Nutrient limitation affects presynaptic structures through dissociable Bassoon autophagic degradation and impaired vesicle release

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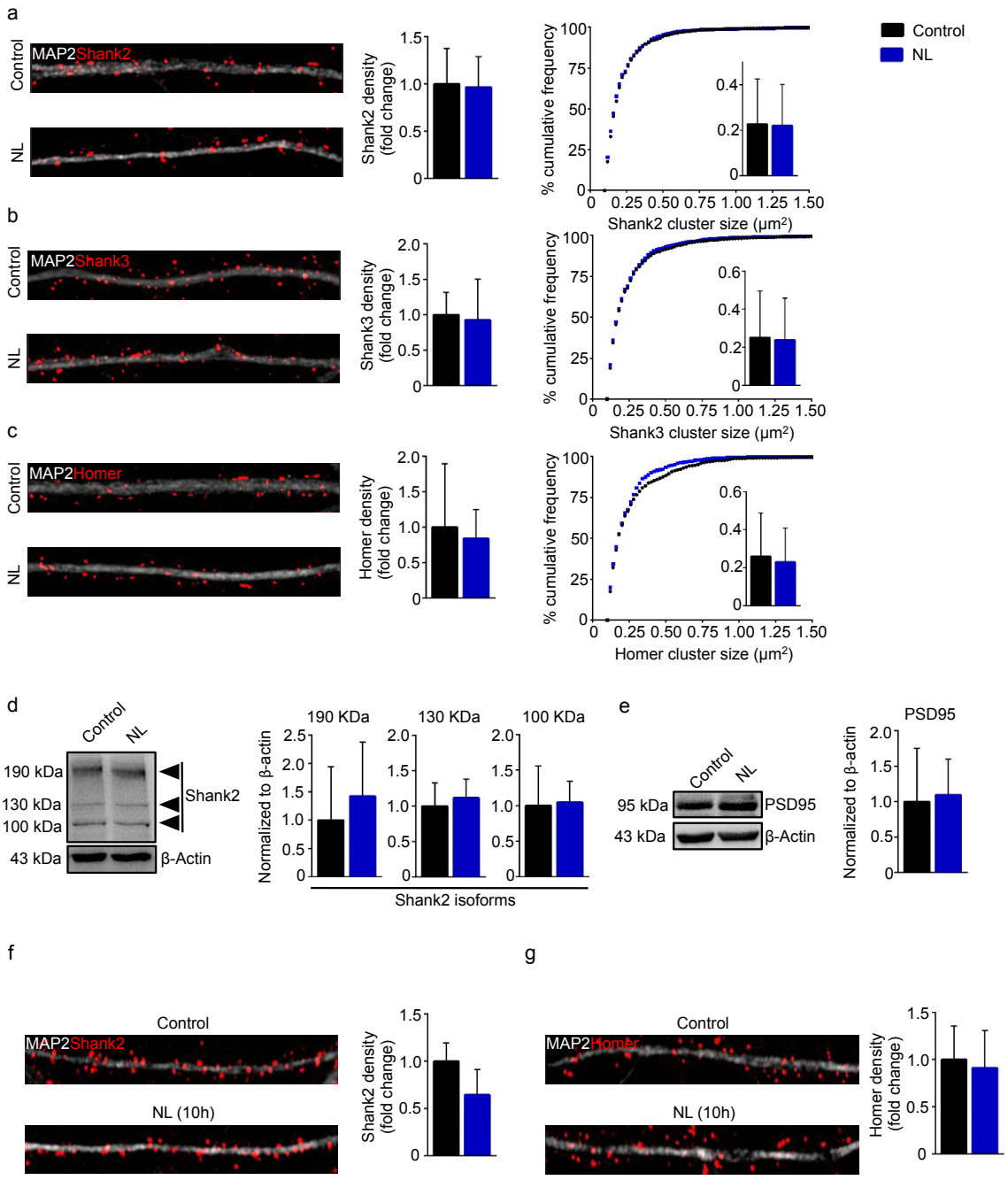
