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Supplementary Materials for

The neuronal pentraxin Nptx2 regulates complement activity and restrains microglia-mediated synapse loss in neurodegeneration

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Supplemental materials and methods

Animals

All animal maintenance and experimental procedures were conducted in accordance with to the policies and procedures described in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committees at the Broad Institute and at JHMI. Animals were group housed and maintained under standard, temperature-controlled laboratory conditions. All mice had free access to water and food and were housed with a 12h:12h light-dark cycle. Neuronal pentraxin 2 (Nptx2) knockout ($Nptx2^{KO}$) mice in congenic C57BL/6J background were obtained from Mark Perrin's lab. PV-Cre mice (stock No: 017320), tdTomato reporter mice (stock No: 007914) and $C1q^{KO}$ mice (stock No: 031675), TauP301S (stock No: 008169) were purchased from Jackson Laboratory. $PSD95\text{-}GFP^{f/f}$ mice were generously provided by Richard Huganir's lab at the JHMI. Throughout the study we used 10-to 12-week-old $Nptx2^{KO}$ mice and 8.5 or 9-month-old P30S mice, unless otherwise stated. Both male and female mice were used for all experiments.

Stable CHO cell line generation and treatment

Chinese hamster ovary (CHO) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco 10569044) supplemented with 10% fetal bovine serum (FBS, Gibco 16000044). CHO cells were incubated at 37 °C in 5% CO2. Stable cell line was generated as follows: A bicistronic expression vector was generated in the pTRE-tight-BI plasmid (Clontech) containing both human *NPTX2* and human *NPTXR* cDNAs. This vector only allows expression of Nptx2 and Nptxr in the presence of doxycycline. This Nptx2-Nptxr expression vector and linear Hygromycin marker (Clontech) were cotransfected into CHO-tetON cells (Clontech). Stably expressing cell lines were selected for by the addition of 1mg/mL hygromycin B to the media and single colonies were picked to generate monoclonal stable cell lines. Cell lines were screened using reverse transcription polymerase chain reaction (RT-PCR) for *NPTX2* and *NPTXR* in the presence and absence of 5ug/mL doxycycline and the cell line showing the strongest doxycycline-dependent expression was selected. The final cell line was passaged using media containing 300 µg/ml G418 (Thermo Fisher, catalog #10131035) and 500 µg/ml hygromycin (Thermo Fisher, catalog #10687010) and induced with 5 µg/ml doxycycline (Thermo Fisher, catalog #J67043.AD). The CHO cell line was incubated with purified C1q (Complement Technology, A099), unbound C1q was washed three times with PBS and cells were fixed with 4% PFA for 12 minutes. After blocking with the blocking buffer [2% bovine serum albumin (BSA), 0.3% Triton-X 100 in PBS], fixed CHO cells were incubated overnight at 4°C with primary antibodies: anti-neuronal pentraxin receptor (Nptxr; 1:500, R&D systems, AF4414) and anti-C1q (1:50, clone JL1, Thermo Fisher, MA1-40311). After three washes with PBS, secondary antibodies (donkey-anti sheep 488, 1:1000, Invitrogen, A-11015; donkey-anti mouse 555, 1:1000, Invitrogen, A32773) were applied and incubated for 1 hour at room temperature. Cells were washed with PBS four times and mounted using the ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, P36935). The primary and secondary antibodies were diluted in the blocking buffer (2% BSA, 0.3% Triton-X 100 in PBS). CHO cells were imaged on a Zeiss LSM 710 confocal microscope.

Neuron-microglia co-cultures

Rat neuronal cultures were prepared from hippocampi of rat embryos on embryonic day 18 (E18) or E19 and plated on poly-D-lysine and laminin coated coverslips at a density of 20,000 cells per well in a 24-well dish and cultured in NbActiv4 medium (BrainBits). 50% of the medium was exchanged with fresh medium weekly. For primary microglial cultures, postnatal (P1-P2) pups were decapitated, and forebrains were triturated with a 5 ml serological pipette and the homogenate was spun at 300xg for 5 min. The supernatant was discarded and the pellet was resuspended with a 1 ml pipette and filtered through a 75 µm filter. Two brains were cultured per 175 cm^2 flask in 40 ml DMEM + 10% FBS. After 24 hours

incubation, flasks were rinsed with Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific, Cat: 14175095) and new media was added. Cultures were grown for an additional 7 to 9 days before microglia were shaken off the astrocyte feeder layer on a rocking platform for 1 h, pelleted and resuspended in NbActiv4 medium and added to neurons in a 1:2 (microglia : neuron) ratio. Primary cells from male and female pups were used. 10,000 primary rat microglia were added to 20,000 primary hippocampal neurons with 40 ng/mL of rat macrophage colony stimulating factor (MCSF) (Peprotech, 400-28-100UG). Microglia were added to days in vitro (DIV) 9 + 3-days post AAV-infected neurons, or DIV12 neurons in the presence of recombinant protein or C1q-blocking antibody. Neurons were infected with AAV-GFP or AAV-Nptx2-V5 at DIV 9 at a concentration of 100 viral genomes per cell (vg/cell). For antibody treatment, 1 µg/ml anti-C1q antibody (Abcam, ab182451) or normal rabbit IgG (Cell Signaling Technology, Cat: 2729) were added to the cultures. For recombinant Nptx2 treatment, 2 µg/ml of recombinant Nptx2 (R&D Systems, CF 7816-NP-050) or BSA were added to the cultures. In case of LPS-induced activation of microglia, LPS was added at a concentration of 1 µg/ml using eBioscience Lipopolysaccharide (LPS) Solution (500X) 24 hours after plating of microglia. 24 hours post treatment with LPS, culture media was collected, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) in PBS for imaging.

After incubation with the blocking buffer (2% BSA, 0.3% Triton-X 100 in PBS), neuron-microglia co-cultures were incubated with primary antibodies overnight at 4°C: anti-MAP2 (1:500, Abcam, ab5392), anti-V5 (1:500, Thermo Fisher, MA5-15253), and anti-ionized calcium binding adaptor molecule 1 (Iba1; 1:500, Wako, 019-19741). Next day, after three washes with PBS, cells were incubated with highly cross-adsorbed Alexa Fluor-conjugated secondary antibodies (1:1000, Goat anti-Chicken 647, Invitrogen, A32933; Goat anti-mouse 488, Invitrogen, A-11001; Goat-anti Rabbit 405, Invitrogen, A48254) at room temperature for one hour, and then washed with PBS. After washing, the coverslip was mounted with mounting medium (Invitrogen, Prolong Gold antifade reagent, P10144). The primary and secondary antibodies were diluted in the blocking buffer. Neuron-microglia co-cultures were imaged on an Andor DragonFly spinning disk confocal microscope. Images were collected, processed using ImageJ, and quantified blind to conditions.

