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Supplementary Information for

Local apoptotic-like mechanisms underlie complement-mediated synaptic pruning

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Supplementary methods

Figs. S1 to S9

Tables S1

Caption for dataset S1

References for SI appendix

Other supplementary materials for this manuscript include the following:

Dataset S1

Supplementary Methods

Animals and Ethics Statement

Adult and newborn (at postnatal day 5) male Crl:NMRI BR mice were used in the experiments (purchased from Toxi-Coop, Budapest, Hungary). Animals were housed under standard laboratory conditions (12:12 h light–dark cycle, with free access to water and food). The care and treatment of all animals conformed to guidelines approved by Council Directive 86/609/EEC and the Hungarian Act of Animal Care and Experimentation (1998, XXVIII) as well as with local regulations for the care and use of animals for research. All efforts were taken to minimize the animals' pain and suffering and to reduce the number of animals used.

Whisker Removal and Isolation of the Barrel Cortices

All of the whiskers on the right-hand side of the mice were plucked and trimmed under isoflurane anesthesia. To maintain sensory deprivation, the possibly regrown whiskers were trimmed 48 h after the initial whisker removal. 72 h after the initial whisker removal, mice were sacrificed via cervical dislocation and their brains were quickly removed and briefly washed in ice-cold artificial cerebrospinal fluid (ACSF). The primary somatosensory barrel cortices from both hemispheres were dissected using vascular landmarks (1). Synaptosome fractions were immediately prepared from the isolated barrel cortices as described below.

Preparation of the Whole Tissue Samples and the Synaptosome Fraction

The mice were sacrificed via cervical dislocation and their brains were quickly removed from their skulls. The whole brains were briefly washed in ice-cold ACSF to remove blood contamination, the cerebral cortices were dissected, and then were collected. In another investigation, transcardial perfusion of mice was performed with ice-cold ACSF after urethane anesthesia. The perfusion procedure

was followed by the rapid removal of the brains, and then the dissection and collection of cerebral cortices.

The preparation of the whole cortical tissue samples was started with their mechanical homogenization using a Sample Grinding Kit (GE Healthcare, Little Chalfont, UK) in lysis buffer (7 M urea, 2 M thiourea, 4% (wt/vol) CHAPS, 20 mM Tris, 5 mM magnesium-acetate). Next, the samples were centrifuged at $14,000 \times g$ at 4 °C for 1 h and the supernatants were acetone-precipitated overnight at -20 °C. The protein precipitates were resuspended in lysis buffer and stored at -80 °C.

The subcellular fractionation has been started immediately after the brain removal. The fraction of synaptosomes (2) was prepared strictly following the protocol published by Phillips et al. (3) and Hahn et al. (4). Briefly, cerebral cortices from both hemispheres were first homogenized in an isoosmotic sucrose solution. Subsequently, samples were ultracentrifuged in a tube with two sucrose media with different densities. Synaptosomes were collected from the interface between the two sucrose layers. The proteins of those synaptosomes which were not fractionated further, were acetone-precipitated overnight at -20 °C, resuspended in lysis buffer and stored at -80 °C.

Preparation of Pre- and Postsynaptic Membrane Fractions

Synaptosome fractions were processed to separate pre- and postsynaptic membranes. A full description of the protocol is presented by Hahn et al. (4). Briefly, synaptosomes were first disrupted with the combination of mechanical homogenization and detergent treatment. Synaptic junctions were isolated with detergent treatment and incubation in a buffer at pH 6. Finally, pre- and postsynaptic membrane elements were separated with a final detergent treatment and incubation at pH 8, followed by ultracentrifugation to pellet the more compact, postsynaptic density-associated postsynaptic membrane, and leaving the presynaptic membrane-associated presynaptic active zone in the supernatant. Proteins of both fractions were acetone-precipitated overnight at -20 °C and resuspended in lysis buffer.

Electron Microscopy

Synaptosome samples were fixed in 2% formaldehyde, 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 30 min at room temperature (RT). Subsequently, the samples were extensively washed in the above buffer and postfixed in 0.5% osmium tetroxide, 0.75% potassium hexacyanoferrate for 45 min, *en bloc* stained with aqueous uranyl acetate for 30 min, dehydrated and embedded in LR White resin (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Ultra-thin sections (70 nm) were collected on formvar coated single slot copper grids (Sigma-Aldrich) and stained with lead citrate for 30 s. Grids were examined with a JEM-1011 electron microscope (JEOL, Tokyo, Japan) operating at 60 kV. Images were taken from different mesh along a longitudinal band to cover the entire width of the specimen.

Isolation of Mouse C1q Protein

Mouse C1q was isolated from Biowest Mouse Serum (Biowest, Nuaille, France) by a 2-step purification method, including affinity and ion-exchange chromatography based on the protocol of Kojouharova (5). Each step of the protocol was carried out at 4 °C. A 10-mL affinity chromatography column was prepared by coupling 15 mg/mL human IgG (human polyclonal IgG1 was a gift of Péter Závodszy (Institute of Enzymology)) to CNBr-activated Sepharose-4B matrix (GE Healthcare) according to the manufacturer's instructions. The IgG affinity chromatography was performed in 20 mM Tris-HCl buffer, pH 7.0 containing 120 mM NaCl and 20 mM EDTA. After thawing, the mouse serum was diluted twice in the equilibration buffer containing 0.2 mM PMSF, filtered through 0.45- μ m syringe filter (VWR International, Radnor, PA, USA) and loaded to the IgG affinity column. Proteins bound to the human IgG were eluted by increasing the ion strength and pH (50 mM Tris-HCl, 1 M NaCl, 20 mM EDTA, pH 10.0) in one step. The C1q-containing fractions were dialyzed overnight against 20 mM HEPES buffer, pH 7.8, containing 60 mM NaCl and 10 mM EDTA. The same buffer was used to equilibrate a 1-mL HiTrap SP HP column (GE Healthcare). The dialyzed sample was loaded to the strong cation exchange column and elution was achieved by using 20-mL increasing NaCl gradient (20

mM HEPES, 700 mM NaCl, 10 mM EDTA, pH 7.8). The mouse C1q was found to elute at ~200 mM NaCl and the C1q-containing fractions were dialyzed overnight against 50 mM NaH₂PO₄ buffer, pH 7.4, containing 100 mM NaCl. Finally, 30% ethylene glycol was added to the isolated mouse C1q and small aliquots of the protein were stored at -80 °C. To test whether the isolated C1q remained intact, we performed analytical size-exclusion chromatography on a 24-mL Superose-6 Prep Grade column (GE Healthcare) equilibrated with the latter dialysis buffer. The mouse C1q was found to be intact, even after thawing the frozen samples. The purity of the protein was also verified by SDS-PAGE.