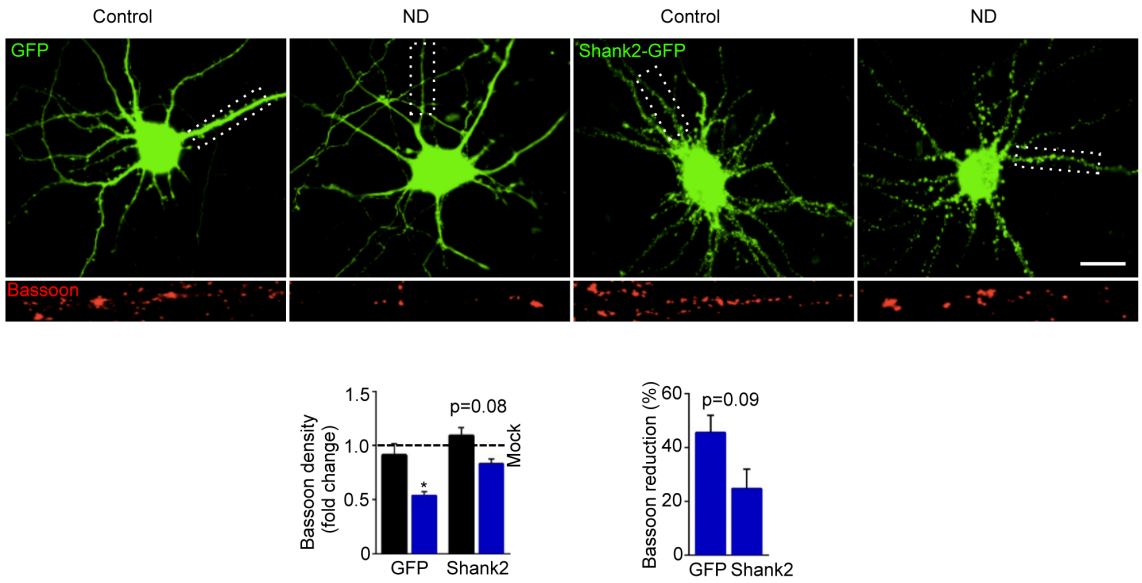
Supplementary material: Supplementary Figures 1, 2, and 3; Movie legends relative to Supplementary Movies 1 – 2; Supplementary Material and Methods; Supplementary References