AAV and Plasmids

For AAVs used in vitro, the sequence of the CamKII promoter was obtained from (*52*). The sequence of miR-204, which we found to reduce AAV transgene expression in mice, was obtained from (*53*). Plasmid Paav-CamKII-Nptx2-V5 was generated by GenScript, by cloning a synthesized CamKII-Nptx2-V5-miR204 DNA fragment upstream of the WPRE-hGHpA sequence of a plasmid derived from plasmid CAG-NLS-GFP (a gift from Viviana Gradinaru, Addgene #104061). Plasmid Paav-CamKII-NLS-GFP was generated by replacing the Nptx2-V5 fragment between the KpnI and EcoRI sites of plasmid Paav-CamKII-Nptx2-V5 with an NLS-GFP fragment excised from plasmid CAG-NLS-GFP. Plasmid sequences were verified by Sanger sequencing. Recombinant AAVs were produced and titered as previously described (*54*). Briefly, HEK 293T/17 cells (American Type Culture Collection (ATCC), CRL-11268) were cultured in DMEM with high glucose, sodium pyruvate, GlutaMAX, and Phenol Red (DMEM, Gibco 10569044) supplemented with 5% FBS (Gibco, 16000044) and 1x MEM Non-Essential Amino Acids Solution (NEAA, Gibco 11140076). Cells were triple transfected with plasmid DNA encoding rep-cap, pHelper, and an ITR-flanked transgene using polyethylenimine (PEI, Polyscience, 24765-1). AAVs were harvested from the cells and the media 3 days post transfection and purified by ultracentrifugation over iodixanol gradients. The concentration of packaged virus genomes was tittered by digital droplet PCR (ddPCR) using ITR primers (forward: 5'-GGAACCCCTAGTGATGGAGTT-3', reverse: 5'-CGGCCTCAGTGAGCGA-3', synthesized by IDT) and probe (5'-FAM-CACTCCCTC-ZEN-TCTGCGCGCTCG-IBFQ-3', synthesized by IDT).

For AAVs used in vivo, pAAV-CaMKII-GFP (Addgene:64545) was purchased from Addgene. pAAV-CaMKII-NPTX2-V5 was generated using NEBuilder HiFi DNA assembly cloning kit (Cat. E5520S). Sequence was confirmed by Sanger sequence. AAVs were prepared by the Janelia Viral Tools facility.

Plate-based binding and complement activation assays

To quantify pentraxin binding to C1q, purified recombinant neuronal pentraxin proteins (4 μg/ml, R&D Systems) or IgM (2 μg/ml, Thermo Fisher Scientific, 31146) were suspended in carbonate buffer (10 Mm $NaHCO_3$; Ph 9.6) and added to 96-well Nunc MaxiSorp Flat-Bottom plates, which were sealed and left to incubate with shaking overnight at 4°C. After immobilization, the plate was washed with 0.05% Tween-20 in PBS (PBST) and blocked with 2% w/v ELISA-Grade BSA in PBS (2% BSA) with shaking for 1 hour at 37°C. The plate was washed with PBST, and purified human C1q protein (2 μg/ml, Complement Technology, A099) was suspended in GVB++ Buffer (Complement Technology, B102) and was added to plate with shaking for 1 hour at 37°C. The plate was washed with PBST and incubated with primary antibody against C1q (JL1, Thermo Fisher Scientific; 1μg/ml) in 2% BSA for 1 hour with shaking at 37°C. The plate was washed with PBST and incubated with species-specific horseradish peroxidase (HRP)-conjugated secondary antibody in 2% BSA with shaking for 1 hour at 37°C. The plate was washed with PBST and chemically exposed by adding 100μl 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (BioLegend, 421501). The reaction was stopped with 100μl Stop Solution 450 nm (2N H_2SO_4) and the optical density $(OD)_{450}$ was recorded using EnVision 2104 multilabel plate reader (Perkins Elmer).

To quantify pentraxins binding to complement proteins, the same protocol as above was used with modifications. Purified human complement proteins (4 μg/mL, Complement Technology C1q: A099; C2: A112; C3: A118; C4: A105) were immobilized and were treated with His-tagged recombinant neuronal pentraxin proteins (4 μg/mL, R&D Systems, NPTX1: 7707-NP-050; NPTX2: 7816-NP-050; NPTXR: 4414-NP-050). The readout was HRP-conjugated anti-His tag antibody (abcam; ab1187 or ab1269) recognizing the pentraxin proteins.

To quantify CCP activity in normal human serum in presence of pentraxins, the same protocol as above was used with modifications. Purified IgM (4 μg/ml, Thermo Fisher, 31146) or BSA was immobilized on the plate. Before addition to the immobilized IgM/BSA, normal human serum (NHS, Complement Technology) was incubated with different concentrations of purified recombinant human pentraxin protein (Nptxr, R&D 4414-NP-050) suspended in GVB++ Buffer (Complement Technology, B102). The mixture was incubated with shaking for 15 min at 37°C and then added to the immobilized IgM/BSA. The control was heat-inactivated NHS and C1q-depleted serum. The readout was a primary antibody against cleaved (active) C3 (Hycult Biotech, HM2257) and a species-specific HRP-conjugated secondary.

To validate the C1q blocking antibody, purified Nptx2 protein (4 μg/mL) or BSA was immobilized on the plate. Mouse serum (Complement Technology, NMS) was incubated with C1q (4.8) antibody (Abcam, ab182451) or rabbit isotype control antibody (CST, Cat:2729) suspended in GVB++ buffer and incubated with shaking for 30 min at 37°C before adding to the Nptx2/BSA coated plate. The readout was a primary antibody against mouse cleaved C3 (Hycult Biotech, HM2257) and a species specific HRP-conjugated secondary.

Protein purification and in vitro pull down

Rat Nptx2 pentraxin domain (X2-PD) was synthesized and codon optimized for expression in *E. coli*. The construct was cloned into the PEG30a-GB1-His and transfected into SHuffle T7 expression competent *E.*

coli (NEB, C3026J). The transfected *E. coli* cell culture was grown at 30 °C until the OD_{600} value reached 0.6. The temperature was reduced to 20°C, and cells were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for 16 h. The cells were harvested and lysed by sonicating in 10mM HEPES (pH 7.3) containing 150mM NaCl, supplemented with 1% Triton X-100. The lysate was clarified by centrifugation at 8000 × *g* for 30 min at 4 °C. The supernatant was filtered and loaded onto a 5ml HisTrap column (GE Healthcare). The column was washed with 10 column volumes of Buffer A (10 mM HEPES, pH 7.3, 0.15 M NaCl, 5 mM imidazole) on an Aktapurifier (GE Healthcare) while collecting fractions. To elute bound protein, a linear gradient from 0 to 100% Buffer B (10 mM HEPES, pH 7.3, 0.15 mM NaCl, 500 mM imidazole) was set over 25 column volumes and protein was fractionated. Fractions were analyzed by SDS-PAGE, and which containing the interested protein were pooled and dialyzed against 2 liters of 10 mM HEPES, pH 7.3, 0.15 M NaCl, 5mM DTT at 4 °C overnight. The following day, the dialyzed proteins were concentrated and loaded onto a Superdex 200 size exclusion column. Fractions were analyzed by SDS-PAGE, and fractions containing pure X2-PD protein were pooled and concentrated.

Rat Nptxr pentraxin domain (XR-PD) was synthesized and codon optimized for expression in *E. coli.* The construct was cloned into the PGEX-6P-1 vector and transfected into Shuffle T7 expression competent *E. coli* from NEB. The *E. coli* growth and induced protein expression was performed in the same manner with X2-PD. The recombinant protein in the supernatant of the cell lysate was mixed with Glutathione Sepharose resin (GE Healthcare) and the mixture was washed extensively in 10mM HEPES pH 7.3. Glutathione S-transferase (GST)-XR-PD was eluted by 10mM GSH and loaded onto a Superdex 75 size exclusion column. Fractions were analyzed by SDS-PAGE, and fractions containing pure XR-PD protein were pooled and concentrated.

