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Supplementary Materials for

Proteomic analysis of S-nitrosylated nuclear proteins in rat cortical neurons

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(available at www.sciencesignaling.org/cgi/content/full/11/537/eaar3396/DC1)

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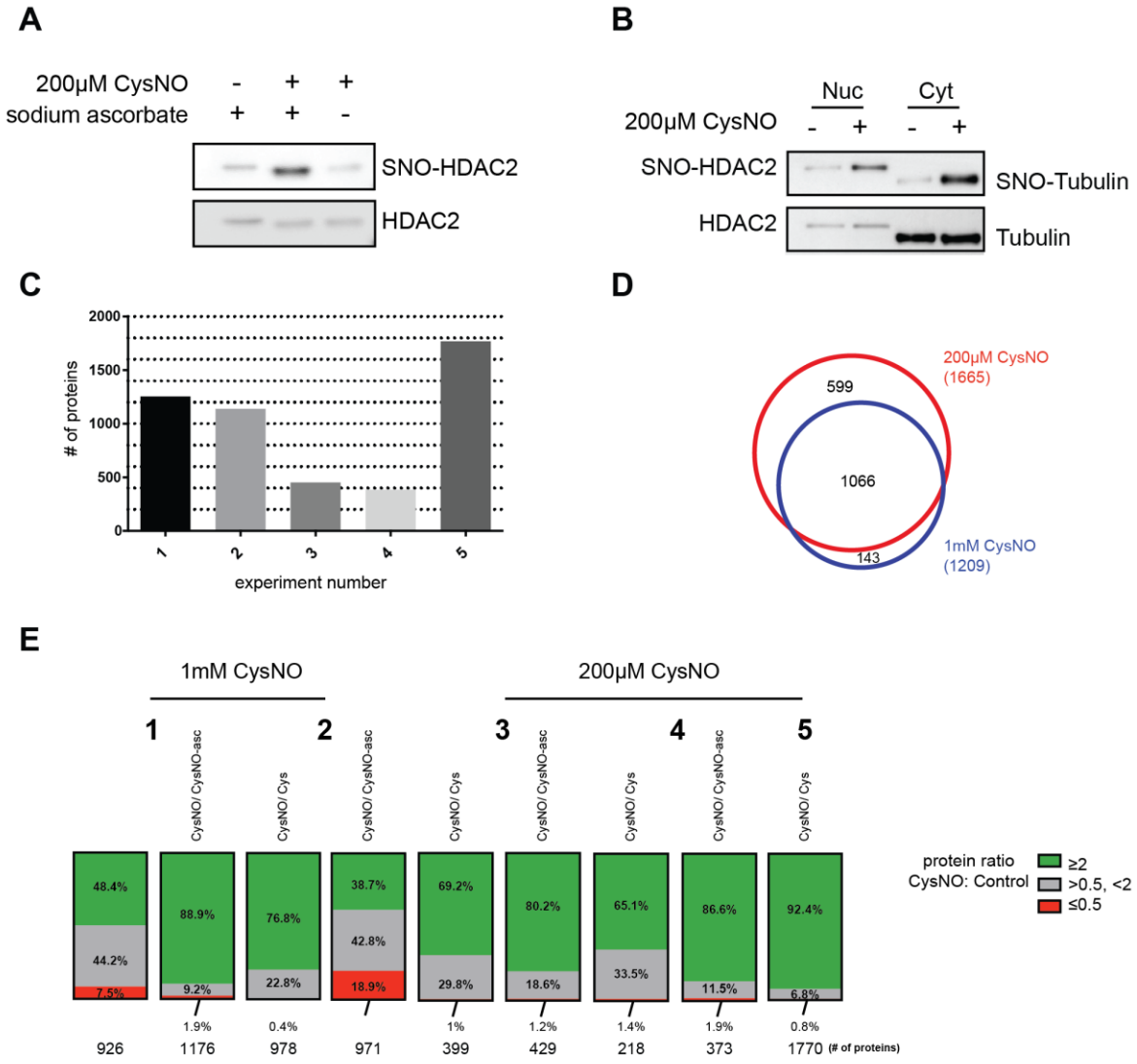
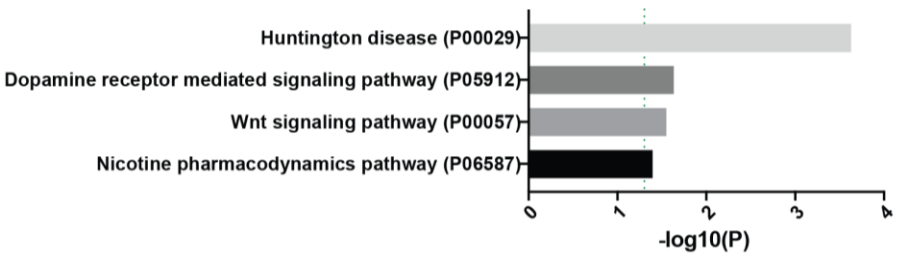
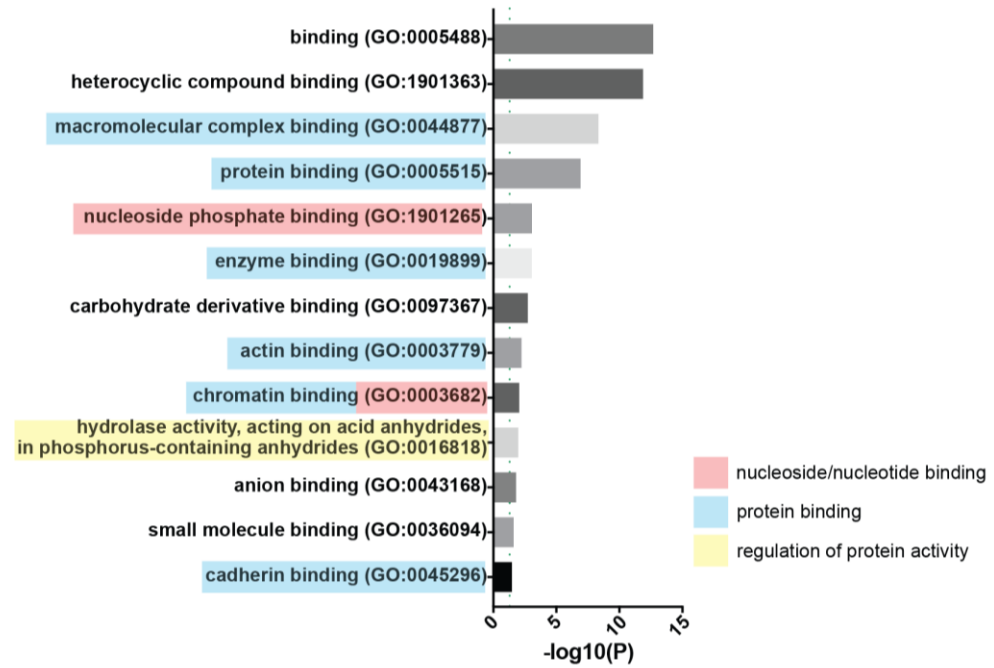