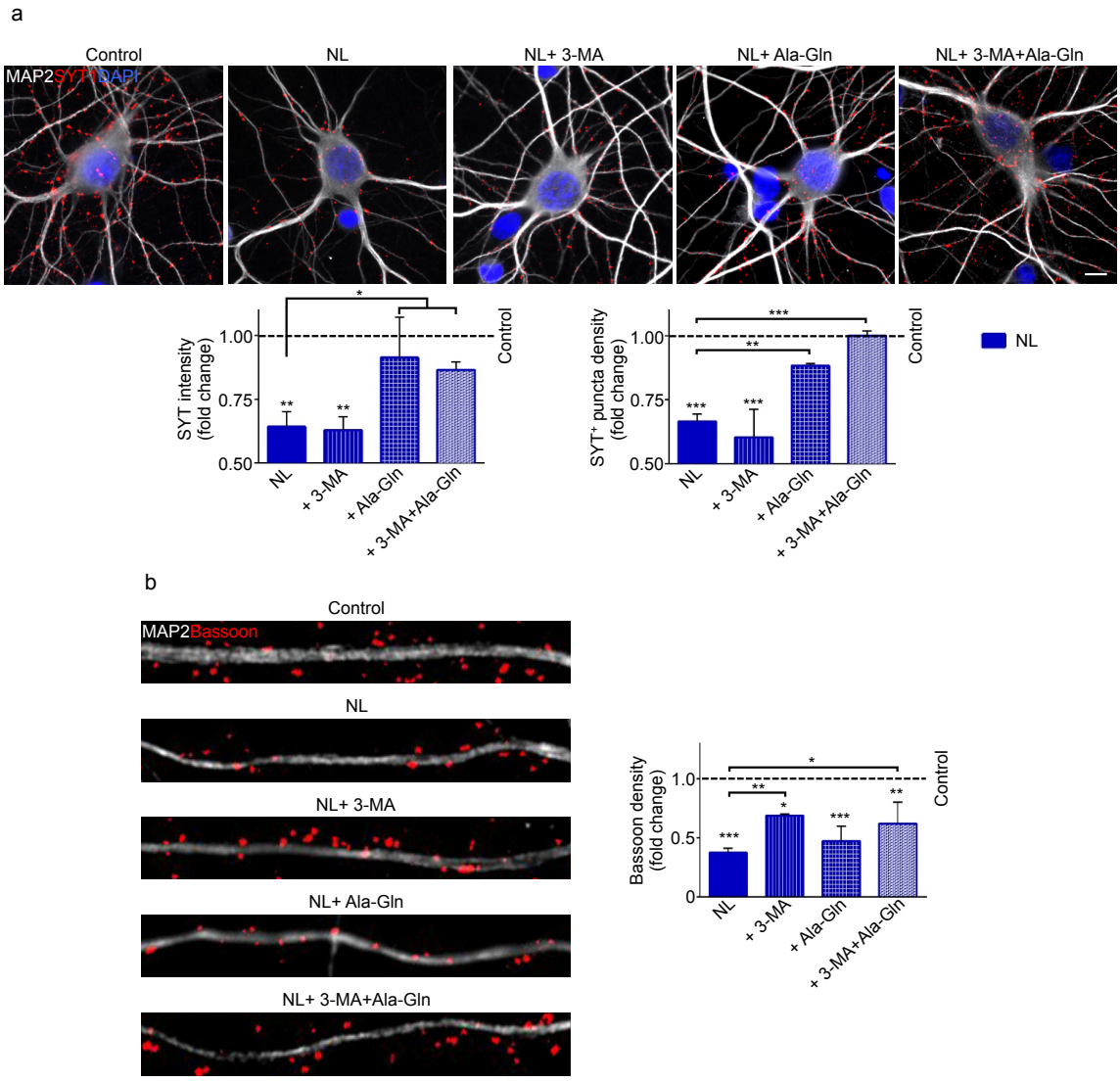


Supplementary Figure S1 The postsynapse show higher stability during NL. (a,b and c) Representative images of dendrites (30 µm) from Control and NL neurons stained against MAP2 and Shank2 (a), Shank3 (b), and Homer1 (c). The composition of the postsynaptic density appears to be preserved upon NL. (d and e) Representative immunoblots and quantitative analysis of Shank2 variants (d) and PSD95 (e). Total levels of both

postsynaptic scaffold proteins are not altered by ND. (f and g) Representative images of dendrites (30 μm) from Control and neurons exposed to 10 hours of NL stained against MAP2 and Shank2 (f) or Homer1 (g). Shank2 is slightly (but not significantly) down-regulated after 10 hours of NL, while Homer1 levels are not altered by the treatment. Experiments were performed in N=3 independent replicates. Data are displayed as Mean±SD.



Supplementary Figure S2 Overexpression of Shank2 does not rescue the NL-related presynaptic phenotype. Representative images of Control and ND neurons transfected with GFP reporter or Shank2-GFP vectors. (Control+GFP vs ND+GFP p=0.0254; Control+Shank2-GFP vs ND+Shank2-GFP p=0.0807; ND+GFP vs ND+Shank2-GFP p=0.0988). Experiments were performed in N=3 independent replicates. Data are displayed as Mean±SD. Scale bar represents 15 µm.