***E. coli* killing assay**

E. coli cells used in these assays were obtained from cultures grown in LB medium. Different amounts of XR-PD (0.5-4 μM), together with 0.25% NHS (Complement technology) or heat-inactivated NHS (incubated at 56°C for 30 minutes) were preincubated for 10 min at 37 °C before adding *E. coli*. After an incubation of 30 min at 37 °C, cells were plated to a LB-agar plate and cultivated overnight at 37 °C before counting colony forming units. Colony forming unit in GVB++ buffer was set as 100%.

Hemolytic assay

To analyze inhibition of hemolysis through the classical pathway, 0.5% NHS with various concentrations of GST or GST-XR-PD were preincubated together for 15 min at 37 °C. After preincubation, the NHS–protein mix was added to the erythrocytes and incubated for additional 30 min at 37 °C. Lysis of erythrocytes was determined by measuring the amount of hemoglobin in the supernatants at 414 nm. Erythrocytes (EA, Complement technology, B200) treated with ddH_2O or incubated with GVB++ buffer served as positive (100% Hemolytic) and negative controls (0% Hemolytic).

Western blot

Brain tissue and cultured cells were lysed in RIPA buffer. Protein samples were boiled in 1x reducing agent and 1x sample buffer (Invitrogen; NP0008) and separated by 4-12% SDS-PAGE (Thermo Fisher Scientific; NP0323BOX, WG1403BOX). Gels were transferred to PVDF membranes (Millipore, IPFL00010) and blocked with 5% milk in 0.1% Tween 20 (v/v) in TBST buffer with rocking for 1 hour at room temperature. Primary antibodies in 1% Milk (w/v) in TBST were incubated with rocking overnight at 4°C. The membrane was washed with TBST and incubated with secondary HRP-conjugated antibodies in 1% Milk with rocking for 1 hour at room temperature. The membrane was washed, exposed with West Pico ECL (Thermo Fisher Scientific; 1863098), and immunoreactivity was detected on ChemiDoc system (Bio-Rad). Analysis was

performed using Image Lab software (Bio-Rad). The following primary antibodies were used: Actin (Millipore Sigma, A5316), dilution 1:10000; CD68 (FA-11, Biorad, MCA1957) dilution 1:500; Nptx2 (Proteintech, 10889-1-AP), dilution 1:1000 or (antibody generated in Paul Worley's lab), dilution 1:1000.

Electron microscopy negative staining

The single C1q protein particles, single Nptx2 protein particles, and C1q-NPTX2 complex was visualized by negative staining and electron microscopy followed the published protocol as described (*55*). Briefly, to visualize C1q and Nptx2 single particles, purified C1q and Nptx2 were diluted to 5 µg/ml in 50 mM HEPES containing 150 mM NaCl; to visualize complex C1q-NPTX2, two proteins was directly incubated at 4°C for 2 hours and diluted to 5 µg/ml in 50 mM HEPES containing 150 mM NaCl. Carbon-coated grids were hydrophilized by glow discharge at low pressure in air. Aliquots of C1q, Nptx2, and C1q–Nptx2 complex were adsorbed onto hydrophilic, carbon-coated grids for 1 min, washed twice with ddH_2O, and stained on a drop of 2% Uranyl Formate (UF) in ddH_2O. Specimens were examined in a Hitachi 7600 TEM at a 300,000x magnification.

ELISA assays

For mouse brain lysates, frozen brain tissue was hand homogenized in lysis buffer (5mM HEPES pH 7.4, 1mM MgCl2, 0.5mM CaCl2, supplemented with protease inhibitors (cOmplete Mini, EDTA-free, Roche, ref: 11836170001). After low speed centrifugation (1,400 xg, 10 min, 4°C), the post-nuclear supernatant fractions were used to measure mouse C1q, C4, C4b, C3b, C3 concentrations using commercial ELISA kits: C1q mouse ELISA (Hycult Biotech, HK211-01), Total C4 (LSBio, LS-F37428-1), C4b (LSBio, LS-F8056-1), C3b (Hycult Biotech, HK216), C3 (Abcam, ab157711) following the manufacturer's instructions. Brain lysate protein concentrations were measured using a BCA kit (Thermo Scientific, ref: 23227). The experimenters were blinded to the genotype.

For Human cerebrospinal fluid (CSF) samples, CSF complement proteins C1q, C3b and Factor B were measured using the following ELISA kits: Human complement C1q (ab170246), Human Complement C3b ELISA kit (ab195461), Human Complement Factor B ELISA Kit (ab137973) from Abcam according to the manufacturer's instructions. All the CSF measurements were performed in duplicate. Experimenters were blinded to all clinical and genetic information.

Proximity ligase assay (PLA)

PLA was used as previously reported with some modification (*56*). Briefly, Nunc MaxiSorp 384-well plate (MilliporeSigma, cat# P6366-1CS) was coated with rabbit anti-Nptx2 (7 µg/ml, 25 µl/well) in carbonate-bicarbonate coating buffer (pH 9.6), and incubated overnight at 4°C. The next day, plates were washed twice with TBST and blocked with 5% BSA + 0.1 mg/ml salmon sperm DNA (Thermo Fisher, cat# 15632011), 100ul/well, at room temperature for one hour. 20 µl of sample or 20ul TBS + 5% BSA + 0.1 mg/ml sperm DNA (as blank) was added to each well and incubated overnight at 4°C. After washing with TBST (5 times, 100 µl/well each time), 20 µl of PLA probe (1 µg/ml of oligo plus probe + 1 µg/ml oligo minus probe in TBS with 5% BSA + 0.1 mg/ml sperm DNA) was added, and incubated at room temperature for one hour. After washing with TBST (5 times, 2 min each, speed 1500 rpm on shaker), 20 µl of ligation mixture (0.4 µl of T4 ligase, 0.2 µl of connector oligo (20 µM), 2 µl of 10x T4 ligase buffer and 17.4 µl of H2O) was added. The plate was incubated at 37°C for 30 min, then at room temperature for one hour. After washing with PBS (3 times), 20 µl of 20mM DTT was added and samples were incubated at room temperature or 37°C for one hour. The eluted ligation products were collected and diluted with 20 µl H_2O. qPCR was performed in 384-well plate (Thermo Fisher, cat# 4309849). The delta-delta Ct method was used for calculating PLA values in qPCR. The sequences of oligos are as follows: Oligo plus (59bp, HPLC purified, 5' add amino-5AmMC6): 5' CGCATCGCCCTTGGACTACGACTGACGAACCGCTTTGCCTGACTGATCGCTAAATCGTG 3'; Oligo

minus (61bp, HPLC purified, 5' add phosphorylation, 3' add amino-3AmMO): 5'TCGTGTCTAAAGTCCGTTACCTTGATTCCCCTAACCCTCTTGAAAAATTCGGCATCGGTGA 3'. The primer sequences are as follows: qPCR Primer 1: 5' CATCGCCCTTGGACTACGA 3'; qPCR Primer 2: 5' GGGAATCAAGGTAACGGACTTTAG 3'. The connector sequence is: 5' TACTTAGACACGACACGATTTAGTTT 3'