Fig. S1. SNORAC screen analysis. (A) Nuclear extracts from cortical neurons were treated with 200 μM Cys or CysNO for 20 min, followed by SNORAC and western blotting of isolates and inputs for HDAC2 (n=2 independent experiments). Sodium ascorbate is required for reduction of S-nitrosothiols. **(B)** Nuclear and cytoplasmic extracts were treated with 200 μM Cys or CysNO for 20 min, followed by western blot for HDAC2 and Tubulin (representative blot of n=3). **(C)** Total number of proteins detected as enriched in CysNO (≥ 2 fold) groups in each experiment. Proteins were compiled from both 'CysNO vs Cys' and 'CysNO vs CysNO w/o ascorbate' for each experiment. **(D)** Comparison of the number of proteins detected as ≥ 2 fold enriched in CysNO versus control groups, in at least 1 experiment. **(E)** Relative proportion of proteins detected as enriched either ≥ 2 fold in CysNO groups (green), unchanged (grey) or enriched ≥ 2 fold in controls (red). CysNO concentration in experiments 1 and 2 is 1 mM CysNO and 200 μM in experiments 3-5.

A


Pathways
Huntington disease (P00029)
Dopamine receptor mediated signaling pathway (P05912)
Wnt signaling pathway (P00057)
Nicotine pharmacodynamics pathway (P06587)
0
1
2
3
4
-log10(P)

B


Molecular Function
binding (GO:0005488)
heterocyclic compound binding (GO:1901363)
macromolecular complex binding (GO:0044877)
protein binding (GO:0005515)
nucleoside phosphate binding (GO:1901265)
enzyme binding (GO:0019899)
carbohydrate derivative binding (GO:0097367)
actin binding (GO:0003779)
chromatin binding (GO:0003682)
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides (GO:0016818)
anion binding (GO:0043168)
small molecule binding (GO:0036094)
cadherin binding (GO:0045296)
0
5
10
15
-log10(P)
nucleoside/nucleotide binding
protein binding
regulation of protein activity

