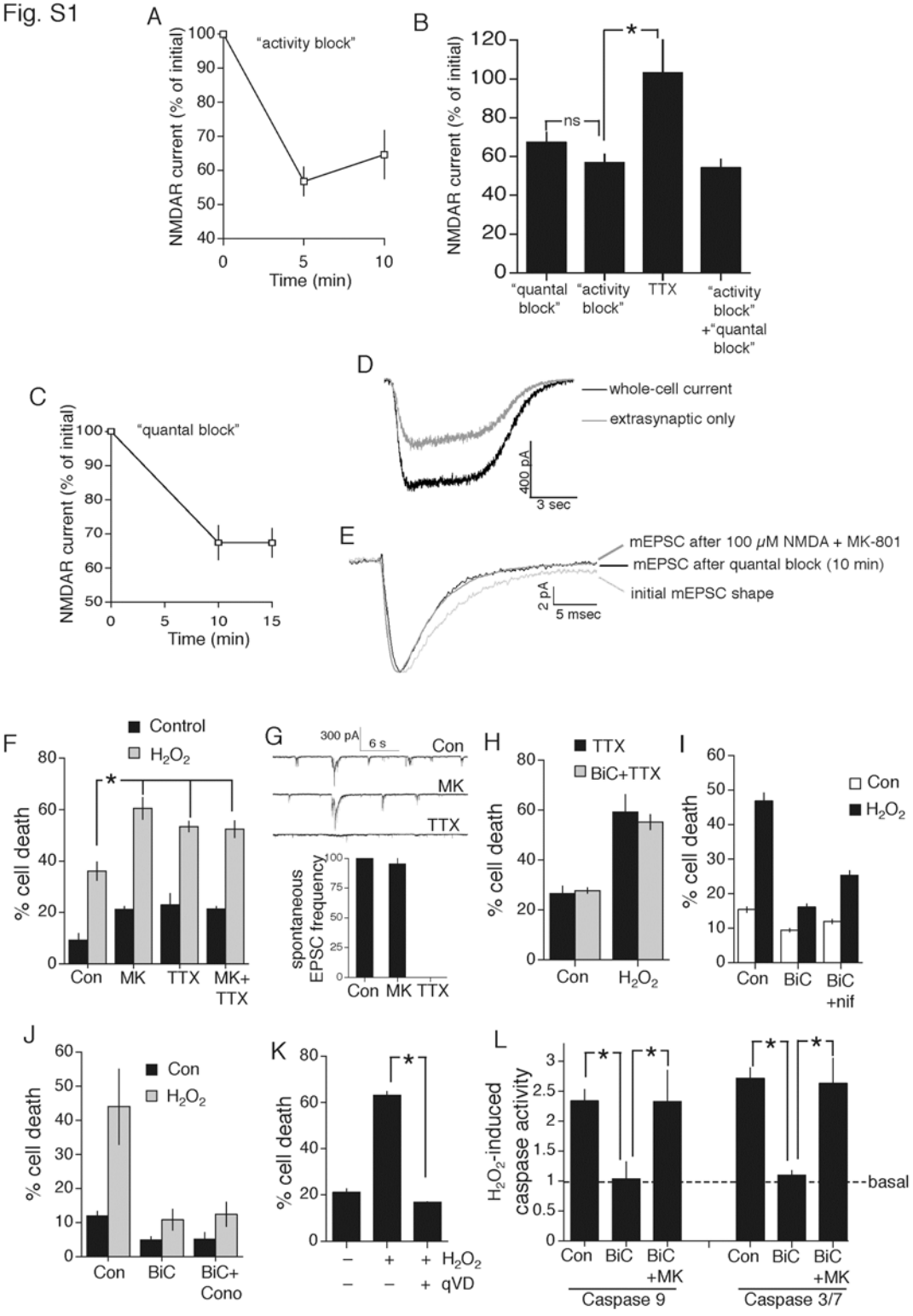

Fig. S1. A) Loss of whole-cell current due to MK-801 exposure of neurons experiencing normal spontaneous synaptic activity. After recording of whole-cell NMDAR currents, neurons were switched to current-clamp mode in normal Mg^{2+}-containing, TTX-free medium in the presence of MK-801 for the indicated 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