Antibody and AAV injections

C1q (4.8) antibody and isotype control antibody injection were performed as described previously with slight modification (*7*). Briefly, the C1q (4.8) antibody and Rabbit IgG isotype control (Invitrogen: Cat 10500C) were concentrated to 6.7 mg/ml. Two- to 3-month-old $Nptx2^{KO}$ mice were anesthetized with 3% isoflurane and placed on a stereotaxic apparatus (RWD) for surgery. All injections were performed with Nanoject II (Drummond scientific company). The injection coordinate is M/L=+/-3.2 mm, A/P=0.02mm, D/V= 2.5mm. Two µl of anti-C1q or isotype control antibody were injected at a rate of 50.6nl/per injection within 3 minutes. The needle was removed 5 minutes after completion of the injection and mice were put back in their cage. Six days after injection, the brains were harvested, and IHC staining, imaging, and data analysis were performed. For ELISA analysis, 1µl of AAV-CamkII-GFP (1*10E13 GC/ml) as control or AAV-CamkII-NPTX2-V5 (1*10E13 GC/ml) was injected into the hippocampus of P301S mice; coordinates: M/L=+/-1.8 mm, A/P=-1.94 mm, D/V= -1.9 mm. For IHC staining analysis, P301S mice were bilaterally injected with AAV-CamkII-GFP (1*10E13 GC/ml, 0.5 µl) as control or AAV-CamkII-NPTX2-V5 (1*10E13 GC/ml, 0.5 µl) in both cortex and hippocampus; coordinates: M/L=+/-2.5 mm, A/P=-2.18mm, D/V= -2.2mm and -1.1mm. For ELISA analysis, mouse brains were harvested 3 weeks after injection. Brains were harvested 6 weeks after injection for IHC staining and analysis.

Immunohistochemistry staining and analysis

$NPTX2^{KO}$ and WT mice were aged between 10-12 weeks for this study. The age of P301S mice has been descripted in the figure legends or indicated in the figures. Mouse brains were harvested following transcranial perfusion with PBS and 4% PFA. Tissue was postfixed in 4% PFA overnight, then washed with PBS and transferred to 30% sucrose solution until the tissue has sunk to the bottom of the tubes. Post-fixed brains were sliced in 35 µm thick sections in a -18°C chamber using a 3050 S Cryostat (Leica). Brain slices were either placed directly on Superfrost Plus slides (VWR) and stored at -80°C until ready for use or stored in PBS at 4°C. Mouse brain slices were blocked with blocking buffer (either 10% normal goat serum, 1% BSA, 0.3% Tween-20 in PBS or 5% BSA, 0.2% Triton X-100 in PBS) for 1 hour, and incubated with primary antibodies overnight. After rinsing with PBS four times, secondary antibodies were applied and incubated for 1 hour at room temperature. Slides were washed and mounted using the ProLong Gold Antifade Reagent with or without 4',6-diamidino-2-phenylindole (DAPI) depending on experiment. The following primary antibodies were used: C1q (clone 4.8, Abcam, 182451), dilution 1:500; CD68 (clone FA-11, Biorad, MCA1957) dilution 1:200; Iba1 (Wako, 019-19741), dilution 1:200; Homer1 (SYSY, 160003 & 160006), dilution 1:500; VGlut1 (SYSY, 135304), dilution 1:2000; Parvalbumin (Millipore, MAB1572), dilution 1:200; Parvalbumin (SYSY, 195006), dilution 1:500; neuronal nuclei (NeuN; clone A60, Millipore, MAB377), dilution 1:500; NeuN (SYSY, 266004), dilution 1:200; GAD67 (clone 1G10.2, Millipore, MAB5406), dilution 1: 500; Gephyrin (clone RbmAb7, SYSY, 147018), dilution 1:500; C3 (Dako/Agilent, A063), dilution 1:500; Synaptophysin (SYSY; 101004), dilution 1:500; oligodendrocyte transcription factor 2 (Olig2; Millipore, MABN50), dilution 1:200; glial fibrillary acidic protein (GFAP; Abcam, ab7260), dilution 1:500; AT8 (biotinylated, Thermo Fisher, MN1020B) 1:200; Nptx2 (generated by Paul Worley's lab), dilution 1:100. Goat-raised highly cross-adsorbed Alexa Fluor-conjugated secondary antibodies (Thermo Fisher, A32723; A32737; A32728; A48282; A32732; A32733; A48282; A48263; A-21449; A-21435; A-21105; dilution 1:500) were used. The primary and secondary antibodies were diluted in blocking buffer. Fluorescence intensity was analyzed using ImageJ (FIJI).

Microglial synapse engulfment was analyzed as previously described (*7*, *8*). Brain sections were imaged on the Zeiss LSM 880 confocal microscope or Andor DragonFly Spinning Disk Confocal Microscope with z-stacks (total z-size up to 15 µm with a step size of 0.3 µm per slice) using 63x oil objectives. Three to 5 region of interests (ROIs) per brain section were imaged and analyzed. CD68-positive lysosomes were 3D-reconstructed using the surface rendering function in Imaris 9.7. Excitatory synapse (Vglut1 and Homer1) and Inhibitory synapse (Gephyrin and GAD67) puncta were detected using the spots function. Fraction of lysosomal synapse puncta were calculated by dividing puncta within the CD68 volume/total number of synapse puncta.

Excitatory and inhibitory synapse density was analyzed using a custom script in Fiji (ImageJ) software followed the published protocol as described (*35*). Briefly, tissue samples were imaged on a Zeiss LSM 880 confocal microscope or Andor DragonFly spinning disk confocal microscope using a 63x oil immersion objective. Images were processed and automatically analyzed using a published script: https://github.com/emiliafavuzzi/synaptic-analyses.

For Homer1-C3 co-localization analysis, images were acquired using a 60x oil-immersion objective on an Andor DragonFly spinning disk confocal microscope and analyzed as described previously (*8*). Maximum intensity projection of a z-stack of two images with 0.5 µm step size was created and colocalization was calculated using the Fiji ComDet v0.5.5 plugin (https://github.com/ekatrukha/ComDet). Puncta were identified using an intensity threshold of 3x standard deviation. Puncta were considered as co-localized if the maximal distance between the Homer1 and C3 spots' centers was less than 3 pixels (300 nm).

