

Proteomic analysis of the human hippocampus identifies neuronal pentraxin 1 (NPTX1) as synapto-axonal target in late-stage Parkinson's disease

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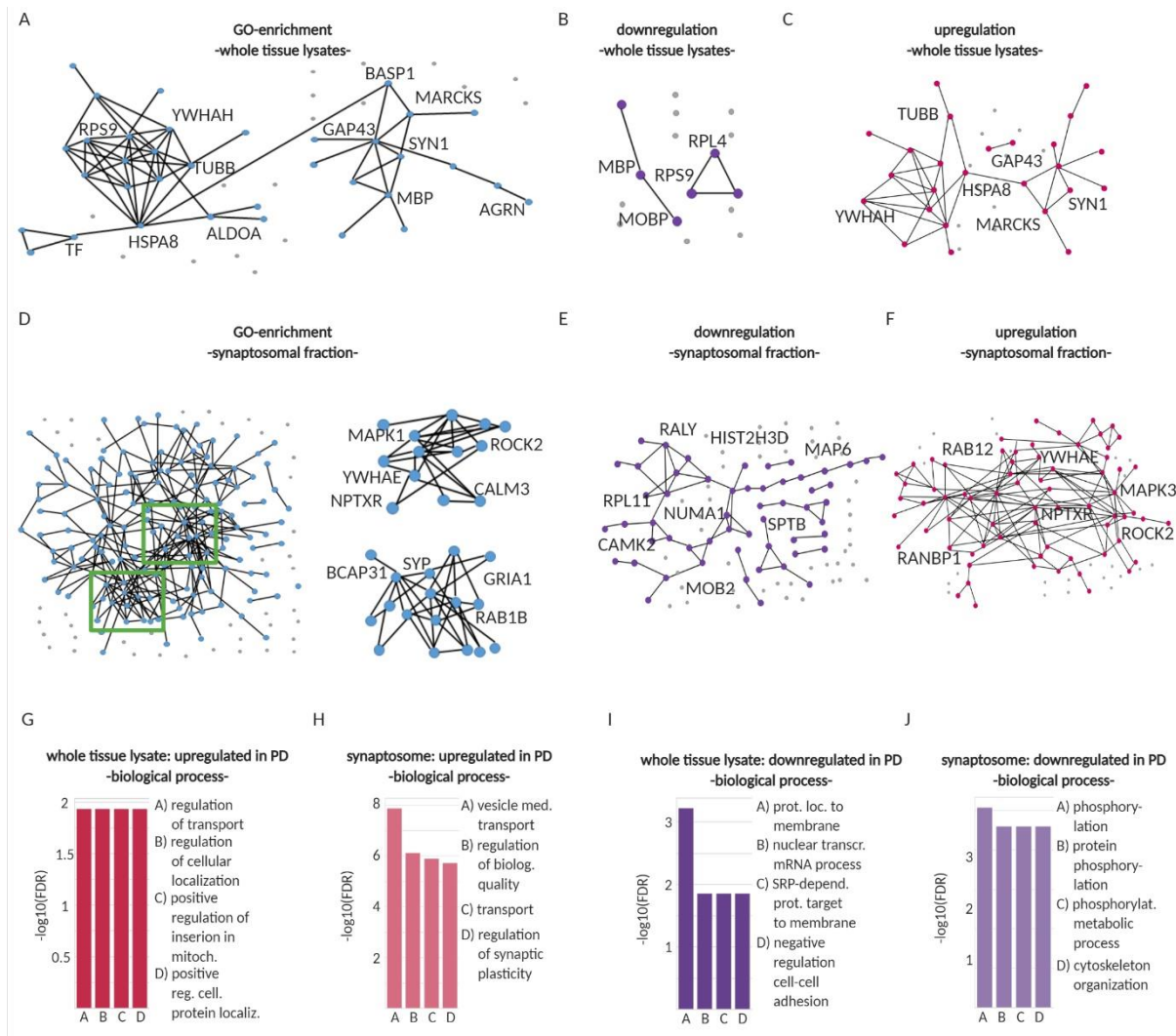
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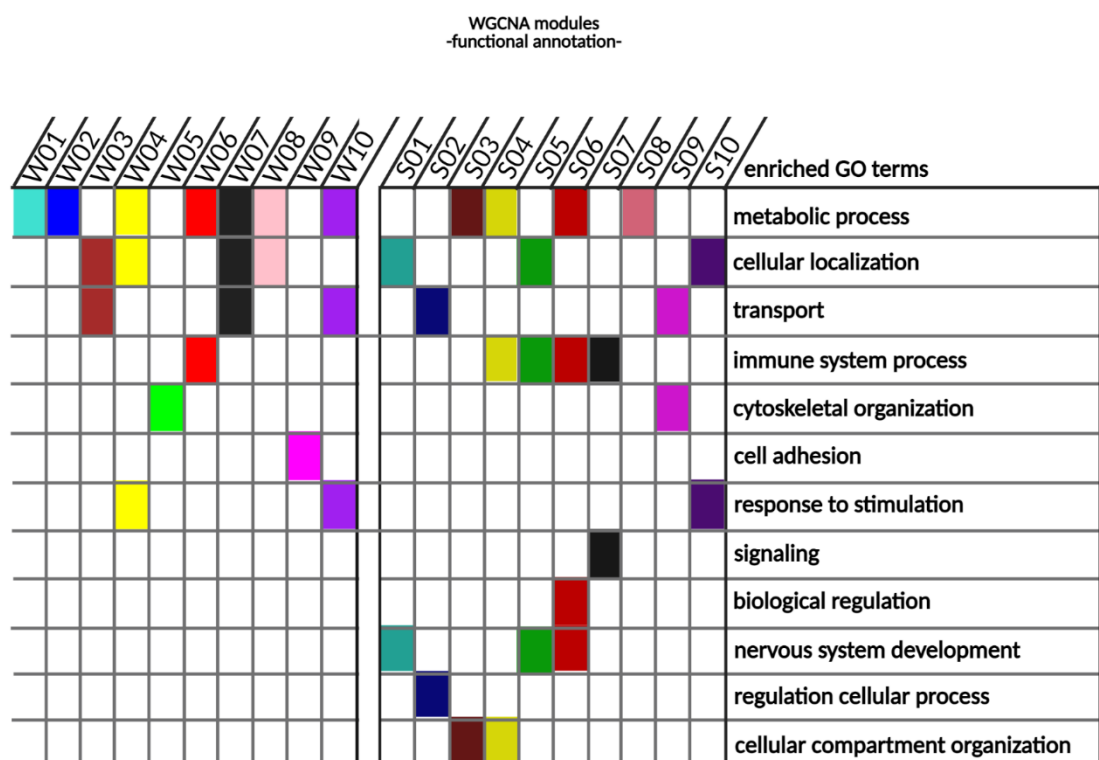
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Supplemental Figure 1: Protein-protein interaction networks and functional annotation analysis. Predicted protein-protein interaction network for differentially expressed proteins of whole tissue lysates (**A-C**) and synaptosomal fractions (**D-F**). The whole network is shown in **A** and **D**, while the network of downregulated proteins is shown in **B** and **E** and the network of upregulated proteins is shown in **C** and **F**. Top 4 GO-biological processes terms significantly enriched, with an FDR <0.05 for the differentially expressed proteins from whole tissue lysates (upregulated in PD in **G**, downregulated in PD in **I**) and synaptosomal fractions (upregulated in PD in **H**, downregulated in PD in **J**).