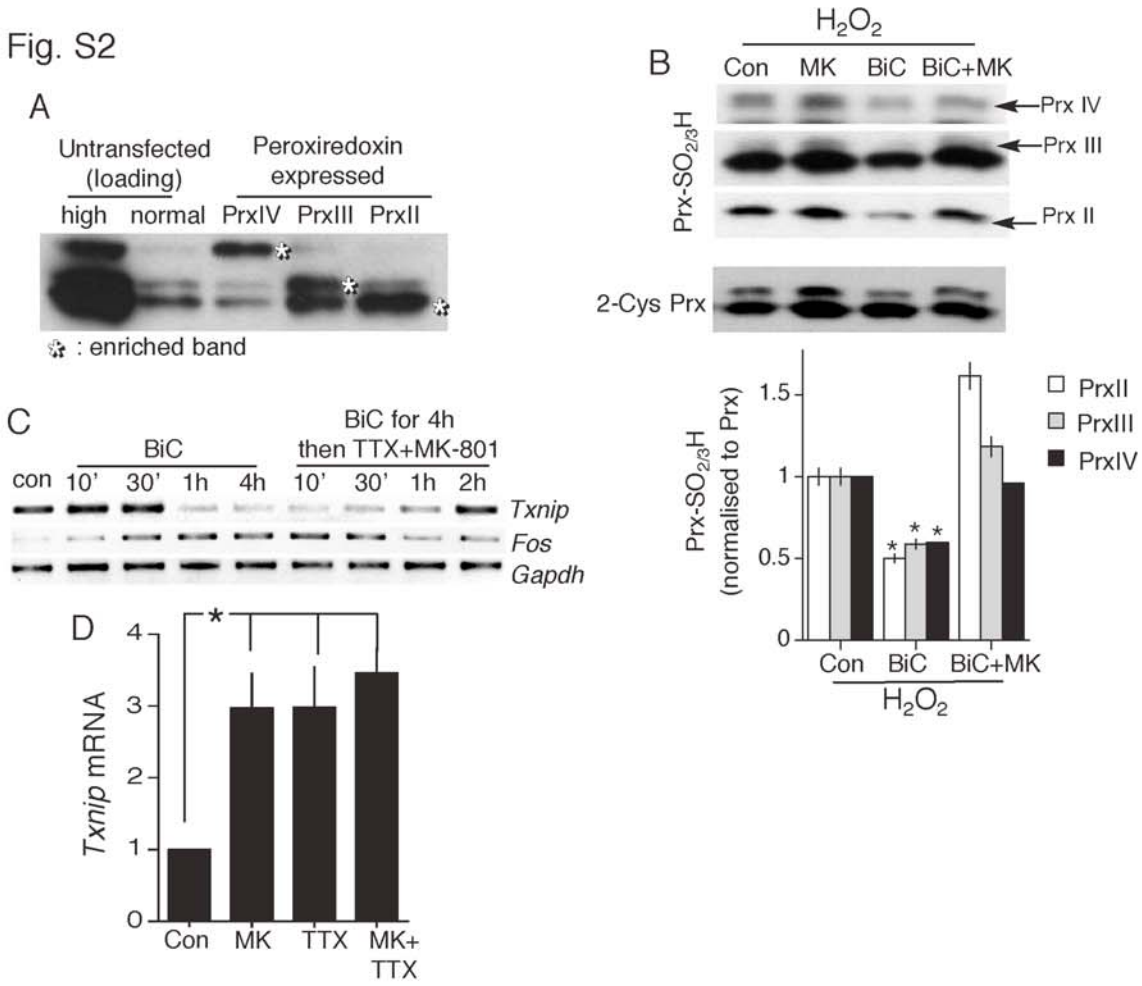
A
Untransfected (loading)
Peroxiredoxin expressed
high
normal
PrxIV
PrxIII
PrxII
: enriched band
B
H_2O_2
Con
MK
BiC
BiC+MK
Prx IV
Prx III
Prx II
Prx-$SO_{2/3}$H
2-Cys Prx
Prx-$SO_{2/3}$H (normalised to Prx)
PrxII
PrxIII
PrxIV
C
BiC
BiC for 4h then TTX+MK-801
con
10'
30'
1h
4h
10'
30'
1h
2h
Txnip
Fos
Gapdh
D
Txnip mRNA
Con
MK
TTX
MK+ TTX
E
NeuN
DAPI
Merged
GFAP
DAPI
Merged
NeuN
GFAP
DAPI
Merged
F
GFAP
DAPI
Merged

Fig. S2. 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.