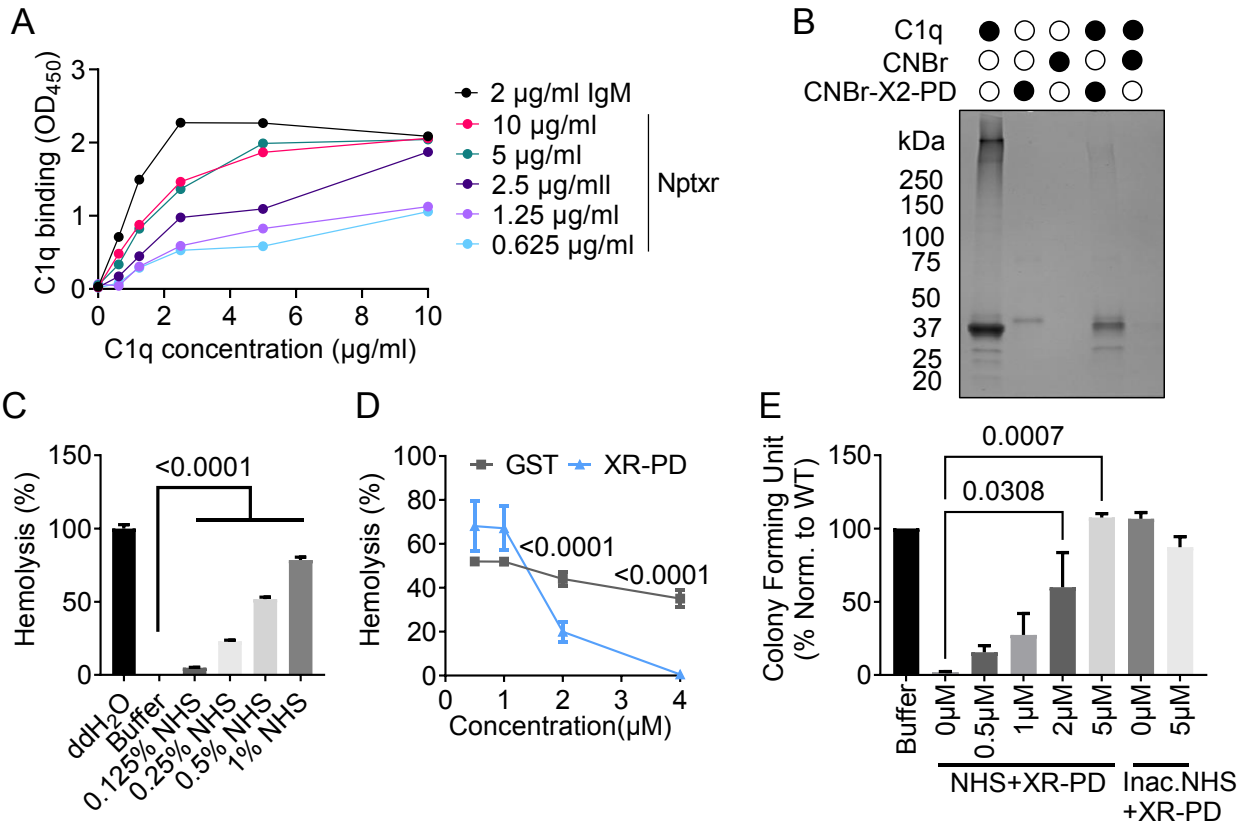

Fig. S1. Neuronal pentraxins interact with C1q and regulate classical complement pathway (CCP) activity in vitro.

(**A**) Microtiter wells were coated with 2 µg/ml IgM or 0.625 to 10 µg/ml Nptxr and incubated with various concentrations of C1q as indicated. Bound C1q was detected using an anti-C1q antibody. OD, optical density. (**B**) Purified Nptx2 pentraxin domain (X2-PD) coupled to CNBr-activated Sepharose 4B was incubated with purified C1q. Proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. Filled circles indicate that reagent was included in the incubation. (**C**) Hemolysis of sheep erythrocytes in presence of increased NHS concentrations was quantified. Lysis of sheep erythrocytes was monitored by measuring released hemoglobin at 415 nm. p values were determined by one-way ANOVA followed by Šidák post hoc test. NHS, normal human serum. (**D**) Purified XR-PD was added to NHS in a CCP-specific buffer. Lysis of sheep erythrocytes was monitored by measuring released hemoglobin at 415 nm. p values were determined by two-way ANOVA followed by Šidák post hoc test. (**E**) Survival of *E. coli* in presence of purified XR-PD in NHS. *E. coli* survival was analyzed by counting colony forming units. Heat inactivated (Inac.) NHS was used as a negative control. p values were determined by one-way ANOVA followed by Šidák post hoc test. Norm., normalized. WT, wild-type. Data are presented as mean ± SEM. Each dot represents data from individual samples.

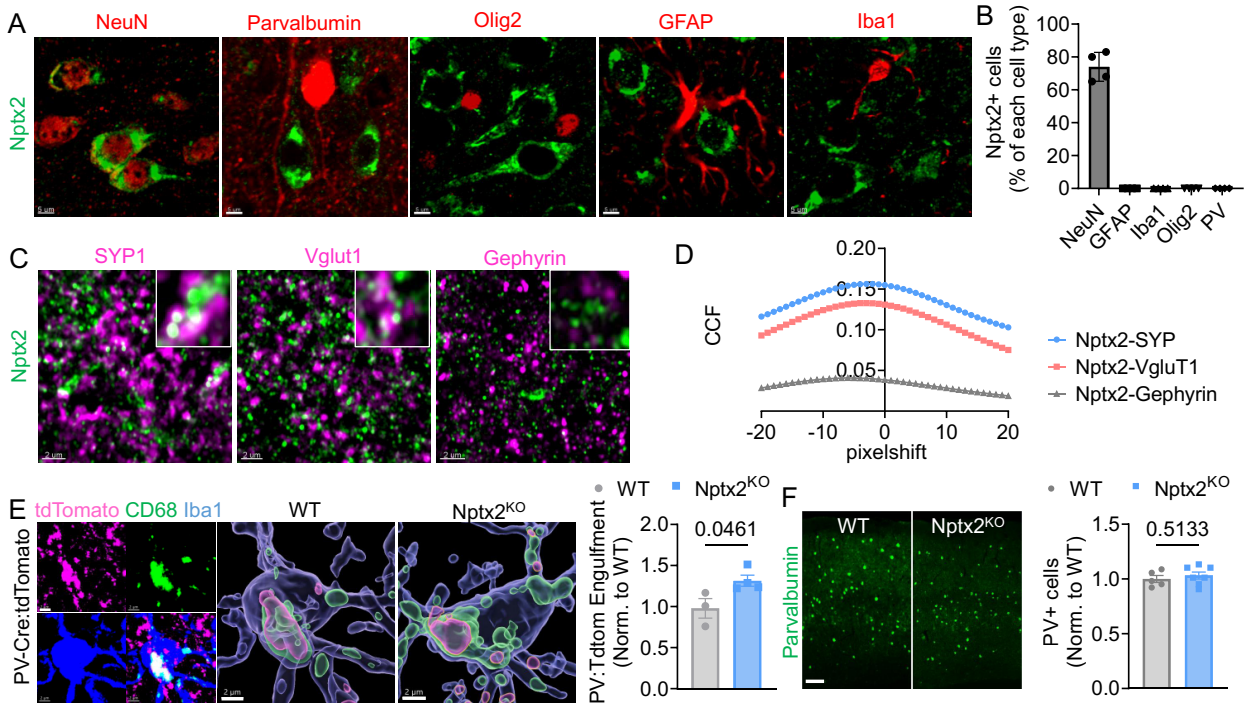

Fig. S2. Nptx2 is expressed by neurons and colocalizes with excitatory synapse.