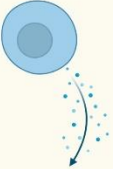
Supplemental Figure 2: Co-expression network functional annotation analysis.
Summary of modules and their biological relevance in both proteomic cohorts. Rectangles colored to respective module depict at least one significantly enriched GO term encompassed in the umbrella terms on the right.

whole tissue lysate: WGCNA co-expression analysis
-biological relevance of modules-

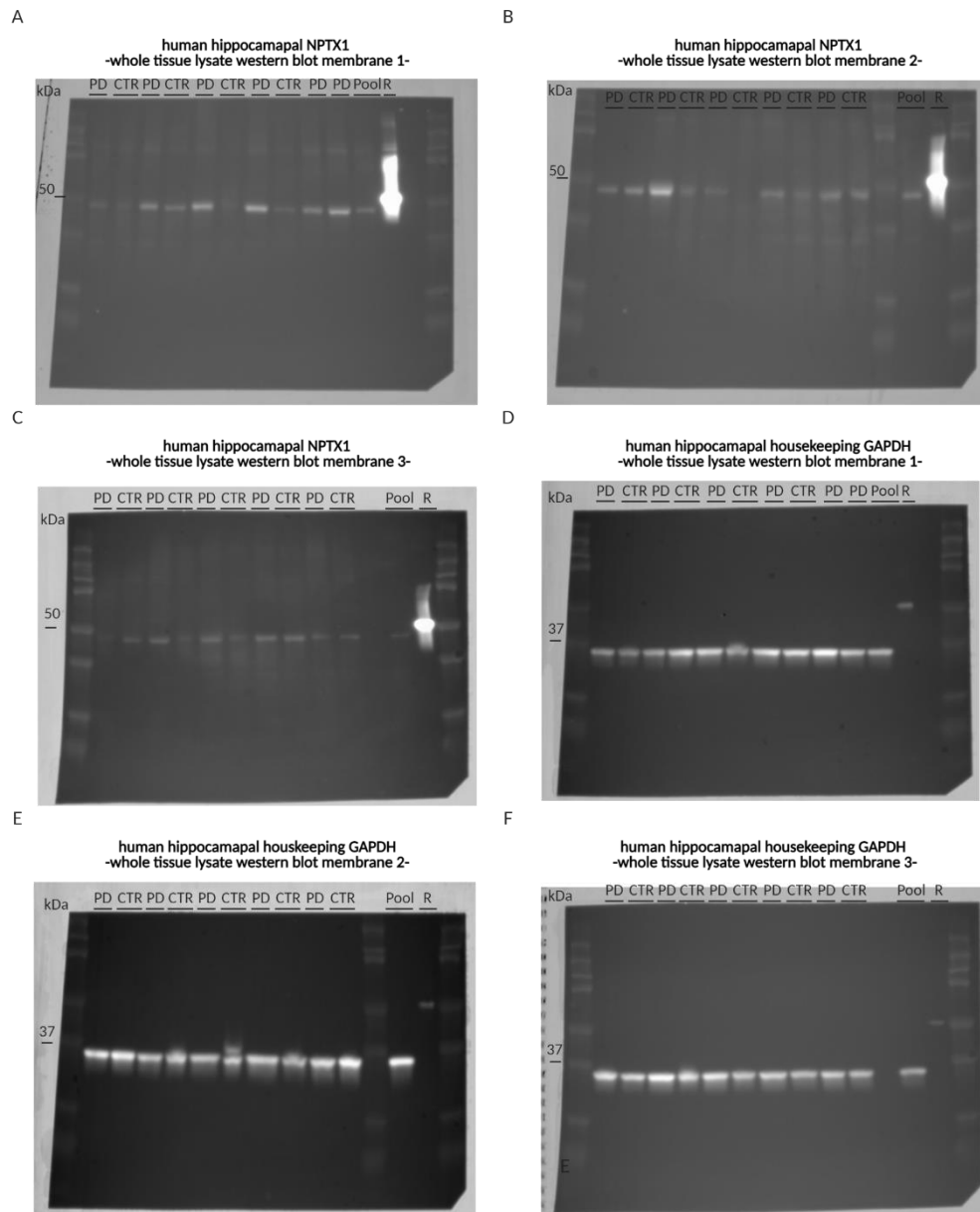
module		description	DE proteins
W01		mitochondria	MPB; YWHAH; LY6H; CCT2; MDH2; MOBP; PPP2R1A; YWHAQ; RAN; GLIPR2; ALDOA; NIPSNAP; PTPRZ1; TF; HSPA8; MYADM; SLC1A4; SLC12A2; DPYSL5; CRIP2
W02		metabolite precursor metabolism	CEND1
W03		synapse	BASP1; YWHAZ; NPTX1; PEA15; SYN1; CORO1A; CAPZA2; ATP6V1B2; ATP6V1H; OXCT1; GANO1; FLOT2; CDH13; NECAB2
W04		intrinsic apoptotic signaling	none
W05		cytoskeleton	BSG; TUBB3
W06		white blood cells	none
W07		synapse	GAP43; RPS16
W08		translation initiation	AGRN
W09		ribosome	H4C1; RPL4; H1; RPS9; CPVL; TUBB
W10		complex I	none