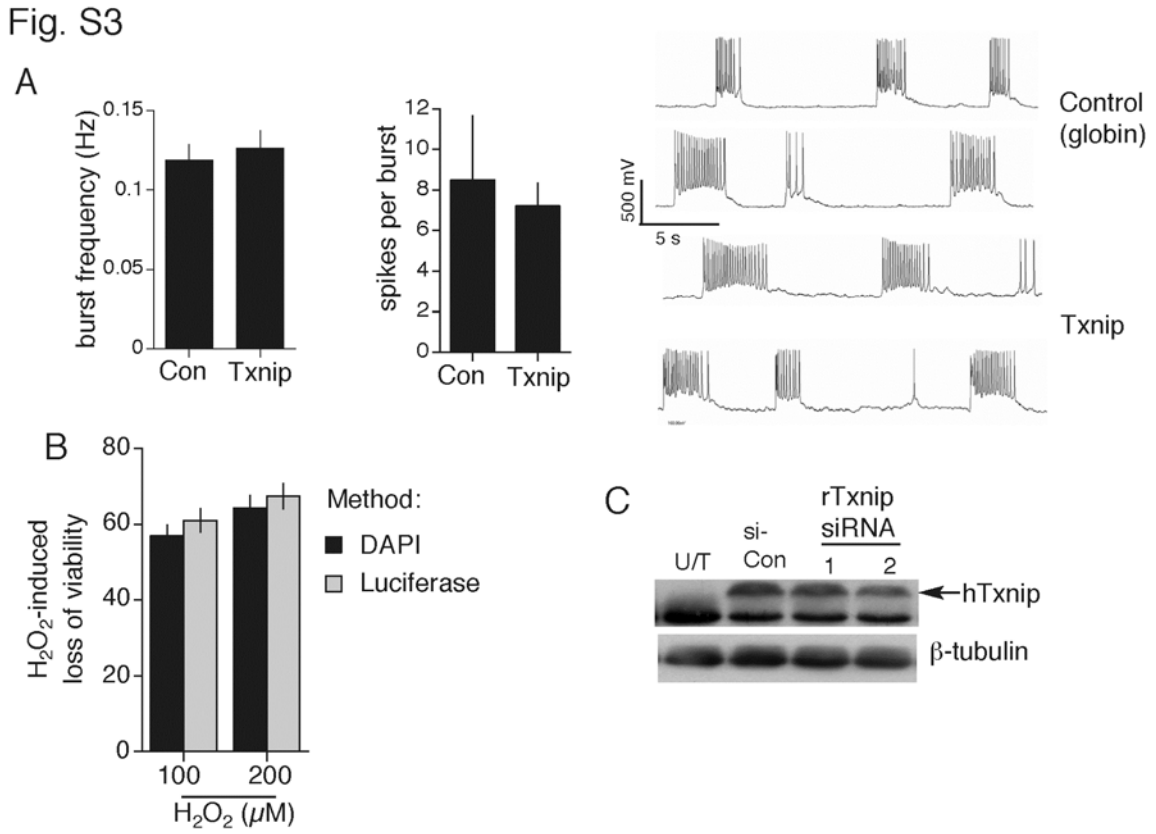

Fig. S3. A) BiC/4-AP-induced bursting was analyzed in neurons transfected with either Txnip- or globin-expression vectors, plus peGFP used to identify transfected cells. BiC/4-AP-induced bursting was recorded under current clamp and the burst frequency (left) and spikes/burst (middle) measured across 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