Western Blot

Protein concentrations of whole tissue samples, synaptosome, and pre- and postsynaptic fractions were determined using 2-D Quant Kit (GE Healthcare). Samples, containing equal amounts of proteins, were diluted with equal volume of two-fold concentrated sample buffer (8% (wt/vol) SDS, 3% (wt/vol) dithiothreitol (DTT), 24% (vol/vol) glycerol, 0.2% (wt/vol) bromophenol blue, 100 mM Tris-HCl, pH 6.8), and incubated for 5 min at 96 °C. Tricine-SDS PAGE was conducted following the protocol of Schagger (6). After gel electrophoresis, proteins were transferred onto Hybond LFP PVDF membrane (GE Healthcare). Blots were stained with the highly sensitive SYPRO Ruby total protein blot stain (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions as loading control when minute amounts of sorted synaptosomal samples were used. Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.05% Tween-20, and then incubated overnight at 4 °C with primary antibodies depending on the experiment as follows: anti-C1qA (1:200 dilution; catalogue number: sc-25856; Santa Cruz Biotechnology, Dallas, TX, USA), anti-synaptophysin (anti-Syp; 1:100 dilution; catalogue number: sc-55507; Santa Cruz Biotechnology), anti-Psd95 (1:2,000 dilution; catalogue number: 04-1066; Merck Millipore, Billerica, MA, USA), anti-cleaved caspase-3 (1:1,000 dilution; catalogue number: STJ97448; St John's Laboratory, London, UK), and anti-actin (1:1,000 dilution; catalogue number: ab1801; Abcam, Cambridge, UK) as loading control. Membranes were washed with 0.05% Tween-20 in TBS, and then

incubated for 2 h at RT with anti-rabbit, Cy5-conjugated and anti-mouse, Cy3-conjugated secondary antibodies (1:2,500-1:2,500 dilution; catalogue numbers: PA45011 and PA43009, respectively; GE Healthcare) depending on the host species of the primary antibodies. Subsequently, membranes were washed with 0.05% Tween-20 in TBS, and the fluorescence was detected with a Typhoon TRIO+ scanner (GE Healthcare) using appropriate lasers and filters. Analysis of images was conducted with ImageJ software (<http://rsbweb.nih.gov/ij>, National Institutes of Health, Bethesda, MD, USA). Statistically significant differences were determined with two-tailed Student's *t*-test of paired or independent samples depending on the experiments.

In order to identify different neuronal pentraxin 1 (Nptx1) protein spots, 2D-immunoblotting was performed. Synaptosomal homogenate containing 1 mg total protein was subjected to traditional 2D gel electrophoresis comprising isoelectric focusing and protein separation by molecular weight (also see the section "Proteomics Experiments"). Subsequently, proteins were transferred to a PVDF membrane. Total protein content was stained using SYPRO Ruby Protein Blot Stain strictly following the manufacturer's instructions. The staining pattern was visualized using a Typhoon TRIO+ scanner. Finally, neuronal pentraxin 1 (Nptx1) protein spots were identified using anti-Nptx1 primary (1:200 dilution; catalogue number: 20656-1-AP; Proteintech, Rosemont, IL, USA), and anti-rabbit, Cy5-conjugated (1:2,500 dilution; GE Healthcare) secondary antibodies as described above, and then visualized using a Typhoon TRIO+ scanner.

Immunolabeling of Synaptosomes and Assessment of Apoptotic Processes and Viability

Synaptosomes, stored at 4 °C, were immunolabeled the next day after the subcellular fractionation. Initially, the synaptosome fraction was immunolabeled in phosphate-buffered saline, which caused significant aggregation of synaptosome sub-populations. This aggregation enabled the visualization of large groups of synaptosomes even by light microscopy. However, after the preliminary investigations of this synaptic fraction, the labeling procedure was carried out in 320 mM sucrose, 1 mM EDTA, 5 mM Tris, pH 7.4 buffer (SET buffer). This medium has been proven to be optimal for the prevention of synaptosome aggregation (7), which would essentially compromise the flow cytometry

experiment. First, synaptosomes were gently fixed with 0.25% formaldehyde for 1 h at 4 °C, which promotes sufficient mechanical stability of the synaptosomes without deteriorating antigen-antibody interactions (8). Aspecific protein binding was blocked by incubating the samples with 1% BSA for 30 min at RT. Synaptosomes were incubated with anti-C1qA (1:30 dilution; catalogue number: sc-25856; Santa Cruz Biotechnology) or anti-C1q (1:30 dilution; catalogue number: 182451; Abcam) primary antibodies for 30 min at RT, followed by two washing steps to remove unbound antibodies. Next, we incubated the suspension for 30 min at RT with anti-rabbit secondary antibody, conjugated with Cy5-fluorochrome (1:1,500 dilution; catalogue number: PA45011, GE Healthcare). When additional labeling of the intracellular Syp and cleaved caspase-3 proteins was conducted, a gentle permeabilization was applied after two additional washing steps via incubation of the samples in the SET buffer with 0.2% Tween-20 for 15 min at 37 °C. Subsequently, permeabilized samples were incubated with either anti-Syp (1:50 dilution; catalogue number: sc-55507; Santa Cruz Biotechnology) or anti-cleaved caspase-3 (recognizing only the ~17 kDa fragment of caspase-3 once activated; Supplementary Fig. S7) (1:100 dilution; catalogue number: STJ97448; St John's Laboratory, London, UK) antibodies depending on the experiment for 30 min at RT, which was followed by two washing steps. Permeabilized samples then were incubated with anti-mouse, Alexa Fluor 488-conjugated secondary antibody (1:1,000 dilution; catalogue number: A11001; Thermo Fisher Scientific), and then synaptosomes were washed. Samples (non-permeabilized and permeabilized as well) were filtered through a 5.0- μ m Durapore membrane filter (Merck Millipore) to remove non-synaptosomal debris and other contaminations. Annexin V labeling was performed before filtering using annexin V-FITC protein (catalogue number: 640906; BioLegend, San Diego, CA, USA) in annexin V binding buffer (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. For viability tests, calcein-AM (catalogue number: C3099; Thermo Fisher Scientific) was applied also before the filtering procedure according to the manufacturer's instructions when experiments were conducted to assess the viability of synaptosomes. Finally, 0.5% Pluronic F-68 detergent (Thermo Fisher Scientific) was added to prevent the aggregation of synaptosomes.

Confocal Microscopy

Examinations on synaptosomes were conducted using a FluoView 500 laser scanning confocal microscope (Olympus Europe, Hamburg, Germany) with an argon-ion laser (with 488 nm excitation wavelength) and with two helium-neon lasers (with 543 and 632 nm excitation wavelengths) operated by the FluoView 5.0 software (Olympus Europe). Image acquisition was performed with a 60x objective (1.1 NA; Plan Apochromat) in DIC and fluorescence modes. Image-processing was conducted using FluoView 5.0 and ImageJ softwares.

