Supplementary information

Shank3 mutation in a mouse model for autism leads to changes in the S-nitroso-proteome and affects key proteins involved in vesicle release and synaptic function

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Animal housing and tissue dissection
Animals were housed at a constant 23 °C in a 12-h light/dark cycle (lights on at 07:00, lights off at 19:00) with ad libitum food and water.
Tissue was obtained from age-matched brains of wild type, and InsG3680(+/+) male mice. Briefly, mice were decapitated, and whole cortex and striatum were micro-dissected, snap-frozen in liquid nitrogen, and stored in -80c until use. For tissue dissection, mice were chosen in an alternate order, and similarly, samples preparation was done in an alternate order.

Materials and reagents. Biotin-PEG3-propionic acid was purchased from ChemPep Inc. Protease cocktail inhibitors were purchased from Sigma. Sequencing-grade modified trypsin from Promega. The High–Performance Liquid Chromatography (HPLC) and the Liquid Chromotography – Mass Spectrometry (LC-MS) solvents were HPLC grade. Acetonitrile (ACN) and distilled water for MS use were purchased from Sigma-Aldrich. Vivapsin 10k molecular weight cut off (MWCO) filters were purchased from Sartorius Stedim NA. Synthesis of SNOTRAP-biotin and NMR analysis are described in details in ref (1). All sample preparation was conducted in dark at room temperature (RT).

Homogenization and preparation of brain tissue for Mass Spectrometry (MS).
Whole cortex and whole striatum were dissected from 6 week-old and 4 month-old WT and InsG3680(+/+) mice, removed and transferred in liquid nitrogen to store in -80 °C until use. We used 2 biological replicates and 3 technical replicates for each biological replicate. Each biological replicate for the cortex was pooling of 3 cortex tissues from 3 mice and for the striatum tissues was pooling of 4 striatum tissues from 4 mice.
Tissues were homogenized into lysis buffer (freshly prepared, 250 mM HEPES-NaOH, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% NP-40, 20 mM IAM, 1% protease inhibitor cocktail (Sigma-Aldrich, Cat. No. P8340)) on ice using a Teflon pestle and a Jumbo Stirrer (Thermo Fisher).
The homogenates were centrifuged at 12,000 g for 10 min at 4 °C and supernatants were collected. The protein concentration was determined by the Bradford assay (Bio-Rad, Cat. No. 500-0006).
Negative controls were generated by treatment with 10 mM TCEP for 30 min at 37°C after sample mixing. Samples were then alkylated with 30 mM IAM in presence of 2.5% SDS in the dark at 37°C. After alkylation, samples were twice washed with 3 times volumes of 8 M Urea (in 50 mM HEPES, pH 7.7) and once with 50 mM HEPES (pH 7.7) by centrifugation at 5,000 g for 30 min at 4°C with 10 K MWCO spin filters (pre-rinsed with water once, Sartorius corporation, Cat. No. VS15T01).
SNOTRAP labeling stock solutions (in 40% ACN) were added to all samples to reach a final concentration of 1.25 mM (in 50 mM HEPES buffer at pH 7.7) to selectively convert SNO to stable disulfide-iminophosphorane. Samples were incubated with SNOTRAP solution at room temperature for 1 hr. After SNOTRAP labeling ,excessive

reagents were removed by three washes with 50 mM HEPES, pH 7.7 buffer with 10 K filters.
After ultrafiltration, 200 µl pre-rinsed Streptavidin agarose beads (Pierce, Cat. No. 20349) were added to each sample and incubated for 1 h at room temperature with gentle shaking. The beads were washed with washing buffer (50 mM HEPES, 150 mM NaCl, 0.1 % SDS, pH 7.7) three times and then with washing buffer (50 mM HEPES, pH 7.7) three times. Proteins were eluted with 10 mM TCEP (in 50 mM HEPES, pH 7.7) and then alkylated with 10 mM IAM. After alkylation, samples were trypsinized (Promega, Cat. No. V5111) at 37°C for 4 h and then desalted with C18 StageTips as described in (2).