Supplemental Figure 3. Biological relevance of identified WGCNA modules for whole tissue lysates. Individual characterization of biological themes was annotated to each WGCNA module based on functional enrichment results. DE proteins present in the respective module are listed.

synaptosomal fraction: WGCNA co-expression analysis
-biological relevance of modules-

module		description	DE proteins
S01 S02 S05 S06		synaptic transmission neuroprojection development	DBN1; WASF1; NPTXR; NAPG; RALY; CRYZ; AUH; HIST2H3A; MYO5A; GOLM1; CPLX2; HCN2; CHGB; SPTB; TKFC; TPM1; MBOAT7; NUMA1; SLC25A23; PDE2A; HECW1; HSPA1B; LUZP1; RAB1A; FBXO2; KATNAL1; CAMK2A
S03 S04		metabolic function	KRT6B; YWHAE; PAFAH1B3; TTC1; NCDN; BCKDK; HSPH1; RAB1B; DNPH1; YWHAH; EEF1B2; RIDA; ROCK2; HUWE1; ECSIT; CPNE8; RHOT1; HNRNPH3; MAPK3; PSMC1; CORO1A; LINGO1; RANBP1; STUB1; AKT1S1; PSMD9; NDUFS7
S07		cell signaling	RCN1; HINT1; PRKCSH; NDRG3; WNK2; ILDR2; EEFSEC; GLYR1; GGT7; MOB2; SKP1; MYL12A; XKR7; NTPCR; LAMTOR1; PALM2; ASNA1; SLC1A3; RAB6A; BCAP31; KCTD16; HAX1; NDRG2; NCALD; CKM; SLC9A3R1; TPD52; RCN2; ANXA7
S08		RNA processing	CALM3; HNRNPUL2; SFPQ; CHTOP; PSAT1; PGRMC1; TOR1AIP1; MAPK1; PDIA3; MPG; RPL11; ZC3HAV; XRCC6 DNAJC5; ILF3; RAB14; CPE; SRRM2; NONO; PSMD3; MECP2; NCOR2; SYNPO2; SCFD1; LMNB2; ELAVL3
S09		cytoskeleton / transport	HPCA; PRKCA; PTMS; ATP6V1H; CSNK1E; MAP7D2; PITPNM1; SYP; SCAMP5; HIST3H2BB; PGLS; ARHGAP1; FDX2; SPTBN4; PPP2R5B; KBTBD11; GRIA1; DDX42; LRPAP1; CLASP1; LENG9; JMY; MAP6; NCAM2; SLC2A3; WNK1; CTD1P1
S10		response to stimulus	ADD3; SVIP; HAPLN1; TESC; ADD1; CDH20; NPTN; PPP4R4; DNER

Supplemental Figure 4. Biological relevance of identified WGCNA modules for the synaptosomal fraction. Individual characterization of biological themes was annotated to each WGCNA module based on functional enrichment results. DE proteins present in the respective module are listed.



Supplemental Figure 5: Complete full-length Western Blot membranes. (A-C) Blots of all 16 PD and 14 CTR samples stained for NPTX1. **(D-F)** The same blots as in A-C stained for GAPDH as housekeeping protein for normalization. A pooled sample (Pool) was loaded onto each gel to account for inter-membrane variation and allow normalization. Recombinant NPTX1 (R) was also loaded onto each gel for reference.

Supplemental Table 1 - Demographic and clinical information of human brain samples used for whole tissue lysates (WTL) and synaptosomal fractions (SF).

	whole tissue lysates (WTL)		PD vs. CTR	synaptosomal fractions (SF)		PD vs. CTR
	CTR	PD		CTR	PD	
sample numbers	14	16	NA	4	6	NA
sex (female/male)	8/6	5/11	0.2685 ^F	1/3	2/4	1.00 ^F
age at death [years]	79.9 ± 3.1 [61–96]	80.6 ± 1.7 [71–93]	0.8536 ^T (DF = 28; t = 0.1862)	85.8 ± 3.6 [88–91]	83.5 ± 2.7 [73–93]	0.7489 ^T (DF = 8; t = 0.3313)
disease duration [years]	NA	14 ± 1.4 [7–25]	NA	NA	15.3 ± 2.5 [9–25]	NA
post-mortem interval [hours]	18.3 ± 1.4 [8–24]	15.6 ± 1.5 [3–24]	0.2123 ^T (DF = 28; t = -1.2763)	14 ± 2.3 [8–18]	16.7 ± 2.1 [10–24]	0.8207 ^T (DF = 8; t = 0.2342)

Means are presented ± SEM and range. CTR: controls; PD: Parkinson's disease; Differences between groups were considered significantly different with $p < 0.05$ according to Fisher's exact test (^F) or two-sample t-test with equal variance assumed (^T).