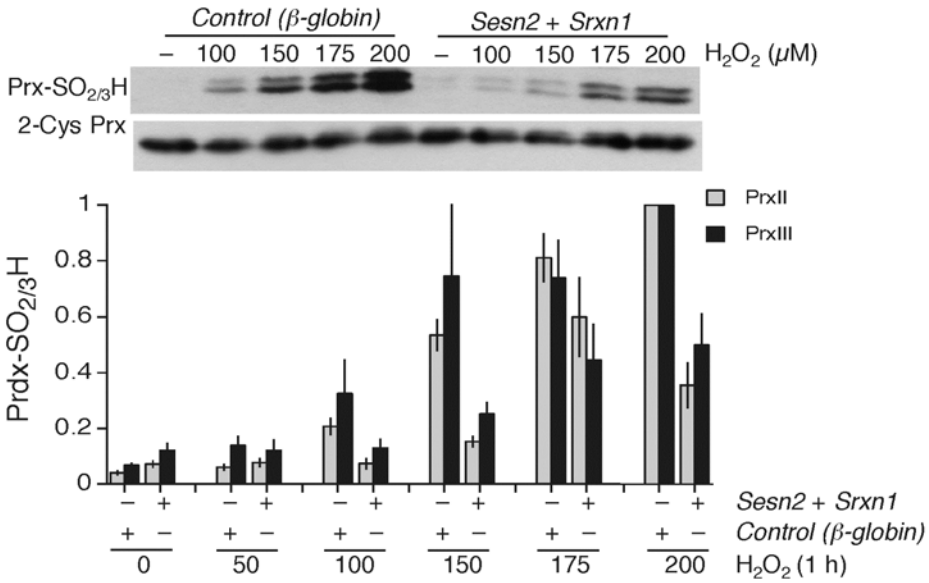

Fig. S4. HEK293 cells were transfected with expression vectors for Sesn2 and Srxn1, or control vector (globin). They were then treated with the indicated doses of H_2O_2 and after 1 h harvested and subjected to western analysis of Prx-$SO_{2/3}$H levels. Upper panel shows example western, lower panel shows analysis (n=3-5 westerns per timepoint).

Fig. S5

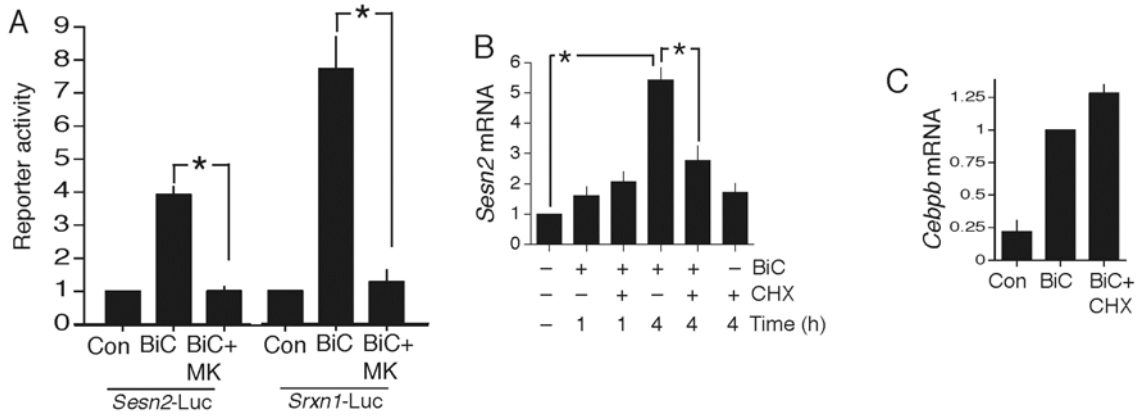

Fig. S5. A) BiC/4-AP induces luciferase-based reporters of both *Sesn2* and *Srxn1* promoters in an MK-801-dependent manner. *p<0.05 (n=5). B) q-RT-PCR of *Sesn2* induction by synaptic activity at 4 h in the presence or absence of cycloheximide (10 μg/ml, n=3). C) q-RT-PCR of C/EBPβ induction by synaptic activity at 1 h in the presence or absence of cycloheximide (n=3).