Mass Spectrometry analysis
Protein digests were analyzed on an Agilent 6550 Nano-HPLC-Chip/MS system, consisting of a micro-autosampler, a capillary and nanoflow pump, and the Chip-Cube that interfaces LC modules and the MS instrument. Water (0.1% FA) and ACN (0.1% FA) were the mobile phases A and B, respectively. Peptide separations were carried out on a Polaris-HR-Chip-3C18 HPLC-Chip (Agilent Technologies, Cat. No. G4240-62030), consisting of a 360 nL enrichment column and a 75 µm× 150 mm analytical column, which both were packed with Polaris C18-A, 180A, 3 µm stationary phase. Peptides were loaded onto the enrichment column from the autosampler at a constant flow of 2 µL/min provided by the capillary pump. A 55-min gradient started at 3% B at 300 nL/min and increased to 30% B from 2 to 35 min, to 60% B at 40 min, to 90% B at 45 min and then was held for 5 min and followed by a 5 min postrun at 3% B. Positive-ion MS spectra were acquired in the 1,700 Da extended dynamic range mode (2 GHz) using the following setting: ESI capillary voltage, 1,960 V; fragmentor, 360 V; Octopole RF peak, 750 V; drying gas, 13 L/min; drying temperature, 225°C. Data were acquired at a rate of 6 MS spectra per second and 3 MS/MS spectra per second in the mass range of m/z 300-1,700 for MS and 50-1,700 for MS/MS and stored in centroid mode. Maximum number of precursors per cycle was 20, with a threshold of 5,000 ions in a precursor abundance-based scan speed in peptide isotope model, with +2, +3 and above charge-state preference, and with active exclusion after 1 spectrum and released after 0.15 min. Fragmentation energy was applied at a slope of 3.1 V/100 Da with a 1.0 offset for doubly charged precursors, 3.6 V/100 Da with a -4.8 offset for triply and multiply charged precursors. Mass accuracy was maintained by using internal reference ion *m/z* 1221.9906. Agilent MassHunter Workstation software was used for data acquisition. Three technical runs were conducted for each sample.

MS data processing
Agilent Spectrum Mill MS proteomics Workbench B.05 was used for peak list generation, database searching, and FDR estimation. Parameters for data extractions were: cysteine carbamidomethylation for fixed modification, precursor MH+ 300 – 8000 Da, scan time range 0-200 min, sequence tag length > 1, merge scans with same precursor m/z +/-30s and +/- 0.05 m/z, default for precursor charge, true for find 12C precursor, MS noise threshold 100 counts. MS/MS spectra were searched against the mouse SwissProt protein database (downloaded on

11/01/2016, 16813 items) with +/-20 ppm precursor ion tolerance and +/-50 ppm fragment ion tolerance. The search included variable modifications of methionine oxidation, protein N-terminal acetylation, deamidation of asparagine, and fixed modification of cysteine carbamidomethylation. For both peptide identification and protein polishing, the FDR was set to 1.2%. The MS/MS spectra were inspected manually to validate the peptide/protein identifications. In addition, for proteins detected in one genotype but not another, the raw LC/MS data-files for the latter were searched manually for the presence of protein-related peptides detected in the former. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier < PXD006907 >.

Western Blot (WB). WB was used to measure levels of proteins mentioned below, supernatant of the homogenizing tissue was diluted with reducing buffer, Laemmli sample buffer (Biorad, 161-0737) and electrophoresed on Tris-HCL 4-20% pre-cast linear gradient gel (Biorad, 4561093) and transferred to PVDF membrane (Biorad, 1620174). The membranes were blocked with 5% milk in PBS-0.05% Tween 20 for 1 hr at RT, incubated with primary antibody overnight at 4 C, and after 6 washes with TBS-0.3%Tween 20, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hr at RT. Protein bands were visualized using ECL reagent. The bands were captured using Fluorchem™ 8900 from Alpha Innotech. Primary antibodies were purchased as follow: Synapsin1 (Invitrogen, 515200, 1/1000 dilution), Phospho-synapsin1-Ser62, Ser67 (Invitrogen, PA1-4698, 1/1000 dilution), GAPDH (Cell signaling, 21185, 1/1000 dilution), CREB (Invitrogen, MA1-083, 1/500 dilution), Phospho-CREB-Ser133- (Invitrogen, PA1-4619, 1/1000 dilution), Pan-Calcineurin A (Cell Signaling, 2614, 1/1000 dilution), Syntaxin1a (Cell Signaling, 13002, 1/1000 dilution), nNOS (Cell signaling, Cat Number 4231S, 1/1000).
Secondary antibody: goat-anti-rabbit (Invitrogen, A11036, 1/20000 dilution) and goat-anti-mouse (Invitrogen, A11001, 1/20000 dilution).. Each group contained 4 mice, each mice represents one biological replicate.