Immunofluorescence Staining of Mouse Brain Sections

Mice were anesthetized with urethane and transcardially perfused first with 0.1 M phosphate buffer (PB), pH 7.4, and then with 2% formaldehyde, 0.1 M PB. Subsequently, the brains were quickly removed and post-fixed in the same fixative solution for 3 h at RT. After replacing the fixative solution to 0.1 M PB, 60 μ m sagittal brain sections were produced with a vibratome. Brain sections were washed with 0.1 M PB and stored in the same buffer supplemented with 0.1% sodium azide at 4 °C. At the beginning of the immunostaining procedure, the 0.1 M PB, 0.1% sodium azide solution was replaced with TBS and sections were blocked in blocking buffer (150 mM NaCl, 50 mM Tris, 100 mM L-lysine, 3% BSA, 0.2% Triton X-100, pH 7.4) for 45 min at RT. For triple immunostaining, sagittal brain sections were incubated in primary antibody buffer (150 mM NaCl, 50 mM Tris, 100 mM L-lysine, 1% BSA, pH 7.4) with anti-C1q (1:1,000 dilution; catalogue number: 182451; Abcam), anti-cleaved caspase-3 (1:100 dilution; catalogue number: STJ97448; St John's Laboratory), and anti-Syp (1:500 dilution; catalogue number: 101 006; Synaptic Systems, Göttingen, Germany) primary antibodies for 48 h at 4 °C. After several washing steps with TBS, the sections were incubated with anti-rabbit, Alexa Fluor 546-conjugated (1:250 dilution; catalogue number: A-11010; Thermo Fisher Scientific), anti-mouse, Alexa Fluor 514-conjugated (1:250 dilution; catalogue number: A-31555; Thermo Fisher Scientific), and anti-chicken, Alexa Fluor 633-conjugated (1:250 dilution; catalogue number: A-21103; Thermo Fisher Scientific) secondary antibodies in TBS for 3 h at RT. Finally, after several washing

steps with TBS, the sections were mounted on glass slides using DAKO fluorescence mounting medium (catalogue number: S3023) and the slides were covered with high precision cover glass (Marienfeld No. 1.5H, catalogue number: 0107222), and then sealed with nail polish.

Processing of the Human Brain Tissue and Immunofluorescence Staining

Brain was removed 2 h 22 min after death, both internal carotid and vertebral arteries were cannulated, and the brain was perfused first with physiological saline (1.5 L in 30 min) containing 5 mL of heparin, followed by a fixative solution containing 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M PB, pH 7.4 (4-5 L in 2 h). The temporal cortex was removed after perfusion and was post-fixed in the same fixative solution overnight without glutaraldehyde (9). Subsequently, 60 μ m-thick coronal sections were prepared from the blocks with a Leica VTS-1000 Vibratome (Leica Microsystems, Wetzlar, Germany) for immunostaining. The immunofluorescence staining was performed essentially the same manner as described above with differences as follows. To carry out the triple immunostaining, anti-C1qC (1:250 dilution; catalogue number: sc-27669; Santa Cruz Biotechnology), anti-cleaved caspase-3 (1:200 dilution; catalogue number: 9661; Cell Signaling Technology, Leiden, The Netherlands; previously validated to be specific to the active form (10)), and anti-Syp (1:250 dilution; catalogue number: 101 006; Synaptic Systems) antibodies were used. Subsequently, anti-goat Alexa Fluor 488-conjugated (1:400 dilution; catalogue number: 705-545-147; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), anti-rabbit Cy3-conjugated (1:400 dilution; catalogue number: 711-165-152; Jackson ImmunoResearch Laboratories), and anti-chicken Cy5-conjugated (1:400 dilution; catalogue number: 703-175-155; Jackson ImmunoResearch Laboratories) secondary antibodies were used. The sections were mounted on glass slides using Aqua-Poly/Mount medium (Polysciences, Warrington, PA, USA), and the slides were covered with cover glass (Menzel Gläser, No. 1; catalogue number: CS2440100), and then sealed with nail polish.

Super-resolution Microscopy (STED, HyVolution 2)

Stimulated emission depletion (STED) super-resolution and HyVolution 2 pseudo-super-resolution (introduced by Leica) imaging was performed with a Leica TCS SP8 STED microscope (Leica Microsystems) using a Leica HC PL APO 100x STED white (1.4 NA) objective. For STED imaging, the stimulated emission depletion of the Alexa Fluor 514 and Alexa Fluor 546 fluorescence was carried out by using a beam shaped continuous wave 660 nm laser. The fluorescence of the Alexa Fluor 633 was detected in confocal mode without depletion. The fluorescence of each dye was detected sequentially by using a hybrid detector and spectral detections. The emissions of the Alexa Fluor 514, 546 and 647 dyes were recorded in the 516-586 nm, 563-633 nm and 651-741 nm range, respectively. The pixel size of the recorded images was 20 nm/pixel. The images were restored by using Huygens Pro deconvolution and were analyzed with Leica LAS X 3.1.1 software.

HyVolution 2 pseudo-super-resolution imaging was carried out on samples stained with Alexa Fluor 488, Cy3, and Cy5 dyes. The fluorescence of each dye was detected sequentially and spectrally by a hybrid detector at 499-569 nm, 565-635 nm, and 650-720 nm wavelengths, respectively. The pinhole diameter was set to 0.6 AU in each channel and the pixel size was 38 nm/pixel of the recorded images. Huygens Pro deconvolution software was used for image restoration and Leica LAS X 3.1.1 software was used for image analysis.

Fluorescence-activated Cell Sorting

The flow cytometry experiments were performed on a BD FACSAria III sorter (BD Biosciences, San Jose, CA, USA) coupled with BD FACSDiva software (BD Biosciences) for instrument operation, data acquisition, and analysis. A 633 nm and a 488 nm laser were utilized for fluorophore-excitation and a 70- μ m nozzle was used to generate single droplets. The applied settings were as follows: forward light scatter (FSC) detector photomultiplier tube (PMT) gain setting = 350 V with a 1.5 neutral density filter; side light scatter (SSC) detector PMT gain setting = 600 V; FSC threshold = 5,000; SSC threshold = 2,000; allophycocyanin (APC) channel PMT gain setting = 508 V;

fluorescein isothiocyanate (FITC) channel PMT gain setting = 455 V. Sample dilution and flow rate were adjusted to optimal event recordings (below 20,000 processed events per sec). The population of synaptosomes was designated based on their FSC and SSC characteristics compared to that of the buffer alone, and further gating of fluorescent and non-fluorescent particles was performed among the members of this population. A fraction of synaptosomes, designated as the negative control, was labeled with the secondary antibody alone and the artificial background fluorescence was excluded according to the fluorescence intensity of this fraction. Fluorescently labeled C1q-tagged and unlabeled untagged synaptosomes were collected into separate tubes in „purity” sorting mode. Three million C1q-tagged and three million untagged synaptosomes were collected for the proteomics experiments and additional six million C1q-tagged and six million untagged synaptosomes for the Western blot investigations. After sorting, the samples were concentrated with 100K Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore) and proteins were precipitated with the chloroform-methanol precipitation procedure (11). Precipitated proteins were resuspended in lysis buffer.

To determine the extent of synaptic co-localization of C1q with apoptotic markers (i.e., cleaved caspase-3 and annexin V), flow cytometry experiments were conducted as well. The amounts of double-negative, C1q-labeled, cleaved caspase-3/annexin V-labeled, and double-positive synaptosomes were calculated from the data of 250,000 synaptosomes/experiment. Four independent experiments utilizing four mice were conducted for each apoptotic marker. Statistically significant differences were determined with two-tailed Student's *t*-test of paired samples.