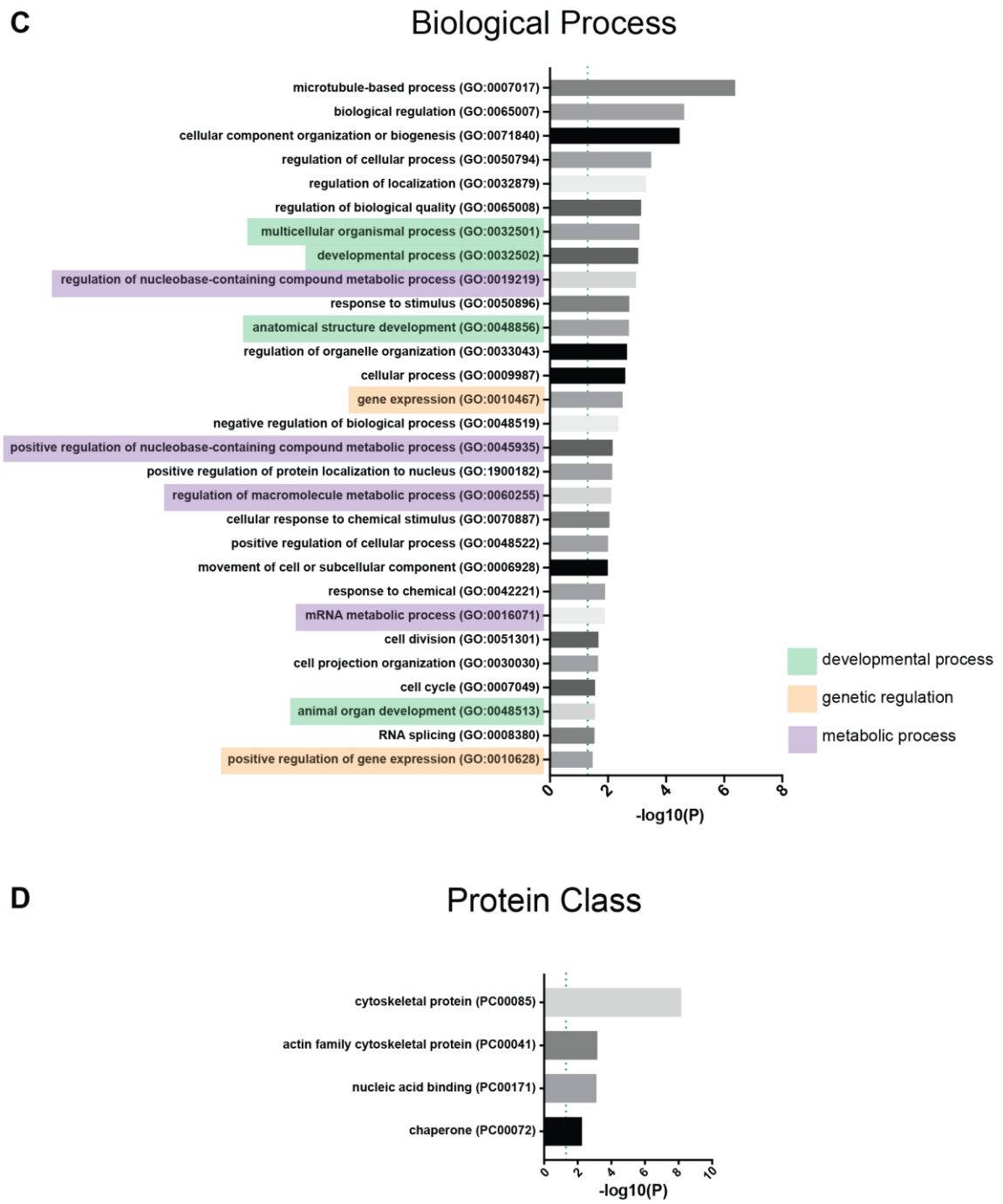


Fig. S2. PANTHER GO overrepresentation analysis. (A-D) Overrepresentation analysis was carried out using S-nitrosylated hits (**table S2**) as the assayed dataset and our nuclear protein dataset (**table S7**) as background. P>0.05 (-log10(P) of 1.3 and above) was considered as statistically significant; all terms displayed here reach this threshold, indicated by dashed green line. Bonferroni correction for multiple testing was carried out. Displayed are the parent terms only. Shown are overrepresented terms for Pathways (A), Molecular Function (B), Biological Process (C) and Protein Class (D). Analysis carried out using PANTHER Overrepresentation Test (release 20170413); PANTHER version 12 Released 2017-07-10, pantherdb.org.

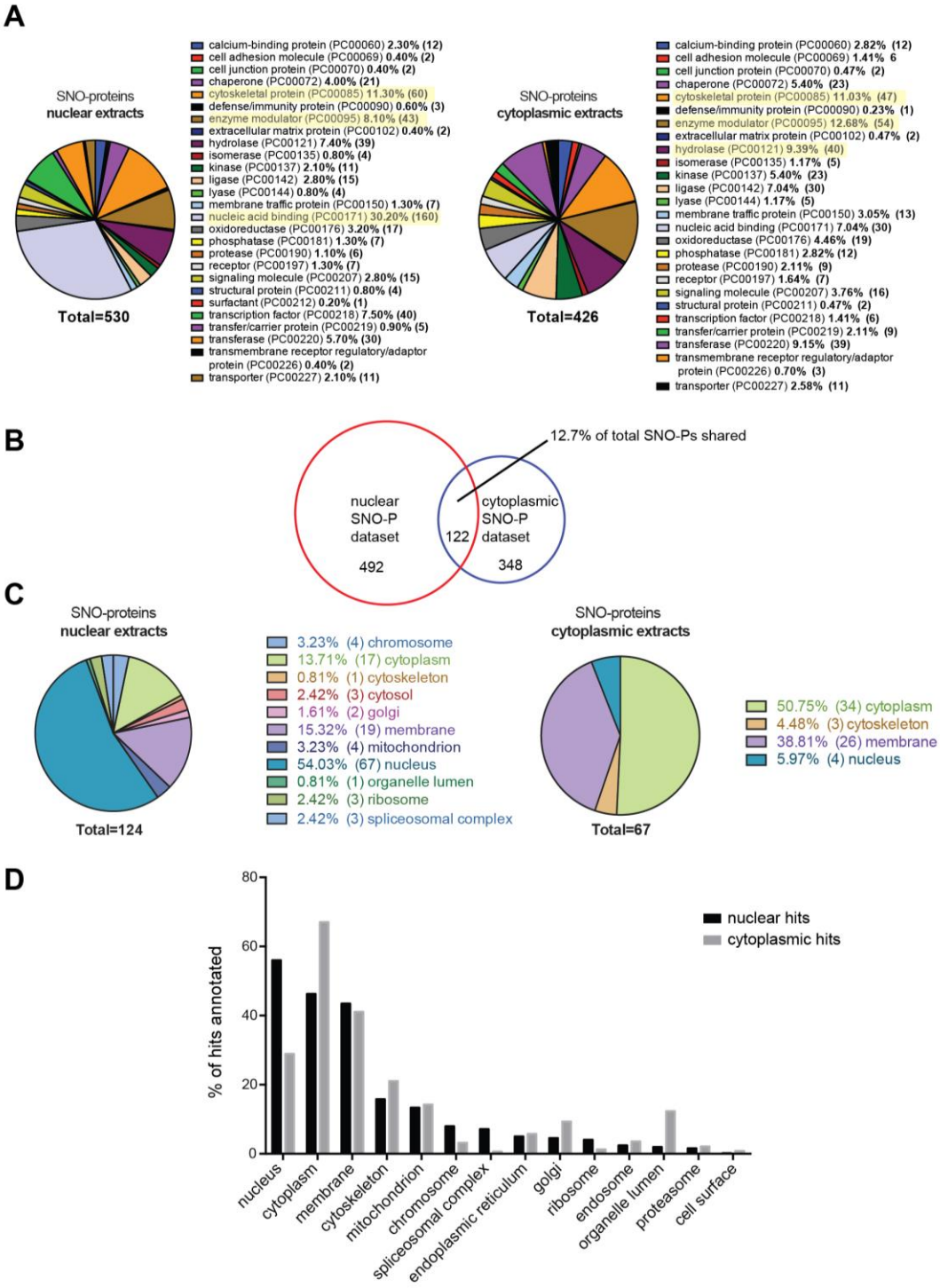


Fig. S3. GO standard classifications. (A-D) GO analysis of S-nitrosylated hits listed in table S2. (A) Protein Class (PANTHER, version 11.1); 530 annotations were assigned for S-nitrosylated proteins identified in nuclear extracts, 426 annotations for cytoplasmic S-nitrosylated proteins. The three most commonly annotated terms for each dataset are highlighted in yellow. (B) Overlap between nuclear and cytoplasmic SNO-P datasets. (C) Exclusive annotations for cellular component (Uniprot terms as assigned by Proteome Discover software). The number of times a particular term is assigned unambiguously, i.e. when no other term is present are shown. (D) Total annotations for cellular component (Uniprot terms, Proteome Discover). The percentage of annotated hits that were assigned each particular term. This includes the majority of cases in which multiple terms were assigned.