(**A**) Representative confocal images are shown of co-immunostained Nptx2 (green) with NeuN, Parvalbumin, Olig2, GFAP or Iba1 (all shown in red) in WT cortical layers II-V. Scale bars are 5 µm. (**B**) The percentage of Nptx2+ NeuN, Parvalbumin, Olig2, GFAP, and Iba1 cells in WT cortical layers II-V was quantified. (**C**) Shown are representative confocal images of co-immunostained Nptx2 with the excitatory synapse marker proteins Synaptophysin 1 (SYP1) or Vglut1, and inhibitory synapse maker Gephyrin in WT cortex. Scale bars are 2 µm. Vglut1, Vesicular glutamate transporter 1. (**D**) Nptx2 colocalization with SYP1, Vglut1, and Gephyrin was quantified. A Pearson's coefficient cross-correlation function (CCF) was used to analyze the colocalization by shifting the magenta channel pixel by pixel with respect to the green channel with ImageJ JACoP plugin. (**E**) Shown are representative images of tdTomato fluorescence and co-immunostained CD68+ microglial lysosomes (green) and Iba1 (blue) in the cortical layers II-V. The three-dimensional surface rendering shows CD68+ microglial lysosomes containing tdTomato in PV-Cre:tdTomato mice in WT and Nptx2^{KO} background. Scale bars are 2 µm. The quantification of tdTomato (Tdtom) volume within CD68+ microglial lysosomes in PV-Cre:tdTomato WT and Nptx2^{KO} brains is shown on the right. Values were normalized to WT mice. p value was determined by unpaired *t* test. n = 3 for WT; n = 4 for Nptx2^{KO}. PV: parvalbumin; KO, knockout. (**F**) Representative images (left) and quantification (right) of PV+ cell density are shown for WT and Nptx2^{KO} cortices. Cell density was normalized to WT mice. p value was determined by unpaired *t* test. n = 5 for WT; n = 7 for Nptx2^{KO}. Scale bar is 100µm. Data are presented as mean ± SEM. Each dot represents data from one mouse.

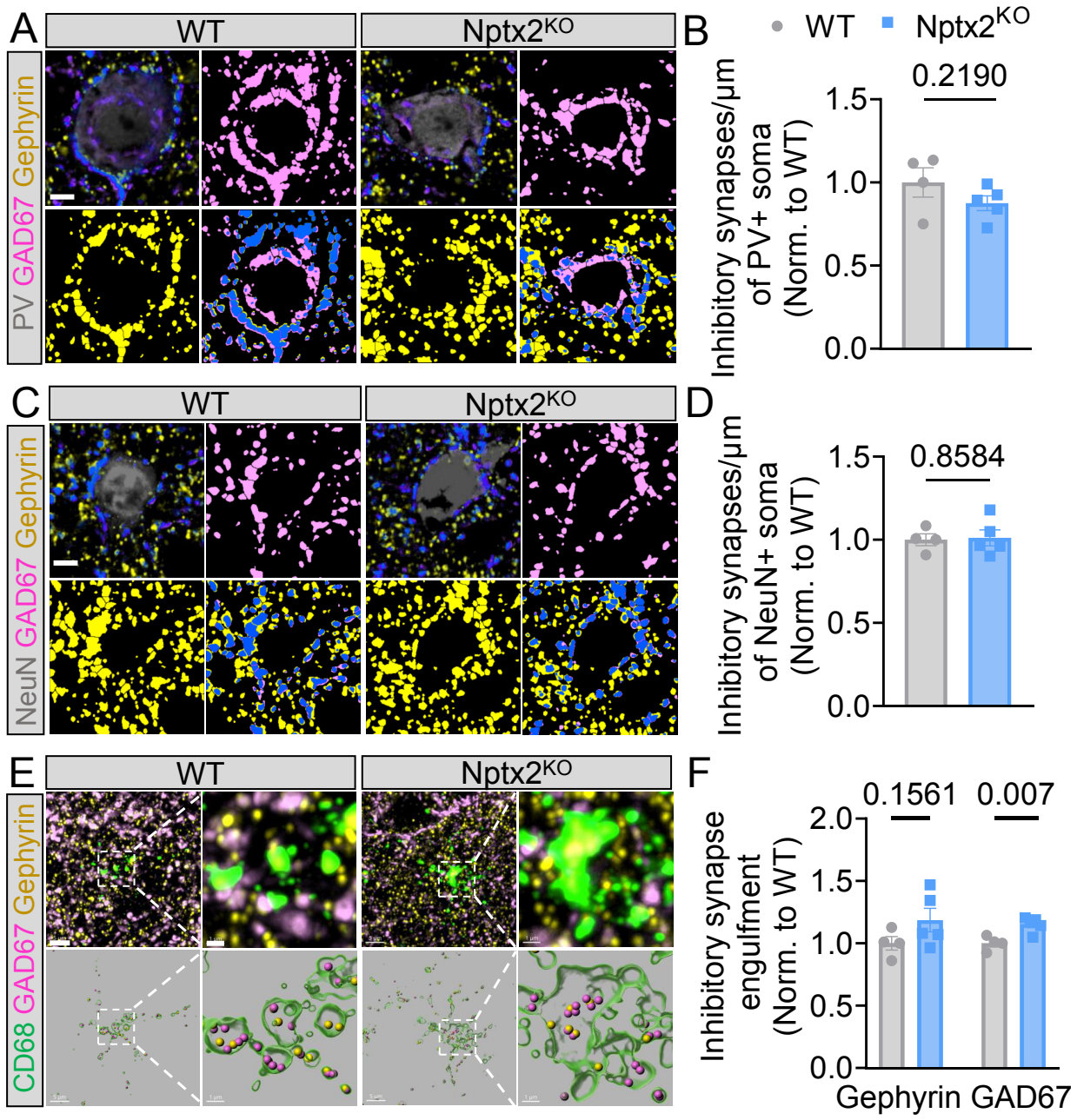

Fig. S3. Inhibitory synapses density is largely unchanged in the cortex of Nptx2-deficient mice.

(**A and B**) Shown are representative confocal images and corresponding masks (A) of PV (gray), Gephyrin (yellow) and GAD67 (pink) in WT and Nptx2^{KO} cortical layers II-V. The bar graph (B) shows inhibitory synapse density around somas of PV+ neurons. P value was determined by unpaired *t* test. n = 4 for WT; n = 5 for Nptx2^{KO}. Scale bars is 5 µm. GAD67, Glutamic acid decarboxylase 67. (**C and D**) Shown are representative confocal images and corresponding masks (C) of NeuN (gray), Gephyrin (yellow) and GAD67 (pink) in WT and Nptx2^{KO} cortical layers II-V. The bar graph (D) shows inhibitory synapse density around somas of NeuN+ neurons. p value was determined by unpaired *t* test. n = 4 for WT; n = 5 for Nptx2^{KO}. Scale bars is 5 µm. (**E and F**) Shown are representative confocal images and 3D rendering of CD68 (green), Gephyrin (yellow) and GAD67 (pink) (E) in WT and Nptx2^{KO} cortical layers II-V. The bar graph (F)

shows the relative amount of Gephyrin and GAD67 puncta within CD68+ microglial lysosomes. p values were determined by unpair *t* test. n = 4 for WT; n = 5 for Nptx2^{KO}. Scale bars are 5 µm, insets, 1 µm. Data are presented as mean ± SEM. Each dot represents data from one mouse.