Flow cytometry data were processed and displayed with FCS Express 6 software (De Novo Software, Glendale, CA, USA).

Proteomics Experiments

Two-dimensional difference gel electrophoresis (2D-DIGE) proteomics examination was conducted to compare the proteomes of sorted C1q-tagged and untagged synaptosomes from six mice. Due to the low protein yield obtainable from C1q-tagged synaptosomes, the 2D-DIGE saturation

labeling technique was chosen, which is suitable for the comparison of proteomes when the total protein amount is around a few μg . Our first investigations indicated that two million synaptosomes provide sufficient amount of protein for this technique.

Six fully independent samples were prepared using six adult male Crl:NMRI BR mice. A detailed protocol has been provided elsewhere (12, 13). All equipment and fluorescent dyes were purchased from GE Healthcare. A brief description is shown below, and experiment-specific details are noted. Proteins from two million C1q-tagged and two million untagged synaptosomes were labeled with Cy5 fluorescent dye, while a pooled sample was prepared containing proteins from one million C1q-tagged and one million untagged synaptosomes together. The latter was labeled with Cy3 dye. C1q-tagged and untagged synaptosome samples were prepared and run on separate gels together with the same amount of a pooled sample. Fluorescence intensity of the Cy5-labeled sample was normalized to the signal of the Cy3-labeled pooled sample on the same gel after the two-dimensional protein separation.

Proteins were first separated according to their isoelectric points, equilibrated under reducing conditions, and then separated according to their molecular weights. Fluorescence intensities of the protein spots were detected with a Typhoon TRIO+ fluorescence scanner. Protein spots were identified, matched, and manually verified as well using the DeCyder 2D Differential Analysis Software. Statistically significant differences were determined with Student's *t*-test of paired samples in the software. Comparisons were performed between C1q-tagged and untagged synaptosomes from the same animals and significance level was set at $P < 0.05$. The significantly altered protein gel spots were manually excised from a preparative gel containing 800 μg total synaptosomal proteins. The gel spots were placed in 1% acetic acid solution and were stored at 4 °C until further protein identification.

For the mass spectrometric protein identification, proteins were digested with Trypsin Gold (Promega, Madison, WI, USA) following the protocol by Shevchenko et al. (14) with minor modifications. The HPLC-MS/MS analysis of the tryptic peptide mixtures was performed using a nanoflow UHPLC system (Dionex UltiMate 3000 RSLCnano System, Thermo Scientific, Sunnyvale, CA, USA) coupled to a high-resolution QTOF mass spectrometer (Maxis II ETD, Bruker Daltonik GmbH, Bremen, Germany). The mass spectrometer was fitted with a CaptiveSpray source and a

nanoBooster tank. The nanoBooster was filled with acetonitrile operating with 0.2 bar N₂-flow. The tryptic peptides were desalted online on an Acclaim Pepmap C18 trap column (100 µm i.d. × 20 mm; Thermo Scientific), and then separated on a reverse-phase Acclaim Pepmap RSLC analytical column (C18, 75 µm i.d. × 150 mm; Thermo Scientific). The elution of the peptides from the analytical column to the emitter tip was achieved using a flow rate of 300 nL/min and a 90 min long gradient going from 2.5% to 45% solvent B (solvent A was water containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid).

Mass spectrometry used electrospray ionization in the positive-ion mode. Signal intensities were measured using single stage mass spectrometry (capillary voltage: 1.2 kV, drying gas flow: 3 L/min, gas temperature: 150 °C). Protein identification was performed using tandem mass spectrometry in AutoMS/MS setup (data-dependent analysis) from the *m/z* range of 300–2,200. Collision gas was N₂ and collision energy was varied in the 10–80 eV range according to predefined charge state-dependent configuration files.

Protein content, identified from data-dependent analysis measurements, was searched against the latest SwissProt sequence database with the taxonomy of *Mus musculus* using Mascot Server v.2.5 (Matrix Science, London, UK). During the searching, one missed cleavage was allowed, carbamidomethyl cysteine was set as fixed modification, and methionine oxidation was allowed as variable modification. Proteins were accepted if a minimum of two unique peptides were identified. Protein data analysis was performed with ProteinScape v 3.0 bioinformatics platform (Bruker, Billerica, MA, USA).

Bioinformatics Analysis

Functional classification of the altered proteins was conducted using the GeneOntology (<http://www.geneontology.org>) and UniProt (<http://www.uniprot.org>) databases as well as with the published literature.

Pathway analyses of the differentially regulated proteins were conducted using the Pathway Studio software (Elsevier Life Science Solutions). Common regulator and common target analyses were

performed using the “Sub-Network Enrichment Analysis” tool where all of the up- or downstream entities and types of connections present in the database were taken into account. Significantly enriched entities ($P < 0.05$, Fisher’s exact test) were ranked based on their calculated P -values.