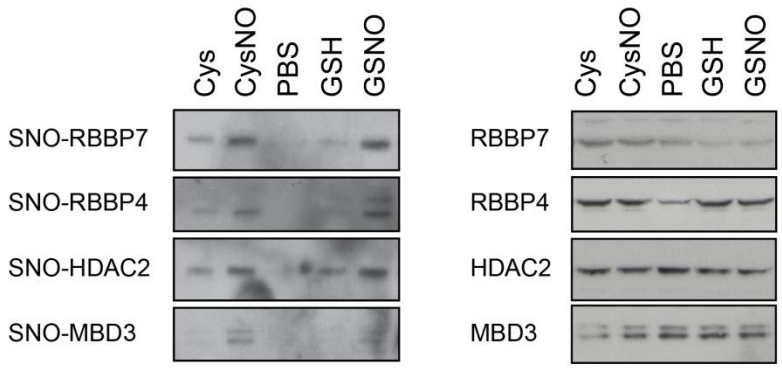


Fig. S4. GSNO-dependent S-nitrosylation of HDAC2, RBBP7, RBBP4, and MBD3. Neurons were treated with Cys, CysNO, GSH or GSNO (200 μM for 20 min) and S-nitrosylated proteins isolated by biotin switch. Western blot was carried out on biotin switch samples and inputs using the indicated antibodies. Blots are representative of n=2 experiments.

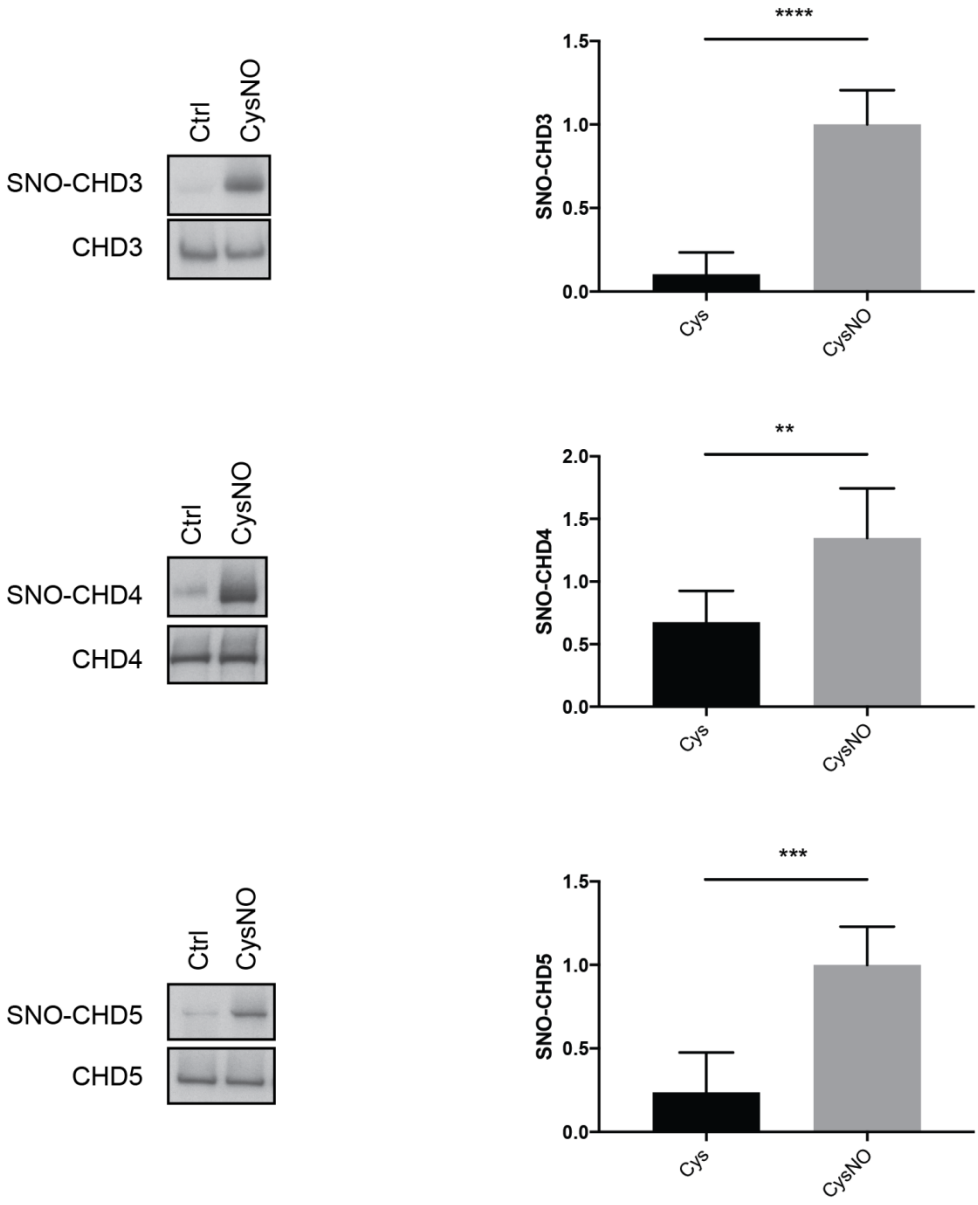


Fig. S5. S-nitrosylation of CHD3, CHD4, and CHD5. Vectors expressing flag-tagged hCHD3, mCHD4 and hCHD4 were transfected in HEK293T cells. After 48 hours, cell lysates were treated with Cys or CysNO (200 µM for 20 min) and subjected to the biotin switch assay using NEM (n=3 experiments) or MMTS (n=3 experiments) blocking conditions. Data were combined for densitometry analysis. Isolated proteins and total inputs were separated by SDS-PAGE followed by immunoblotting using a flag antibody. Densitometry analysis was carried out using ImageJ. SNO-signals were normalised to total inputs and expressed as fold change relative to CysNO. Data are mean +/- SEM (n= 6 experiments), **p<0.01, ***p<0.001, and ****p<0.0001 by unpaired t-test.