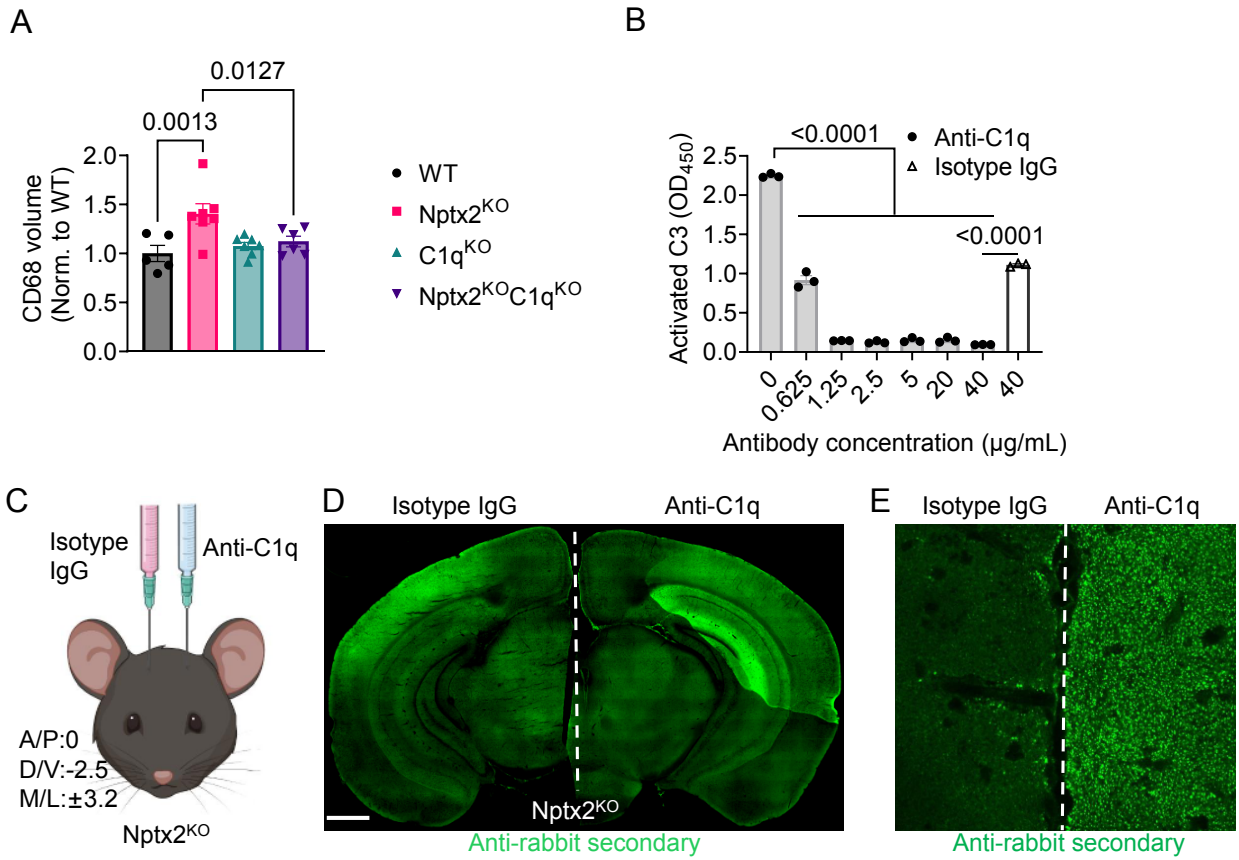

Fig. S4. Characterization and injection of a C1q-blocking antibody into Nptx2^{KO} mice.

(**A**) Shown is the volume of CD68+ microglial lysosomes in the cortical layers II-V of mice with indicated genotypes. p values were determined by one-way ANOVA followed by Šidák post hoc test. n = 5 for WT; n = 7 for Nptx2^{KO}; n = 7 for C1q^{KO}; n = 6 for Nptx2^{KO};C1q^{KO}. (**B**) Nptx2-coated microtiter plates were incubated with 5% NHS supplemented with different concentrations of anti-C1q antibody. Complement activation and deposition was measured using an activated C3 specific antibody. Isotype IgG was used as negative control. p values were determined by one-way ANOVA followed by Šidák post hoc test. (**C**) Shown is a schematic of stereotactic antibody injection. Coordinates correspond to the injection site. (**D**) Shown is a representative image of a Nptx2^{KO} brain injected with anti-C1q antibody and isotype IgG and immunostained with an Alexa-conjugated anti-rabbit secondary antibody. Scale bar is 1000 µm. IgG, Immunoglobulin G. (**E**) Shown are representative confocal images from a Nptx2^{KO} brain cortex injected with anti-C1q and isotype IgG. Injected antibodies were detected with an Alexa-conjugated anti-rabbit secondary antibody. Note that, whereas the immunoreactivity in the isotype IgG injected hemisphere is low and dim, the anti-C1q injected hemisphere shows bright puncta that is a characteristic C1q pattern. Data in (A) and (B) are presented as mean ± SEM. Each dot represents data from individual samples.

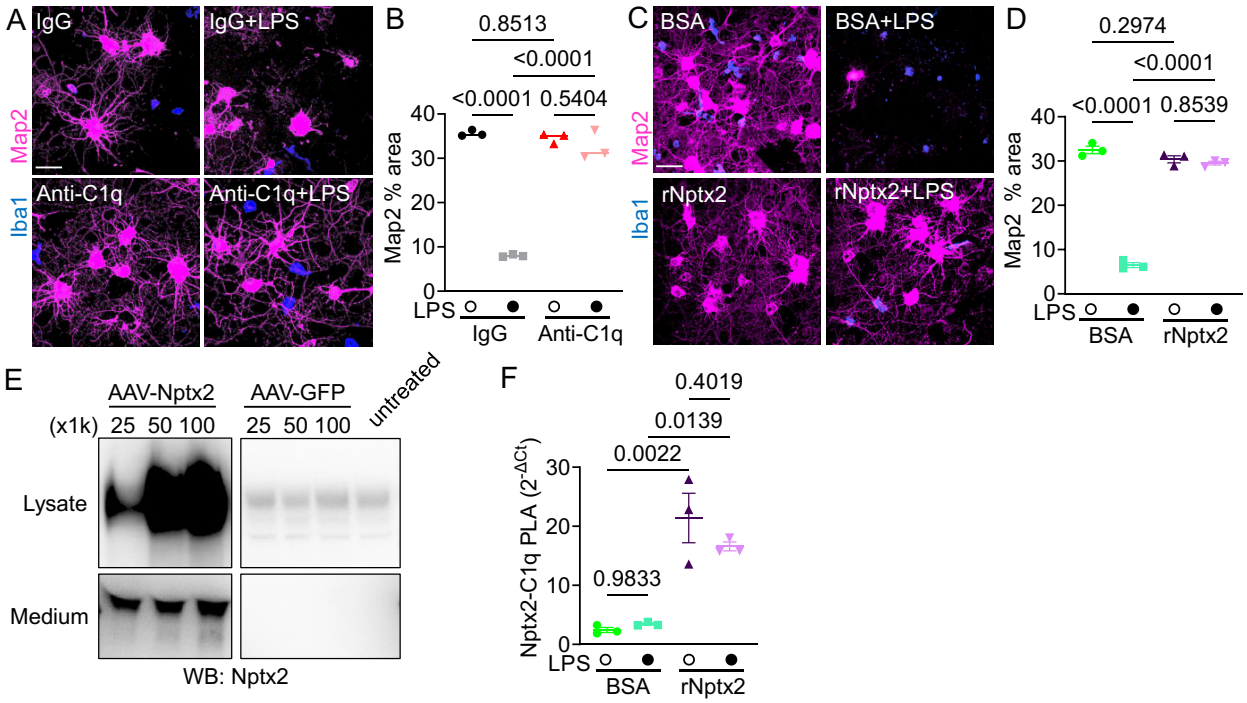

Fig. S5. C1q-blocking antibody and recombinant Nptx2 prevented neuronal loss in LPS-stimulated neuron-microglia co-cultures.