Supplementary Figures and Tables

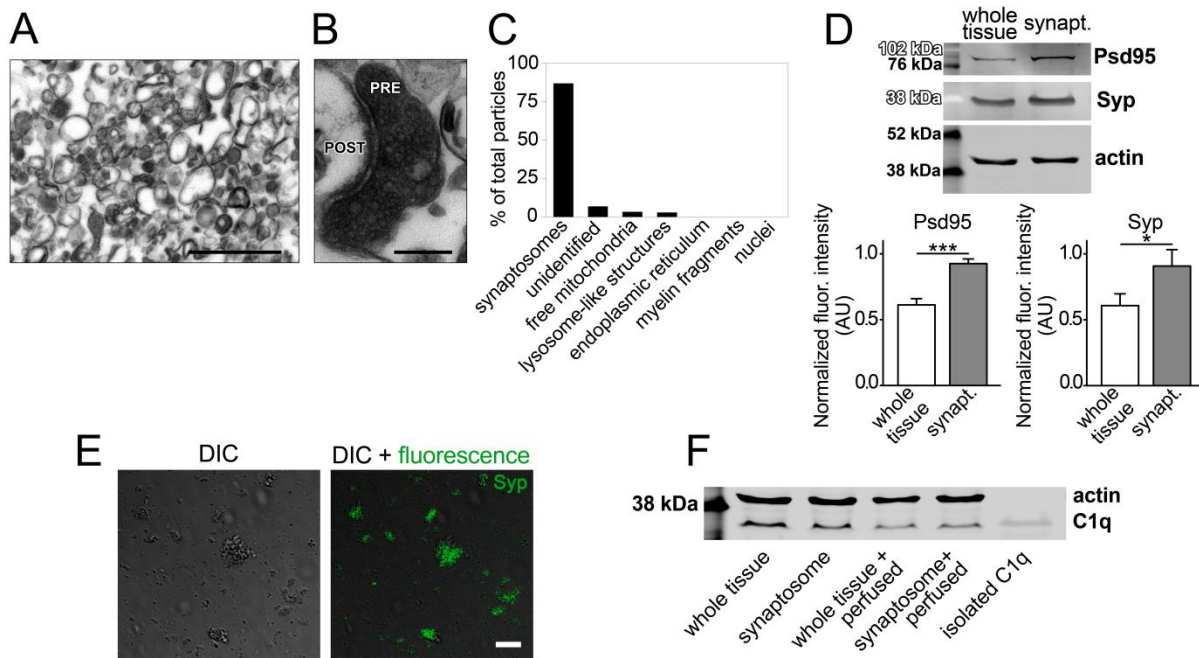


Fig. S1. Characterization of the synaptosome fraction. (A, B) Electron micrographs of the synaptic fraction. Crowded membranous structures are visible (A), corresponding to detached synaptic vesicle-filled presynaptic nerve terminals (PRE) connected to the postsynaptic compartment (POST) (B). (C) Manual analysis of the electron micrographs of the synaptosome fraction. The results are presented from thorough analysis of 5972 particles from 40 different images. (D) Western blot (WB) image and bar graphs showing enrichment of the synaptic markers Psd95 and Syp in the cortical synaptosome fraction compared to their levels in the whole cortical tissue homogenate ($n = 5$ biologically independent samples; fluorescence intensities were normalized to actin). (E) Light microscopy examination of synaptosomes. The differential interference contrast (DIC) image is presented both separately and overlaid with the fluorescence image showing Syp-positive synaptosomes. Synaptosomes became highly aggregated in PBS. (F) WB image demonstrating the presence of C1q in the cerebral cortices of healthy adult mice ($n =$ at least 6 biologically independent samples). Means \pm S.E.M. are shown. Scale bars are $2 \mu\text{m}$ (A), $0.25 \mu\text{m}$ (B), $20 \mu\text{m}$ (E).

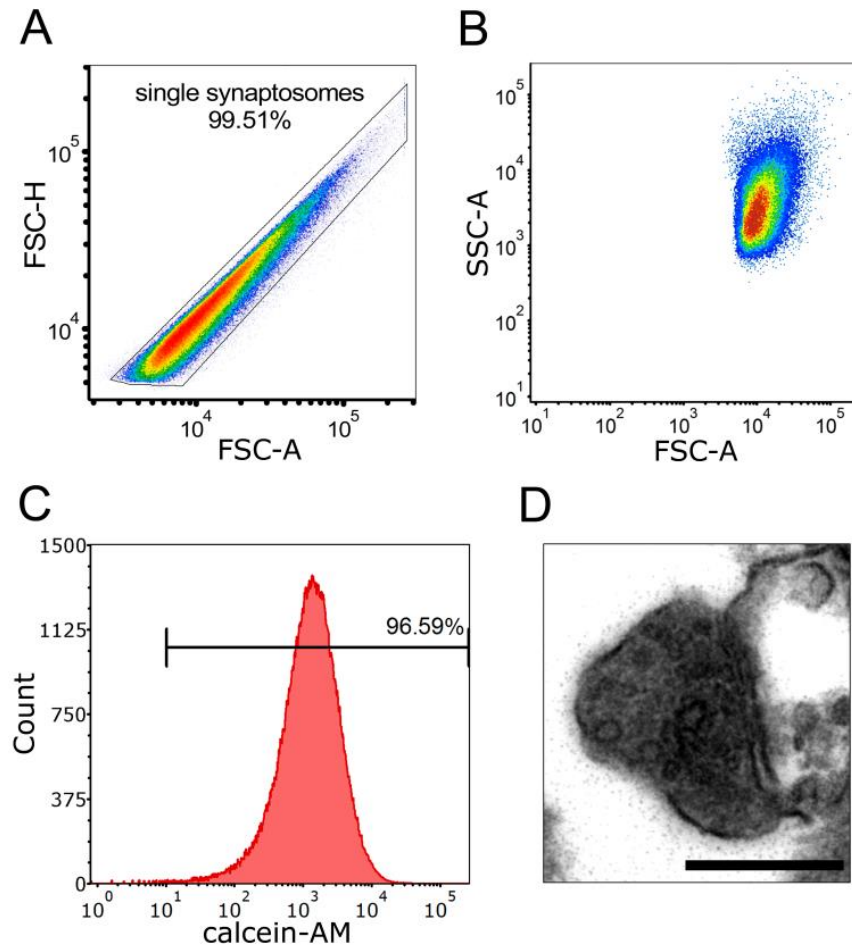


Fig. S2. Flow cytometry analysis and sorting of synaptosomes. (A) Synaptosomes were analyzed individually without the presence of aggregates, as plotting their forward light scatter area (FSC-A) signal against their forward light scatter height (FSC-H) signal resulted in a diagonal distribution indicating the absence of synaptosome aggregates (representative image of at least 5 experiments). (B) Dot plot showing the homogeneous population of synaptosomes, considering their size (FSC-A axis) and inner complexity (side light scatter area (SSC-A) axis) (representative image of at least 15 independent experiments). (C) Representative recording showing that synaptosomes were almost completely labeled with the viability dye calcein-AM (representative image of 4 independent experiments). (D) Electron micrograph of a sorted intact synaptosome. Scale bar is 0.25 μm.

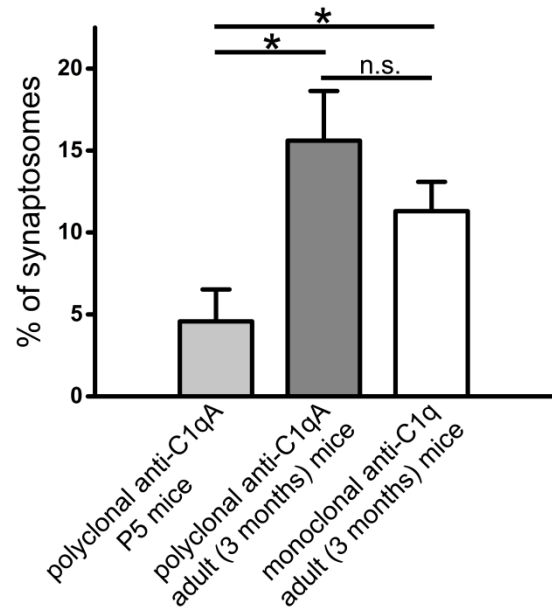


Fig. S3. The percentage of C1q-positive synaptosomes under different conditions assessed using flow cytometry. Cerebral cortical synaptosomes isolated from mice at postnatal day 5 (P5) ($n = 12$ mice, pooled in groups of 3 before subcellular fractionation) and adult (3 months old) mice ($n = 4-6$ mice per experimental condition) were separately immunolabeled with two anti-C1q antibodies (polyclonal anti-C1qA and monoclonal anti-C1q) using the same antibody dilution. Means \pm S.E.M. are shown. * $P < 0.05$, two-tailed independent Student's t -test.