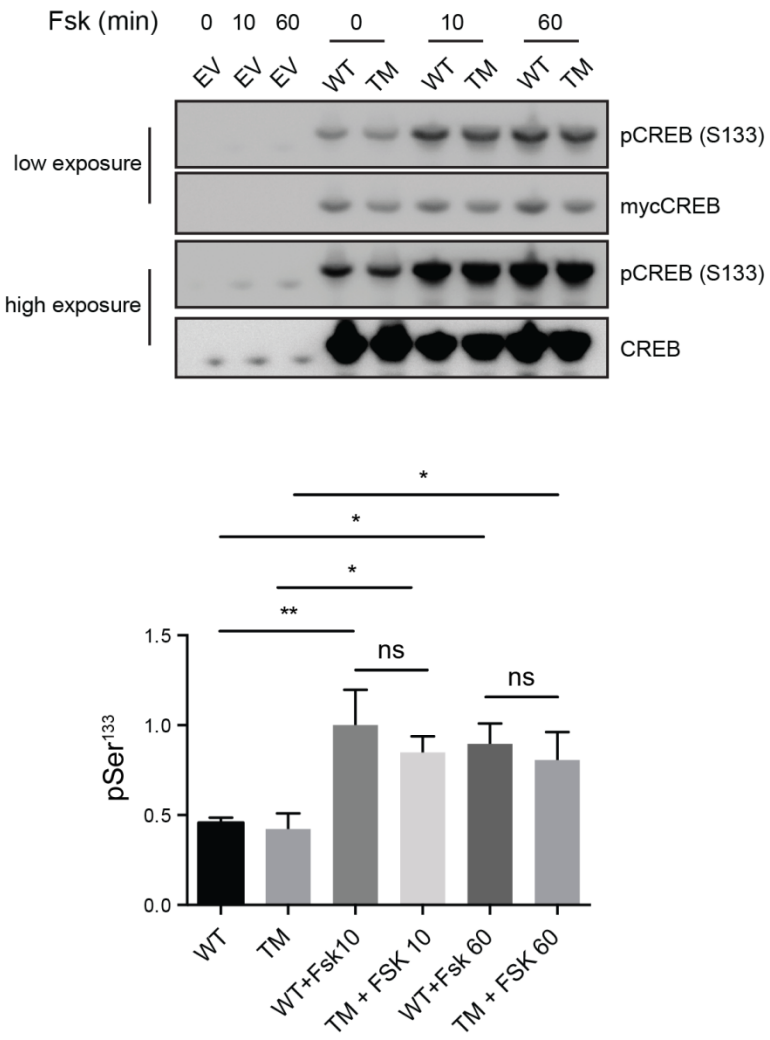


Fig. S6. CREB phosphorylation at Ser^{133} is unaffected in the C300/310/337S mutant. HEK293T cells were transfected with empty vector (EV), myc-tagged wild-type (WT) or C300/310/337S mutant (triple mutant; TM) CREB and after 48 hours, were treated with forskolin (50 µM for 10 or 60 min). Proteins from cell lysates were separated by SDS-PAGE followed by western blotting using an antibody against CREB phospho-Ser^{133} (pS133). Expression levels of transfected plasmids were assessed with myc antibody and endogenous CREB levels were assessed using a CREB antibody. Densitometry analysis on western blots was carried out using ImageJ; pS133 signals were normalised to mycCREB, and values expressed as fold change of the pS133 signal in WT mycCREB upon 10 min FSK treatment. Data are mean +/- SEM. from n=3 independent experiments. *p<0.05, **p<0.01, and ns= not significant by one-way ANOVA, Fisher's LSD.