Supplemental Table 2

Supplemental File

Supplemental Table 3

Supplemental File

Supplemental Table 4

Supplemental File

Supplemental methods

Human postmortem hippocampal samples acquisition and processing

Frozen human hippocampal samples were obtained from the Parkinson's UK Brain Bank (Imperial College London, London, England), 16 PD and 14 respective age-correlated control samples (CTR), all of which were handled and stored under controlled temperature conditions. The samples were conceded to the Department of Neurology of the University Medical Center Göttingen, Göttingen, Germany, and the ethical approval was given by the Multicenter Research Ethics Committee (07/MRE09/72) as well as the Ethics Committee of the University Medical Center Göttingen (18/3/17).

Brain blocks were processed in a cryostat chamber kept at -20 °C for tissue sampling. Needle biopsies of ~13 mg were obtained from each frozen tissue block using a 20 G Quincke Spinal Needle (Becton Dickinson, cat.no. BEC-405245). In addition, a sub-cohort of samples was

chosen based on the amount of tissue available for a synaptosomal fraction (SF) isolation. Blocks of tissue from 6 PD samples and 4 CTRs had enough mass for a sufficient yield of enriched synaptic proteins in an isolated synaptosomal fraction. The selected sample blocks were removed with a scalpel, collecting ~600 mg of tissue. Needle biopsy samples were lysed in a Urea/Thiourea/Chaps lysis buffer, homogenized and sonicated twice at 30s intervals. Protein quantification was performed with the modified-Bradford Roti-Nanoquant Kit (Carl-Roth GmbH, cat. no. K880.3).

Data-independent acquisition mass spectrometry (DIA-MS) of whole tissue lysate (WTL) samples

A peptide library was generated pooling 80 µg per sample and separated into eight fractions using a reversed phase spin column (Pierce High pH Reversed-Phase Peptide Fractionation Kit, cat. no. 84868, Thermo Fisher Scientific). All samples were spiked with a synthetic peptide standard used for retention time alignment (iRT Standard, Schlieren).

Protein digests were analyzed on a nanoflow chromatography system (Eksigent nanoLC425) hyphenated to a hybrid triple quadrupole-TOF mass spectrometer (TripleTOF 5600+) equipped with a Nanospray III ion source (Ionspray Voltage 2400 V, Interface Heater Temperature 150°C, Sheath Gas Setting 12) and controlled by Analyst TF 1.7.1 software build 1163 (all AB Sciex). In brief, peptides were dissolved in loading buffer (2% acetonitrile, 0.1% formic acid in water) to a concentration of 0.3 µg/µl. For each analysis 1.5 µg of digested protein was enriched on a precolumn (0.18 mm ID x 20 mm, Symmetry C18, 5 µm, Waters, Milford/MA, U.S.A) and separated on an analytical RP-C18 column (0.075 mm ID x 250 mm, HSS T3, 1.8 µm, Waters) using a 90 min linear gradient of 5-35 % acetonitrile/0.1% formic acid (v:v) at 300 nl min⁻¹.

Qualitative LC/MS/MS analysis was performed using a Top25 data-dependent acquisition method with an MS survey scan of m/z 350–1250 accumulated for 350 ms at a resolution of 30,000 full width at half maximum (FWHM). MS/MS scans of m/z 180–1600 were accumulated for 100 ms at a resolution of 17,500 FWHM and a precursor isolation width of 0.7 FWHM, resulting in a total cycle time of 2.9 s. Precursors above a threshold MS intensity of 125 cps with charge states 2+, 3+, and 4+ were selected for MS/MS, the dynamic exclusion time was set to 30 s. MS/MS activation was achieved by CID using nitrogen as a collision gas and the manufacturer's default rolling collision energy settings. Three technical replicates per reversed phase fraction were analyzed to construct a spectral library.