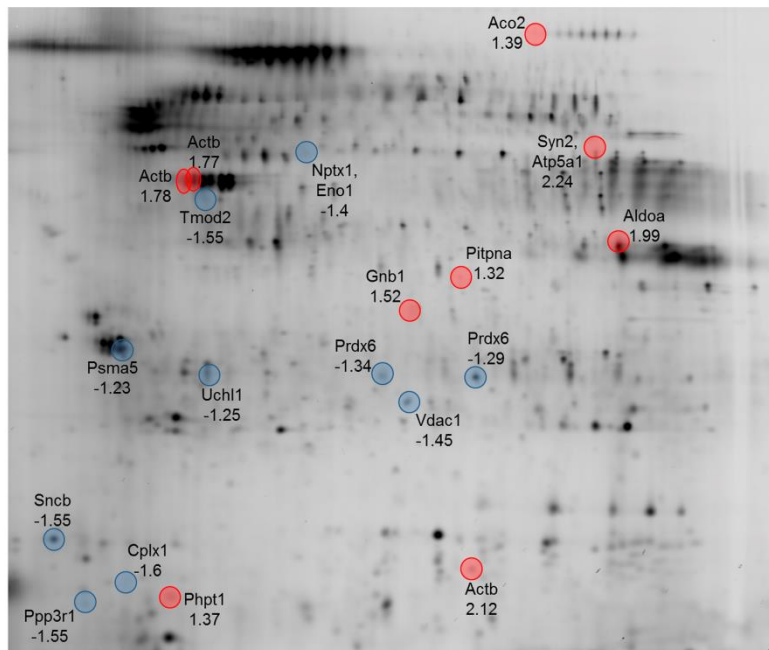


Fig. S4. Results of 2D-DIGE experiments. Representative gel image from the 2D-DIGE analysis of sorted synaptosomes showing the positions and level changes of altered proteins (representative image of 6 independent experiments).

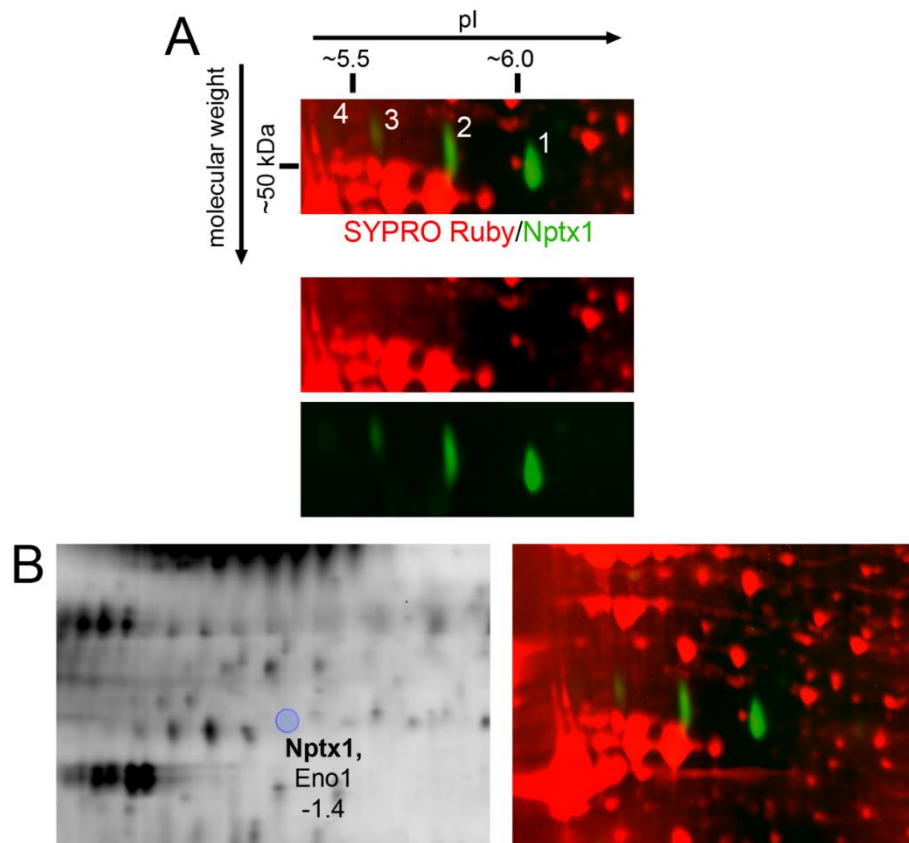


Fig. S5. The identification of post-translationally modified forms of Nptx1. (A) Proteins of the synaptosome homogenate were separated using 2D gel electrophoresis. After blotting of the proteins, the membrane was stained with the total protein stain SYPRO Ruby (red) and labeled with anti-Nptx1 antibody (green). According to our results, the post-translationally modified forms of the synaptic Nptx1 are present (4 distinguishable Nptx1 protein spots are observable), which mostly differ in their isoelectric points (representative image of 4 independent experiments). (B) Comparison of the 2D-DIGE proteomics and 2D-immunoblot results revealed that the downregulated Nptx1 form is only one but the most abundant form of the four Nptx1 spots. The amounts of the other forms were too low to be quantified in the proteomics study.

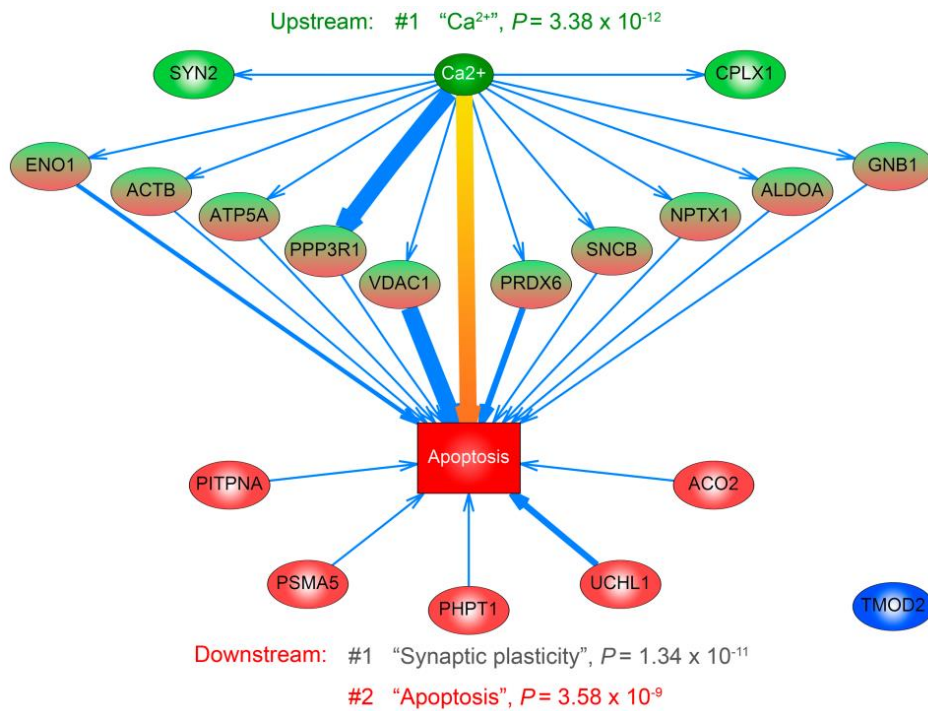


Fig. S6. Bioinformatics analysis of the protein network altered upon C1q-tagging. “Ca²⁺” is the most significant upstream regulator, whereas the process of “Apoptosis” is the second highest-ranked downstream target of the set of altered proteins according to the bioinformatics analysis (using Fisher’s exact test). The thickness of an edge is proportional to the number of the corresponding references.