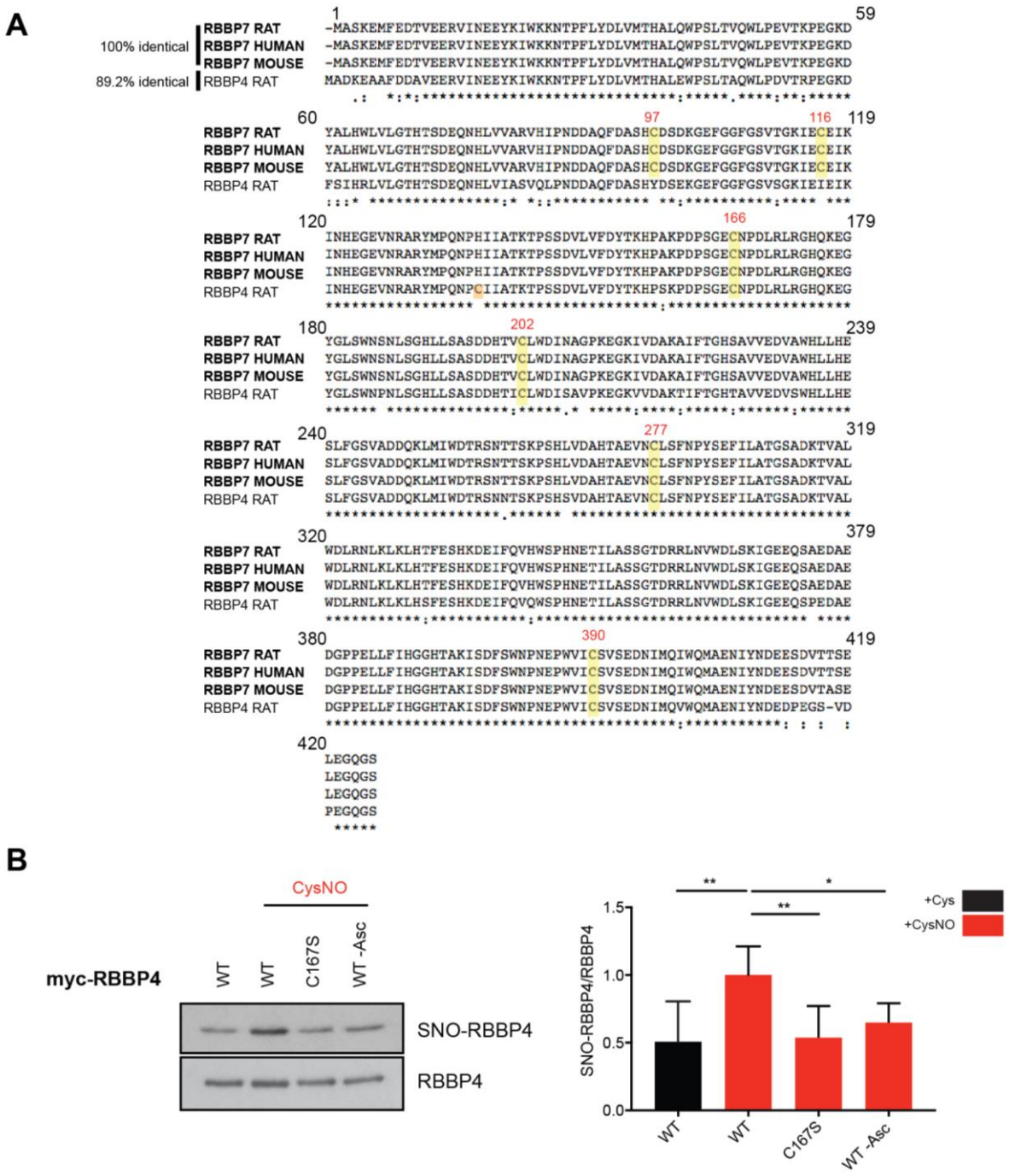


Fig. S7. RBBP7/4 sequence conservation and SNO-site analysis for RBBP4 and MBD3. (A) Sequence homology between rat, human and mouse RBBP7 and rat RBBP4. Sequence alignment carried out using Clustal Omega (Embl-Ebi**). (B)** Vectors expressing myc-tagged wild type rtRBBP4 (WT) or RBBP4 Cys^{167} to serine mutant (C167S) were transfected in HEK293T cells. 48 hours after transfection cells were treated with Cys or CysNO and lysates subjected to biotin switch assay. Isolated proteins and total inputs were separated by SDS-PAGE followed by immunoblotting using a myc antibody. Densitometry analysis was carried out using ImageJ. SNO-signals were first normalised to total inputs and expressed as fold change relative to WT +CysNO. All data are shown as mean +/- SEM. *p<0.05, **p<0.01 by one-way ANOVA compared to WT +CysNO column; Fisher's LSD. (B) n=6 independent experiments.

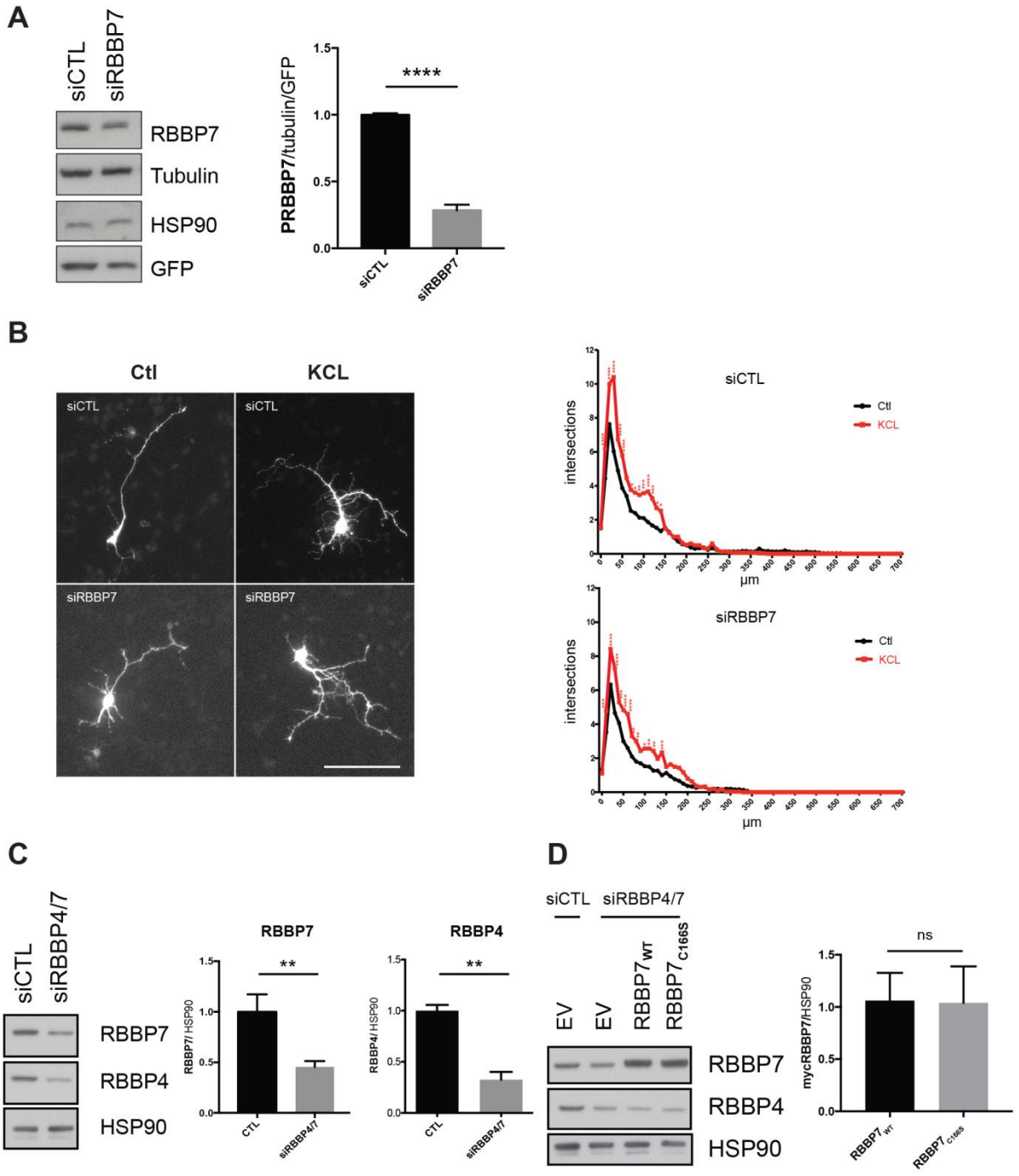


Fig. S8. RBBP7 knockdown and dendritogenesis analysis. (A). RBBP7 knockdown in N2a cells. N2a cells were transfected with a GFP expression vector together with 200 nM of either control siRNA (CTL) or targeting RBBP7 (siRBBP7). Cells were harvested 48 h after transfection. Western blot analysis of RBBP7, tubulin, HSP90 and GFP levels. Densitometry analysis of western blots was carried out using ImageJ. RBBP7 signal normalised to tubulin and GFP. **p<0.01 unpaired t-test (n=3). **(B)** RBBP7 knockdown on activity-induced dentritogenesis in cortical neurons. Control siRNA (CTL) or siRNA against RBBP7 (siRBBP7) was transfected into E15 mouse cortical neurons alongside a GFP expression vector and mycEV. Neurons were maintained in normal media (Ctl) or in the presence of 50 mM KCl (KCl) for 2 days then immunostained using an anti-GFP antibody. Images were analysed using Fiji Sholl plugin. Maximal projections of representative neurons, scale bar