Supplementary Figure S3 Autophagy inhibition does not modify the rescue of synaptic vesicle cycling in response to metabolites re-administration (a) Anti-Synaptotagmin-1 antibody uptake assay was performed in Control and NL neurons co-treated for the last 2 hours of NL protocol with 3-MA, Ala-Gln, and the combination of the two (3-MA+Ala-Gln). Ala-Gln and 3-MA+Ala-Gln exert a comparable rescue of SV release, while 3-MA alone shows no effect. (Syt1 intensity: Control vs NL p=0.0025; Control vs NL+3-MA p=0.0021; NL vs NL+Ala-Gln p=0.0299; NL vs NL+3-MA+Ala-Gln p=0.0481; NL+3-MA vs NL+Ala-Gln p=0.0277; NL+3-MA vs NL+3-MA+Ala-Gln p=0.0481. Syt1

numbers: Control vs NL $p<0.0001$; Control vs NL+3-MA $p<0.0001$; NL vs NL+Ala-Gln $p=0.0021$; NL vs NL+3-MA+Ala-Gln $p<0.0001$; NL+3-MA vs NL+Ala-Gln $p=0.0003$; NL+3-MA vs NL+3-MA+Ala-Gln $p<0.0001$). (b) Representative images of dendrites (30 μm) from Control and NL neurons. Cells were co-treated for the last 2 hours of NL protocol with 3-MA, Ala-Gln, and the combination of the two and immunostained against MAP2 and Bassoon. 3-MA and 3-MA+Ala-Gln similarly increase the levels of dendritic Bassoon, while Ala-Gln alone is ineffective (Control vs NL $p=0.0002$; Control vs NL+3-MA $p=0.0243$, Control vs NL+Ala-Gln $p=0.0007$; Control vs NL+3-MA+Ala-Gln $p=0.0078$; NL vs NL+3-MA $p=0.0018$; NL vs NL+3-MA+Ala-Gln $p=0.0424$). Experiments were performed in N=3 independent replicates. Data are displayed as Mean±SD. (*$p<0.05$, **$p<0.01$,***$p<0.001$). Scale bar represents 10 μm in (a).

Supplementary Movie M1 Representative 3D reconstruction of a synapse from Control neurons

Supplementary Movie M2 Representative 3D reconstruction of a synapse from ND neurons

Supplementary Material and Methods

Primary neuronal culture

Briefly, embryonic brains were dissected out and placed in Hanks' Balanced Salt Solution w/ $CaCl_2$ w/ $MgCl_2$ (HBSS, Gibco) at 4°C; hippocampi were manually dissected under stereomicroscopic guidance. Dissected hippocampi were incubated for 15 minutes with 0.25% trypsin-EDTA (1x) (Gibco) at 37°C and 5% CO_2 under gentle shakings. The tissues were then washed once with Dulbecco's Modified Eagle Medium high glucose (4.5 g/L) (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, Sigma), 1% penicillin/streptomycin (P/S, Gibco) and 1% GlutaMAX (100x, Gibco) and mechanically dissociated in in Neurobasal Medium (1x, Gibco) supplemented with 1% P/S, 1% GlutaMAX and 2% B27 (50x, Gibco). The cell suspension was cleared by filtering on a 100µm mesh filter, and resuspended in Neurobasal. Dissociated cells were then plated on poly-L-lysine-coated (Sigma-Aldrich) glass coverslips or culture dishes. For transmission electron microscopy (TEM), cells were plated on sapphire discs coated with carbon (using a BAF 300 electron beam evaporation device) (Balzers) followed by poly-L-lysine coating. Cells were maintained in Neurobasal medium supplemented with 2% B27, 1% GlutaMAX and 1% P/S at 100 U/ml (henceforth NB^+ medium) at 37°C in 5% CO_2; medium was half-renewed weekly.