Quantitative DIA-MS data were acquired using 65 variable size windows (Zhang, et al., 2015) across the 400-1,050 m/z range. Fragments were produced using rolling collision energy settings for charge state 2+, and fragments acquired over an m/z range of 350–1400 for 40 ms per segment. Including a 100 ms survey scan this resulted in an overall cycle time of 2.75 s. Two replicate injections were acquired for each biological sample. Proteins were identified using ProteinPilot Software version 5.0 build 4769 (AB Sciex) at “thorough” settings. A total of 386,088 MS/MS spectra from the combined qualitative analyses were searched against the UniProtKB human reference proteome (revision 04-2018, 93,661 entries) augmented with a set of 52 known common laboratory contaminants to identify 2,528 proteins substantiated by 17,479 distinct peptides and 187,465 PSM, respectively, all at global False Discovery Rates of 1% (Suppl. Table 2). Spectral library generation and peak extraction were achieved in PeakView Software version 2.1 build 11041 (AB Sciex) using the quantitation microApp version 2.0 build 2003. Following retention time correction using the iRT standard, peak areas were extracted using information from the MS/MS library at an FDR of 1% (Lambert, et al., 2013). The resulting peak areas were then summed to 7.299 peptide and 2.089 protein quantitations at 1% FDR, which were used for further statistical analysis.

Differential expression analysis of WTL samples

Normalized protein abundance counts for each patient were log transformed for further analysis. The limma R package (Smyth, 2005) (RRID:SCR_010943) was used for differential expression analysis, fitting an $Y \sim \text{control} + \text{PD}$ model using lmFit and differential expression computed using limma's eBayes function. Benjamini-Hochberg correction was used to adjust p-values for multiple testing. Proteins with an adjusted p-value < 0.05 were considered statistically differentially expressed and employed for downstream analyses based on differential results.

Western Blot experiments

Samples were lysed in RIPA cell lysis buffer (Pierce, Thermo Fisher Scientific, cat.no. 89900). Protein concentrations were determined using BCA protein assay kit (Pierce, Thermo Fisher Scientific, cat.no. 23227). Equal amounts of protein and protein ladder PageRuler (Thermo Fisher Scientific, cat.no. 26616) were loaded into 12% Mini-PROTEAN TGX gels (BioRad, cat.no. 4561043) run and transferred to a PVDF membrane (Amersham, GE Healthcare, cat.no. 10600101). Membranes were blocked in 5% dry milk in TBST for 1h and washed three times for 15 minutes each. Primary antibodies for NPTX1 (1:100, Becton Dickinson, RRID:AB_397755), SYN1 (1:1,000, Synaptic Systems, RRID:AB_11042000) and/or GAPDH (1:1,000, HyTest, RRID:AB_1616722) were incubated overnight at 4 °C. Membranes were washed three times for 15 minutes each and secondary antibodies (goat-anti-rabbit-HRP, RRID:AB_2099233; and horse-anti-mouse-HRP, RRID:AB_330924, 1:10,000, Cell Signaling) were incubated for 1.5 h at RT. Membranes were developed using ECL Prime Western Blotting Detection Reagent (TH Geyer) and visualized with FusionPulse (Vilber). Signals were quantified with EvolutionCapt (Vilber) and normalized to housekeeping gene GAPDH signals as well as to the signal of a pooled sample for analysis comparing multiple membranes.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Fresh RNA was isolated from each midbrain sample from the discovery cohort for the experimental validation of selected targets from small RNA and total RNA sequencing results (see RNA isolation method above). RNA samples were reverse transcribed using the Quantitect RT Kit (Qiagen, cat.no. 205311). Quantitative real time polymerase chain reaction (qRT-PCR) experiments were performed in 45 cycles on a QuantStudio5 system (Applied Biosystems), using the Quantitect SYBR Green PCR Master Mix (Qiagen, cat.no. 204143). All primers were designed with the GeneGlobe Design and Analysis Hub platform from Qiagen. Abundance was normalized with expression values of applicable housekeeping genes (HKG) (i.e. GAPDH) and relative expression levels (RELs) were calculated using the ΔCt method ($\Delta\text{Ct} = \text{Ct}_{\text{GeneOfInterest}} - \text{Ct}_{\text{HKG}}$; $\text{REL} = 2^{-\Delta\text{Ct}}$). Statistical analysis for qRT-PCR data was performed using GraphPad Prism 9 (RRID:SCR_002798). Data were shown as mean $\pm$ SEM. Normality tests were performed (D'agostino and Pearson and Shapiro-Wilk tests), and differences between PD and CTR cohorts were analyzed by Mann-Whitney-U tests. For outlier detection, the 'Robust regression and Outlier removal' (ROUT) test in GraphPad Prism 9 was applied (Q at 1%).