WB detection of *S*NO-proteins in Mouse Brains.
To detect *SNO*-proteins in the cortex by WB, WT and KO cortex tissues were labeled with SNOTRAP, pulled down by streptavidin and eluted with TCEP as described above. The eluted SNO-proteins were transferred into PVDF membranes, and the blot was probed against the targeted antibody as described above.

Immunohistochemistry (IHC). Immunohistochemical staining of mouse brain sections was performed as the following. Nitrotyrosine antibody (Millipore, AB5411, 1/200 dilution) was used. Formalin-fixed paraffin-embedded (FFPE) tissue sections were deparaffinized in 100% Xylene (Sigma-Aldrich) and then washed in 100%, 90% and 70% ethanol and ddH2O. Sections were then boiled in the citrate based antigen retrieval buffer (Dako, pH=6.0, S1700) at 95 °C for 20 minutes. The tissue sections were washed in 1X PBS and blocked with 3% BSA in PBS-Triton X100 (0.3% v/v) overnight. Tissue sections were incubated with primary antibody,

washed in PBS-Tween 20 (0.05% v/v) and incubated with Secondary antibody: goat-anti-rabbit (Invitrogen, A11036, 1/5000 dilution) and goat-anti-mouse (Invitrogen, A11001, 1/5000 dilution) at RT for 1 hour. Sections were washed in PBS-Tween 20 and counterstained with DAPI. The antibodies for the colocalization are NeuN (Millipore, catalog number MAB377, 1/500) and nNOS (Cell signaling, Cat Number 4231S, 1/1000). Each group contained 9 mice, when each mouse represents one biological sample.

GSNO analysis

For GSNO internal standard synthesis, sample preparation for GSNO quantification, and ESI + -QqQ-MS analyses, see reference (3). Each group contained 3 mice, each mice represents one biological replicate.

Statistics and Bioinformatics

To find the functional enrichment of Cellular Compartments (CC), Biological Processes (BP) and KEGG pathways we uploaded the SNO-proteins into The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (DAVID) Bioinformatics Recourses (version 6.8, https://david.ncifcrf.gov) (4). P values for term enrichment were modified from the Fisher Exact p value (4). GO and KEGG pathway enrichment analyses were performed by using the functional annotation tool in DAVID. The Benjamini-Hochberg correction (5)was used on the p-value to generate false discovery rate (FDR), and terms with FDR values below 0.1 were accepted. STRING (version 10.0) was used to analyze the protein-protein interaction of SNO-proteins (http://string-db.org) (6). High reliability interactions (score > 0.7) from neighborhood, gene fusion, co-occurrence, co-expression, experiments, databases and textmining lists were kept. To visualize the protein-protein interaction we used Cytoscape plug-in software (version 3.3.0). One tailed t-test was conducted with P-value<0.05. Mean and Standard Error of the mean (S.E.M) were calculated in the WB and IHC experiments. Sample sizes were chosen on the basis of preliminary experiments and our experience with similar experiments. Blind-coded experiments were done, in which the researchers who obtained the data were unaware of the specific genotype of mice.

Mathematical models

In order to roughly estimate the proportion of SNOTRAP-detectable proteins present in our SNOTRAP eluates, we fit mathematicalmodels.
Two free parameters: *n*, the number of detectable proteins and *p,* the fraction identified stochastically within each replicate, were estimated independently for each condition by a least-squares fit to the equation

$$f(x) = n - (n * (1 - p)^x)$$

where *x* is a number of replicates and *f(x)* is the average number of SNO-proteins identified across that many replicates.

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