Primary antibodies list

Ms anti-Bassoon (SAP7F407, Enzo Life Sciences; 1:1000 in ICC), Rb anti-NR1 (G 8913, Sigma-Aldrich; 1:400 in ICC), Ck anti-MAP2 (CPCA-MAP2, EnCor Biotechnology; 1:1000 in ICC), Rb anti-Akt (C67E7; 1:1000 in WB), Rb anti-phospho-Akt (Thr308)

(D25E6; 1:1000 in WB) (all Cell Signaling), Rb anti-Atg5 (ab108327; 1:250 in ICC), Rb anti-mTOR (ab32028; 1:1000 in WB), Rb anti-mTOR (phospho S2448) (ab109268; 1:1000 in WB), Ms anti-p62 (ab56416; 1:500 in ICC and 1:1000 in WB), Ms anti-PSD95 (ab2723; 1:1000 in WB) (all Abcam), Gp anti-Piccolo (142 104; 1:500 in ICC), Rb anti-Bassoon (141 002; 1:500 in ICC), Ms anti-Gephyrin (147 011; 1:500 in ICC), Gp anti-Homer1 (160 004; 1:500 in ICC), Gp anti-MAP2 (188 004; 1:500 in ICC) (all Synaptic Systems).

Western Blot

Cells were lysed and sonicated in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Na-DOC, 100 mM NaOrthovanadate, 1% NP40 and protease/ phosphatase inhibitors). 10µg of proteins were resolved on 10% SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare) with a Trans-Blot Turbo device (BioRad). The membranes were blocked in 5% BSA (in TBS) and incubated with primary antibodies for 12 hours at 4°C. Membranes were washed 3 x 30 min in TBS+0.2% TWEEN (Carl Roth), incubated with HRP-conjugated secondary Ab (DAKO), and washed 3 x 30 min before HRP signal was detected using ECL detection kit (Thermo Fischer) and a MicroChemi 4.2 device (DNR Bio Imaging System). For quantification, Gel-analyzer Software 2010a was used.

Immunostaining

Cells were fixed in ice-cold methanol (-20ºC) for 5 minutes or in (4% paraformaldehyde/4% sucrose for the Synaptotagmin-1 antibody uptake, Shank2-GFP

transfection and LifeAct infection experiments. Cells were permeabilized and blocked 10% Goat serum/ 0.2% Triton-X-100 in PBS and thereafter incubated with primary antibodies for 48 hours. Cells were washed 3 x 30 min times in PBS and then incubated with the secondary antibodies (Alexa Fluor® 488, 568 or 647-conjugated, Molecular Probes) for 2 hours. Cells were washed 3 x 30 min in PBS before mounting with VectaMount (Vector labs) containing 4′,6-diamidino-2-phenylindole (DAPI) onto microscope glass slides.

Neuron transfection

Neurons were transfected with 1 μg full-size ProSAP1/Shank2 fused to GFP[1] or the GFP-reporter vector (VEC-PRT-0005/0006) using Optifect reagent (Invitrogene) according to the manufacturer's recommendations at DIV10. Culture medium was replaced with fresh medium 12 hours later.

Electron Microscopy

Samples were high pressure frozen without prefixation with liquid nitrogen at a pressure of 2100 bar using a Wohlwend HPF Compact 01 high-pressure freezer (Engineering Office, M. Wohlwend), freeze-substituted in 0.2% osmium tetroxide, 0.1% uranyl acetate, 5% dH2O in acetone and embedded in epoxy resin. For regular TEM 70-100 nm-thick sections were cut using an Ultracut UCT ultramicrotome (Leica) equipped with a diamond knife (Diatome). The sections were stained with 0.3% lead citrate.

Supplementary References

1. Proepper C, Johannsen S, Liebau S, et al. Abelson interacting protein 1 (Abi-1) is essential for dendrite morphogenesis and synapse formation. *EMBO J* 2007; 26: 1397-1409.