100 μM. 3 biological replicates were carried out and 10 neurons analyzed per experiment (30 neurons in total). Data are mean values for number of intersections (y axis) against distance from soma (x axis; μm). Readings were taken every 10μm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 two-way ANOVA with Sidak's test for multiple comparisons. Red stars indicate KCl value is significantly greater than Ctl. **(C)** Confirmation of RBBP4/7 double knockdown. N2a cells were co-transfected with GFP expression vector and 400 nM total RNA of either control siRNA (CTL) or siRNA's against RBBP4 and RBBP7 in a 1:1 ratio (siRBBP4/7). Cells were harvested 48 hours after transfection and analyzed by Western blot. Western blotting of RBBP7, RBBP4 and HSP90 are shown. Densitometry analysis of western blots carried out using ImageJ. RBBP7 and RBBP4 levels were normalized to HSP90. All data are means +/- SEM. **p<0.01, unpaired t-test (n=3). **(D)** Expression of siRNA-resistant myc-RBBP7^{WT} RBBP7^{C166S}. N2a cells were transfected with either myc-EV, siRNA-resistant myc-RBBP7^{WT} or RBBP7^{C166S} and siRNA CTL or the double knockdown siRBBP4/7. 48 h after transfection cells were harvested and levels of RBBP7, RBBP4, HSP90 analyzed by Western blot. RBBP7 levels in RBBP7^{WT} and RBBP7^{C166S} conditions were quantified by densitometry, using HSP90 as a loading control. ns= not significant by an unpaired t-test. Data are means +/- SEM from n=3 experiments.

Table S1. Numerical summary of quantitative MS data of CysNO-treated nuclear extracts from E17 rat cortical neurons. Table shows the number of proteins detected by quantitative mass spectrometry analysis either as enriched in CysNO (≥2 fold versus controls), unchanged (<2 fold and >0.5 fold enriched in CysNO versus controls), or enriched in controls (≥2 fold versus CysNO). The data were obtained from 5 independent experiments. Controls are either cysteine only (Cys) or CysNO without addition of ascorbate (CysNO minus asc) as indicated. Please note that ascorbate is required for the detection of S-nitrosylated proteins with SNORAC.

Treatment	# proteins detected ≥2 fold enriched in cysNO	% of total	# proteins detected unchanged	% of total	# proteins detected ≥2 fold in control	% of total	total number of proteins detected
1mM CysNO vs Cys (Exp 1)	448	**48.4**	409	**44.2**	69	**7.45**	926
1mM CysNO vs CysNO minus asc (Exp 1)	1046	**88.9**	108	**9.18**	22	**1.87**	1176
1mM CysNO vs Cys (Exp 2)	751	**76.8**	223	**22.8**	4	**0.41**	978
1mM CysNO vs CysNO minus asc (Exp 2)	371	**38.2**	416	**42.8**	184	**18.9**	971
200µM CysNO vs Cys (Exp 3)	276	**69.2**	119	**29.8**	4	**1**	399
200µM CysNO vs CysNO minus asc (Exp 3)	344	**80.2**	80	**18.6**	5	**1.17**	429
200µM CysNO vs Cys (Exp 4)	142	**65.1**	73	**33.5**	3	**1.38**	218
200µM CysNO vs CysNO minus asc (Exp 4)	323	**86.6**	43	**11.5**	7	**1.88**	373
200µM CysNO vs Cys (Exp 5)	1635	**92.4**	121	**6.84**	14	**0.79**	1770
Average	593	**71.8**	177	**24.4**	34.7	**3.88**	804

Table S2. Collated hits of CysNO-treated neuronal nuclear extracts. S-nitrosylated proteins in CysNO-treated nuclear extracts from E17 rat cortical neurons. Proteins listed were detected by quantitative mass spectrometry analysis as enriched in CysNO (≥2 fold average) in 3 or more biological replicates. The value for each protein is the ratio of detection in CysNO vs control (either Cys or CysNO-asc as indicated). Values of 100 were assigned when the protein was detected in a treated sample but not in control samples. The average fold increase is displayed in green and takes into account only experiments in which the protein was detected. Gene ontology annotation was assigned by Proteome Discoverer (Thermo Fisher). Table is provided as an .xls file in the online supplementary materials.

Table S3. Altered stringency S-nitrosylated list 1. Proteins detected by quantitative mass spectrometry analysis as enriched in CysNO (≥1.3 fold average) in 3 or more biological replicates. Proteins already detected in the high stringency dataset were not included. The displayed value for each protein is the ratio of detection in CysNO vs control (either Cys or CysNO-asc as noted). Values of 100 were automatically assigned when the protein was detected in a treated sample but not detected in control samples. The average fold increase is displayed in bold and only takes into account experiments in which the protein was detected. Gene ontology annotation was assigned by Proteome Discoverer (Thermo Fisher). Table is provided as an .xls file in the online supplementary materials. Please, note that this

analysis may contain false positives, as histones that do not contain cysteines were also detected (highlighted in red).

Table S4. Altered stringency S-nitrosylated list 2. List of nuclear proteins from E17 rat cortical neurons detected by quantitative mass spectrometry analysis as enriched in CysNO (≥1.5 fold average) in 2 or more biological replicates. Proteins already detected in the high stringency dataset were not included. The displayed value for each protein is the ratio of detection in CysNO vs control (either Cys or CysNO-asc as noted). Values of 100 were automatically assigned when the protein was detected in a treated sample but not detected in control samples. Gene ontology annotation was assigned by Proteome Discoverer (Thermo Fisher). Table is provided as an .xls file in the online supplementary materials. Please, note that this analysis may contain false positives, as histones that do not contain cysteines were also detected (highlighted in red).

Table S5. Basally SNO-Ps. List of nuclear proteins from E17 rat cortical neurons detected at comparable levels in CysNO vs Cys samples (>0.5,<2), but increased in CysNO compared to CysNO w/o ascorbate (≥2 fold). Proteins were included if they behaved as described in ≥75% detection. These proteins are likely basally S-nitrosylated in untreated conditions and are not affected by CysNO. Experiments 1 to 4 were used for this analysis due to the presence of both Cys and CysNO without ascorbate controls. Table is provided as an .xls file in the online supplementary materials.