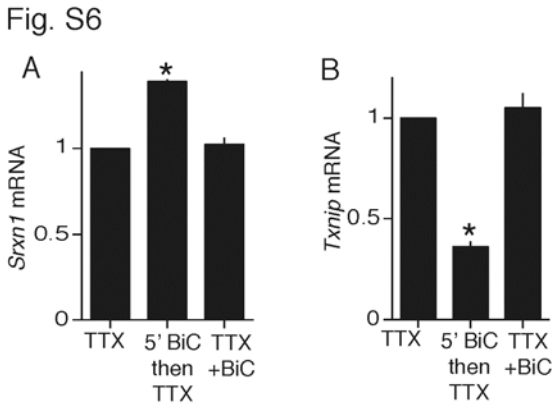

Fig. S6. A) Analysis of *Srxn1* expression in neurons stimulated with BiC/4-AP for 5 min, after which activity was blocked by the addition of TTX. Expression was analyzed at 4 h and compared to TTX-treated neurons and to neurons treated with BiC/4-AP 2 min *after* TTX treatment. *$p<0.05$ (n=3). B) As for (A) but *Txnip* levels were analyzed at 1 h. *$p<0.05$ (n=3)

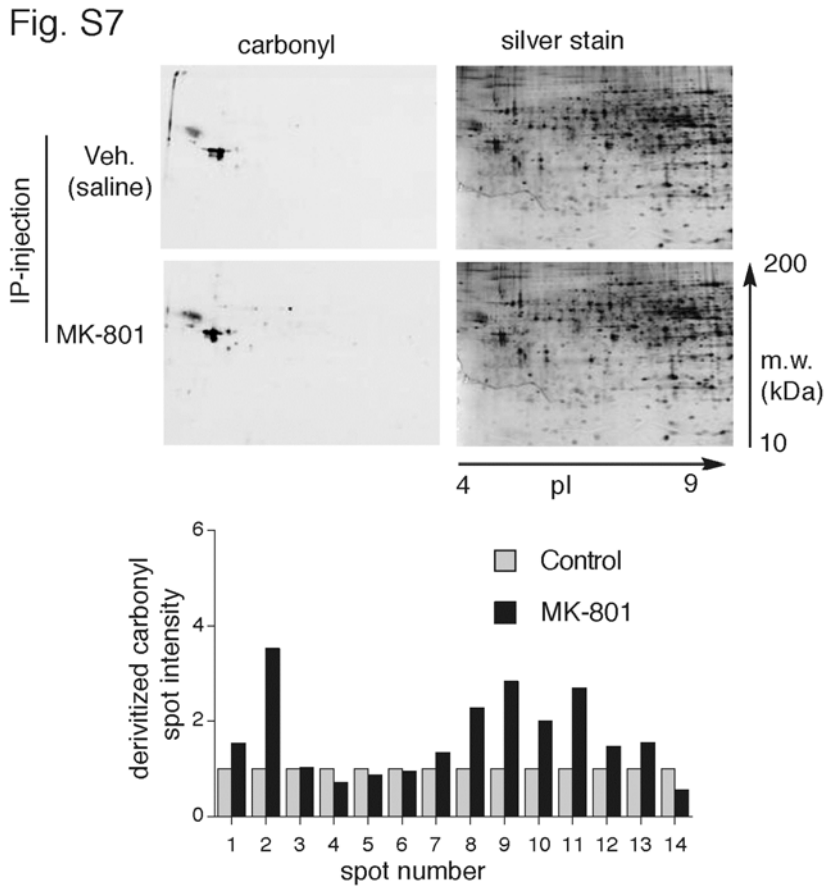

Fig. S7. Analysis of carbonyl content of 2-D separated proteins from the cortices of adult mice subjected to IP-injection of MK-801. Carbonyl content (left) was detected using an antibody specific to the dinitrophenylhydrazone-derivatized residues. Blotted 2-DE gels were stained with silver staining 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

Table T1. 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

Table T2. Information regarding the human samples analysed for Txnip expression