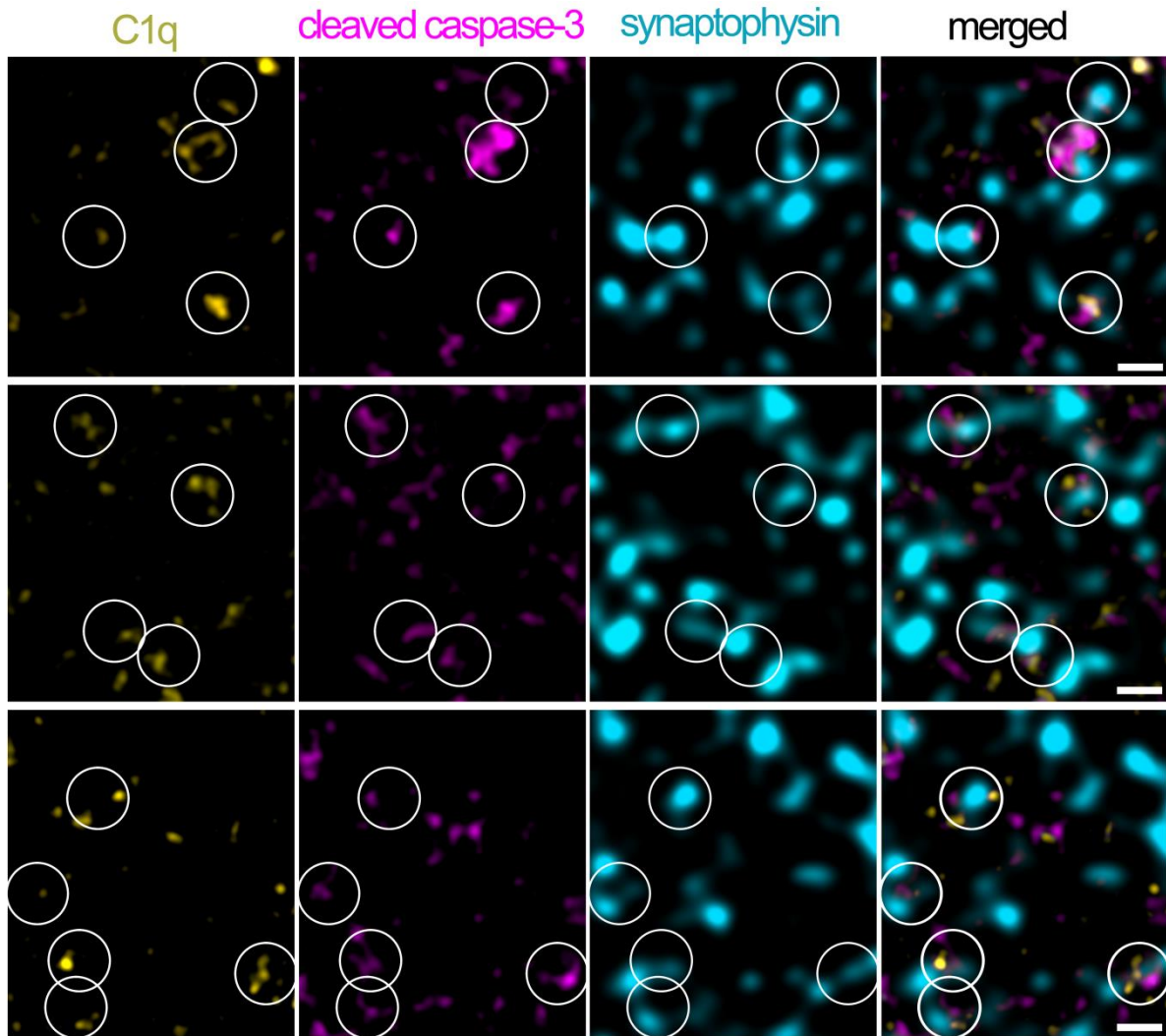


Fig. S7. STED-confocal combined microscopy images showing co-localization of C1q, cleaved caspase-3 and Syp on mouse brain sections. The circles indicate examples of co-localization between C1q, cleaved caspase-3 (both were visualized using STED super-resolution microscopy) and Syp (visualized using confocal microscopy) in the molecular layer of the hippocampal dentate gyrus. Scale bar is 0.5 μm .