Table S6. Proteins detected as unchanged across conditions. List of nuclear proteins from E17 rat cortical neurons detected in CysNO-treated neuronal extracts as unchanged across conditions (>0.5, <2 fold enriched in CysNO versus controls). Proteins were included if they behaved as described in ≥75% detections. These proteins likely represent background signal due to non-specific binding to the beads. Experiments 1 to 4 were used for this analysis due to the presence of both Cys and CysNO without ascorbate controls. Table is provided as an .xls file in the online supplementary materials.

Table S7. Nuclear extracts background data set. Nuclear extracts from untreated E17 rat cortical neurons cultured for 4 days in vitro and subjected to LC MS/MS. Proteins detected in this analysis are listed alphabetically. Table is provided as an .xls file in the online supplementary materials.

Table S8. SNO-Ps in CysNO-treated neuronal cytoplasmic extracts. S-nitrosylated proteins in CysNO-treated cytoplasmic extracts from E17 rat cortical neurons. Proteins listed here were detected by quantitative mass spectrometry analysis as enriched in CysNO versus Cys (≥2 fold). Gene ontology annotation was assigned using PANTHER (pantherdb.org). S-nitrosylated protein hits are listed alphabetically. Gene ontology annotation was assigned by Proteome Discoverer (Thermo Fisher). Listed is the average quantitative value for each protein in CysNO vs Cys, from 2 technical replicates. Table is provided as an .xls file in the online supplementary materials.

Table S9. SNO-Ps involved in transcription. Gene ontology annotation was assigned using PANTHER (pantherdb.org) for the term “transcription, DNA dependent” (GO:0006351). Proteins are listed in alphabetical order. Table is provided as an .xls file in the online supplementary materials.

Table S10. SNO-Ps involved in chromatin organization. Gene ontology annotation was assigned using PANTHER (pantherdb.org) for the term “chromatin organization” (GO:0006325). Proteins are listed in alphabetical order. Table is provided as an .xls file in the online supplementary materials.

Table S11. Cysteine-containing SNO peptides detected in CysNO-treated nuclear extracts. Shown is LC MS/MS data for all cysteine-containing peptides detected upon

CysNO treatment of nuclear extracts from E17 rat cortical neurons. Data is from SNO-site analysis of experiment 5. See also table S12 (SNO-peptides detected in Cys) and table S13 (SNO-sites assigned to quantitatively validated SNO-proteins). Table is provided as an .xls file in the online supplementary materials.

Table S12. Cysteine-containing SNO peptides detected in Cys-treated nuclear extracts. Shown is LC MS/MS data for all cysteine-containing peptides detected upon Cys treatment of nuclear extracts from E17 rat cortical neurons. Data is from SNO-site analysis of experiment 5. See also table S11 (SNO-peptides detected in CysNO) and table S13 (SNO-sites assigned to quantitatively validated SNO-proteins). Table is provided as an .xls file in the online supplementary materials.

Table S13. SNO-Ps with their corresponding SNO sites. SNO-peptides detected in CysNO from experiment 5 (see table S11) were assigned to quantitatively validated SNO-proteins (see table S2). Displayed are the SNO-proteins with corresponding SNO-sites, with cysteine numbers listed. Table is provided as an .xls file in the online supplementary materials.

Table S14. Altered stringency S-nitrosylated list 1, paired with SNO sites.
SNO-peptides detected in CysNO from experiment 5 (table S11) were assigned to reduced stringency hit list 1 (table S3). Displayed are SNO-proteins with corresponding SNO-sites. Table is provided as an .xls file in the online supplementary materials.

Table S15. Altered stringency S-nitrosylated list 2, paired with SNO sites.
SNO-peptides detected in CysNO from experiment 5 (table S11) were assigned to reduced stringency hit list 2 (table S4). Displayed are SNO-proteins with corresponding SNO-sites. Table is provided as an .xls file in the online supplementary materials.

Table S16. Treatment groups for MS analysis of 1 mM CysNO-treated neuronal nuclear extracts. Treatment groups are for experiments 1 and 2, in which nuclear extracts from rat cortical neurons were treated with Cys, CysNO, or CysNO without ascorbate. Please note that ascorbate is required for the detection of S-nitrosylated proteins with SNORAC.

Exp. 1	**Exp. 2**
A1. 1 mM Cys	B1. 1 mM Cys
A2. 1 mM CysNO	B2. 1 mM CysNO
A3. 1 mM CysNO without ascorbate	B3. 1 mM CysNO without ascorbate

Table S17. Treatment groups for MS analysis of 200 µM CysNO-treated neuronal nuclear extracts. Treatment groups are for experiments 3 and 4, in which nuclear extracts from rat cortical neurons were treated with Cys, CysNO, or CysNO without ascorbate. Please note that ascorbate is required for the detection of S-nitrosylated proteins with SNORAC.

Exp. 3	**Exp. 4**
C1. 200µM Cys	D1. 200µM Cys
C2. 200µM CysNO	D2. 200µM CysNO

C3. 200μM CysNO without ascorbate	D3. 200μM CysNO without ascorbate

Table S18. Labeling scheme for dimethyl labeling of 200 μM CysNO-treated neuronal nuclear extracts. Treatment groups for dimethyl labelling of experiments 3 (C1-C3) and 4 (D1-D3), in which nuclear extracts from rat cortical neurons were treated with Cys, CysNO, or CysNO without ascorbate. Please note that ascorbate is required for the detection of S-nitrosylated proteins with SNORAC. L; light, M; medium.

Sample	**Label**	**M:L pairs**
C1. 200μM Cys	L	80 μL of C1 (L) + 80 μL of C2 (M) 80 μL of C3 (L) + 80 μL of C2 (M)
C2. 200μM CysNO	M	
C3. 200μM CysNO without ascorbate	L	
D1. 200μM Cys	L	80 μL of D1 (L) + 80 μL of D2 (M) 80 μL of D3 (L) + 80 μL of D2 (M)
D2. 200μM CysNO	M	
D3. 200μM CysNO without ascorbate	L	

Table S19. Treatment groups for MS analysis of 200 μM CysNO-treated neuronal and cytoplasmic nuclear extracts. Treatment groups for experiment 5 on Cys or CysNO-treated nuclear extracts from rat cortical neurons and experiment 1 on Cys or CysNO-treated cytoplasmic extracts from rat cortical neurons.

Nuclear extracts (Exp. 5)	**Cytoplasmic extracts (Exp. 1)**
1. 200μM Cys	3. 200μM Cys
2. 200μM CysNO	4. 200μM CysNO