Immunohistochemistry

Paraffin-embedded hippocampal tissue sections of one PD and one CTRL were deparaffinized and pre-treated according to standard IHC protocol. A detailed protocol can be read in Fard et al., 2017. Primary NPTX1 antibody (1:100, Becton Dickinson, RRID:AB_397755) was incubated over night at room temperature. For visualization, the HRP-based EnVision Kit (Dako, Code K4003) was used.

Primary hippocampal neuron cultures

Primary hippocampal neurons were prepared from mouse pups at postnatal day 0 (P0). Briefly, the hippocampus of C57BL/6J newborn pups (P0) was collected in ice-cold CMF and incubated with trypsin (Sigma Aldrich, cat.no. T2600000) at 37 °C for 12 minutes. DNase was added (Sigma Aldrich, cat.no. DN25), samples centrifuged for 1 minute and the supernatant exchanged with fetal calf serum (FCS) (Biochrom, cat.no. S 0115) to inactivate residual trypsin. Cells were dissociated via trituration with a fire-polished Pasteur pipette and pelleted down. Cell quantities were determined using a Neubauer counting chamber and 200,000 cells were seeded per well in 24-well plates (Sarstedt, cat.no. 83.3922.005) or 1,000,000 cells per well in 6-well plates (Sarstedt, cat.no. 83.3920.005). The cells were kept in neurobasal medium with 1% Glutamax (Gibco, cat.no. 35050061), 1% Pen/Strep (Gibco, cat.no. 15140122), 1.3% Glucose 45% (Merck, cat.no. SIALG8769) and 2% B27 (Gibco, cat.no. 17504044) and incubated in a 5% CO₂ , 95% humidity and 37°C. Half of the medium was changed the day after seeding. Fresh medium was added weekly.

Recombinant NPTX1 treatment

Starting from DIV01, 100 µl medium was removed from each well with seeded cells and placed individually into Eppendorf tubes. Next, 0.23 µg recombinant NPTX1 (R&D Systems, cat.no 7707-NP-050) were added to each tube, briefly vortexed and placed back into the incubation chamber to warm up. The medium with recombinant NPTX1 was then returned individually to the corresponding culture wells. The treatment was administered daily for a week until DIV07.

Immunocytochemistry

Coverslips in plates were fixated for 10 minutes in 4% PFA at room temperature (RT) and washed with PBS. 25 mM Glycine in PBS was added for 20 min and washed with PBS. Blocking solution (10% NHS, 5% BSA, 0.3% Triton, 25 mM Glycine in PBS) was added and incubated for 1.5 h at RT. Antibody solutions at 1:50 for NPTX1 (Becton Dickinson, RRID:AB_397755) and 1:500 for SYN1 (Synaptic Systems, RRID:AB_11042000) were incubated over night at 4°C. Coverslips were washed three times with PBS. AlexaFluor AffiniPure fluorescent dye (1:250, anti-rabbit, Biozol) or Cy3 AffiniPure fluorescent dye (1:250, anti-mouse, Jackson ImmunoResearch, AB_2338447) conjugated secondary antibodies were incubated at RT for 1.5h. Coverslips were washed three times with PBS. Nuclei were counterstained with DAPI at RT for 2 minutes.

Synapse morphology assessment

Using custom-written Matlab (RRID:SCR_001622) scripts rectangular areas containing numerous synapses were selected manually. No blinding was performed during data analysis. A threshold was then applied to determine the regions of interest that contain signals above background, initially set to the mean plus standard deviation of the signal within the rectangular area. The threshold was optimizable by user input. The regions of interest (ROI) above this threshold were fitted to ellipses and considered to be synapses. Their different characteristics, including mean intensity, area, major axis and minor axis were calculated using the *regionprops* function of Matlab [R2020b], as described in the package documentation.

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