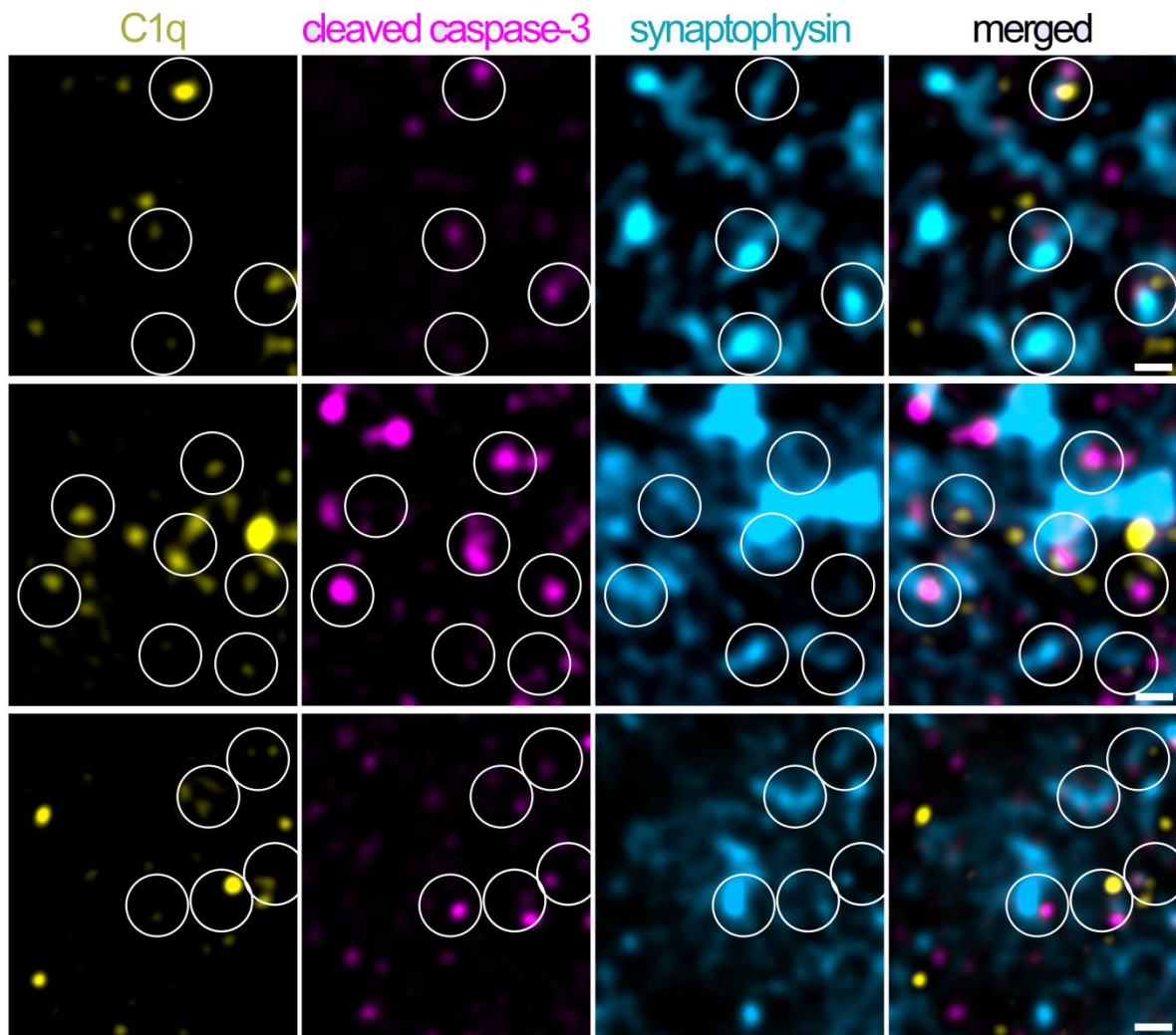


Fig. S8. HyVolution 2 pseudo-super-resolution confocal microscopy images showing co-localization of C1q, cleaved caspase-3 and Syp on human brain sections. The circles indicate examples of co-localization between C1q, cleaved caspase-3 and Syp in the temporal cortex (Brodmann area 38). Scale bar is 0.5 μm .

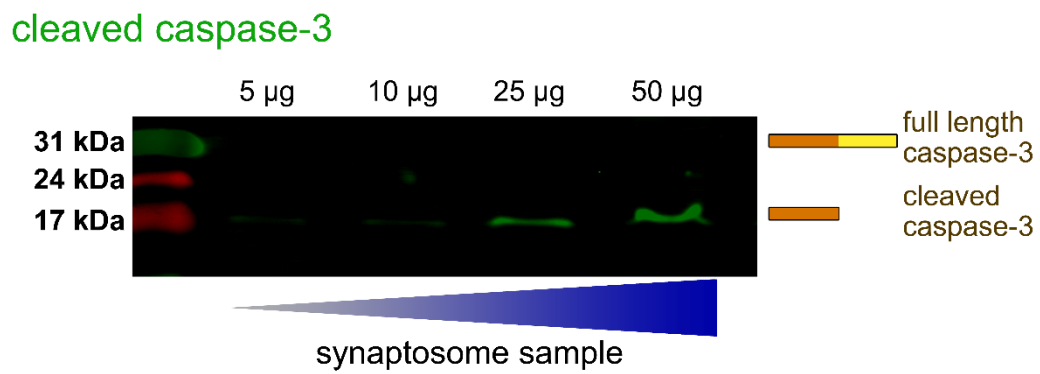


Fig. S9. Validation of the specificity of the used anti-cleaved caspase-3 antibody. Western blot image demonstrating that the used anti-cleaved caspase-3 antibody recognizes specifically the ~17 kDa cleaved fragment of the activated caspase-3 but not the inactive intact caspase-3 with molecular mass of ~31 kDa.

#	Fold change (C1q-tagged/untagged)	P-value	Protein name	Gene name	Molecular mass (kDa)	Calculated theoretical pI	Number of identified unique peptides	Sequence coverage (%)	Primary cellular function
1	-1.55	0.00071	Beta-synuclein	Sncb	14	4.4	10	91	Synaptic transmission
2	-1.4	0.00079	Alpha-enolase	Eno1	47.1	6.4	7	27.4	Glycolysis
			Neuronal pentraxin-1	Nptx1	47.1	6.1	5	15.7	Synaptic transmission
3	1.32	0.0039	Phosphatidylinositol transfer protein alpha isoform	Pitpna	31.9	6	6	18.1	Signal transduction
4	-1.29	0.0062	Peroxiredoxin-6	Prdx6	24.9	5.7	18	75	Response to oxidative stress
5	1.78	0.011	Actin, cytoplasmic 1	Actb	41.7	5.3	22	50.7	Microfilament constituent
6	-1.55	0.012	Tropomodulin-2	Tmod2	39.5	5.3	11	42.7	Microfilament organization
7	1.52	0.012	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	Gnb1	37.4	5.6	4	16.8	Signal transduction
8	2.24	0.014	Synapsin-2	Syn2	63.3	8.6	11	21.8	Synaptic vesicle exocytosis
			ATP synthase subunit alpha, mitochondrial	Atp5a1	59.7	9.2	9	21.3	Energy metabolism
9	1.77	0.018	Actin, cytoplasmic 1	Actb	41.7	5.3	12	31.7	Microfilament constituent
10	2.12	0.021	Actin, cytoplasmic 1	Actb	41.7	5.3	2	7.2	Microfilament constituent
11	-1.6	0.025	Complexin-1	Cplx1	15.1	4.9	4	33.6	Synaptic vesicle exocytosis

12	-1.23	0.027	Proteasome subunit alpha type-5	Psm5	26.4	4.7	5	22.4	Protein degradation
13	-1.25	0.035	Ubiquitin carboxyl-terminal hydrolase isozyme L1	Uchl1	24.8	5.1	18	80.7	Protein degradation
14	-1.55	0.038	Calcineurin subunit B type 1	Ppp3r1	19.3	4.6	21	73.5	Signal transduction
15	1.39	0.039	Aconitate hydratase, mitochondrial	Aco2	85.4	8.1	9	14.1	Carbohydrate metabolism
16	1.37	0.039	14 kDa phosphohistidine phosphatase	Phpt1	14	5.3	7	46	Unknown
17	1.99	0.044	Fructose-bisphosphate aldolase A	Aldoa	39.3	8.3	17	49.2	Glycolysis
18	-1.34	0.045	Peroxiredoxin-6	Prdx6	24.9	5.7	16	63.8	Response to oxidative stress
19	-1.45	0.048	Voltage-dependent anion-selective channel protein 1	Vdac1	32.3	8.6	6	15.5	Ion transport

Table S1. List of proteins identified at significantly altered levels. Note that the same protein could be present in different protein spots if the level of different post-translational modifications of it exhibited statistically significant differences. In addition, more than one identified protein could contribute to the fluorescence intensity level of the same spot.

Dataset S1. Result of the bioinformatics analysis using Pathway Studio. (S1A) The top 15 highest ranked entities (according to their Fisher's exact test *P* values) up- and downstream of the altered proteins. (S1B) Manually validated references provided by Pathway Studio about the connections between the altered proteins and the process of apoptosis or Ca²⁺.

Supplementary References

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