(**A**) Shown are representative images of immunostained Map2 (pink) and Iba1 (blue) in neuron-microglia co-cultures treated with LPS or vehicle control for 24 hours in the presence of control IgG or anti-C1q antibodies. Scale bar is 50 µm. Map2, Microtubule-associated protein 2; LPS, Lipopolysaccharides. (**B**) The percentage of Map2+ area in indicated cultures is shown, where filled circles indicate LPS treatment. Three independent neuron-microglia co-cultures were used for the experiment. p values were determined by two-way ANOVA followed by Tukey post-hoc test. (**C**) Shown are representative images of immunostained Map2 (pink) and Iba1 (blue) in neuron-microglia co-cultures treated with LPS or vehicle control in the presence of BSA or recombinant Nptx2 (rNptx2). Scale bar, 50 µm. (**D**) The percentage of Map2+ area in indicated cultures is shown. Filled circles indicate BSA or rNptx2 treatment. Three independent neuron-microglia co-cultures were used for the experiment. p values were determined by two-way ANOVA followed by Tukey post-hoc test. (**E**) A representative Western blot shows Nptx2 abundance in lysate and culture medium of neurons infected with different virus titers (genome vector/per cell) of AAV-Nptx2 or AAV-GFP. AAV, adeno-associated virus; GFP, green fluorescent protein (**F**) Nptx2-C1q complex abundance in culture medium was analyzed by proximity ligation assay (PLA). Filled circles indicate BSA or rNptx2 treatment. Media from three independent neuron-microglia co-cultures were used. p values were determined by two-way ANOVA followed by Tukey post-hoc test.

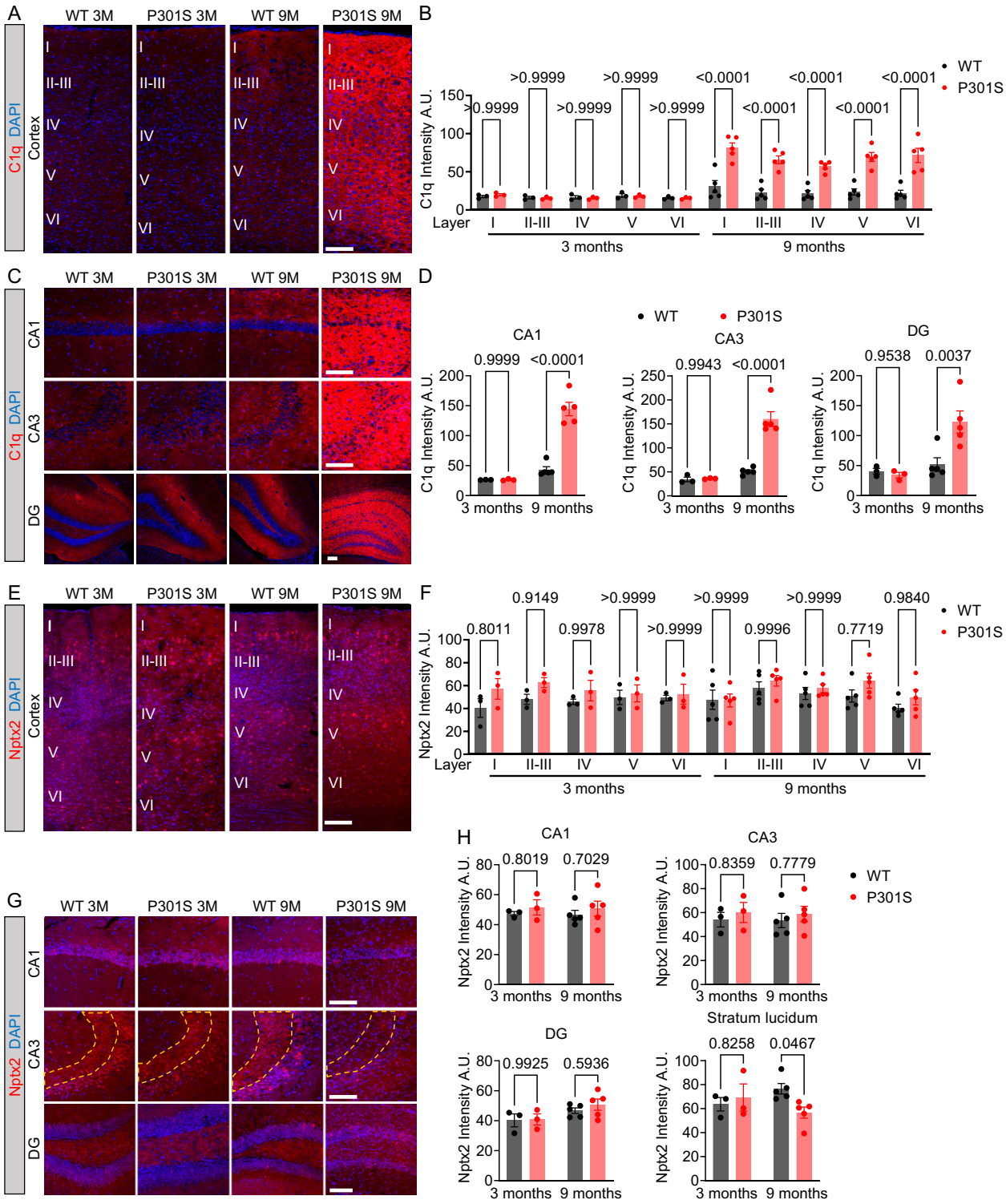

Fig. S6. Nptx2 and C1q intensity in 3- and 9-months-old WT and P301S brains.

(**A to D**) Shown is representative C1q immunostaining (A and C) and quantified immunofluorescence intensity in cortical layers (B) and hippocampal regions (D) from WT and P301S mice at 3- and 9-months. Scale bars are 100 µm. p values were determined by unpaired *t* test. WT 3 months, n = 3; P301S 3 months, n = 3; WT 9 months, n = 5; P301S 9 months, n = 5. A.U., arbitrary units. (**E to H**) Shown is representative

Nptx2 immunostaining (E and G) and quantified immunofluorescence intensity in cortical layers (F) and hippocampal regions (H) from WT and P301S mice at 3- and 9-months. Scale bars are 100 µm. p values were determined by unpaired *t* test. The dotted yellow lines indicate the stratum lucidum in the hippocampus. WT 3 months, n = 3; P301S 3 months, n = 3; WT 9 months, n = 5; P301S 9 months, n = 5.

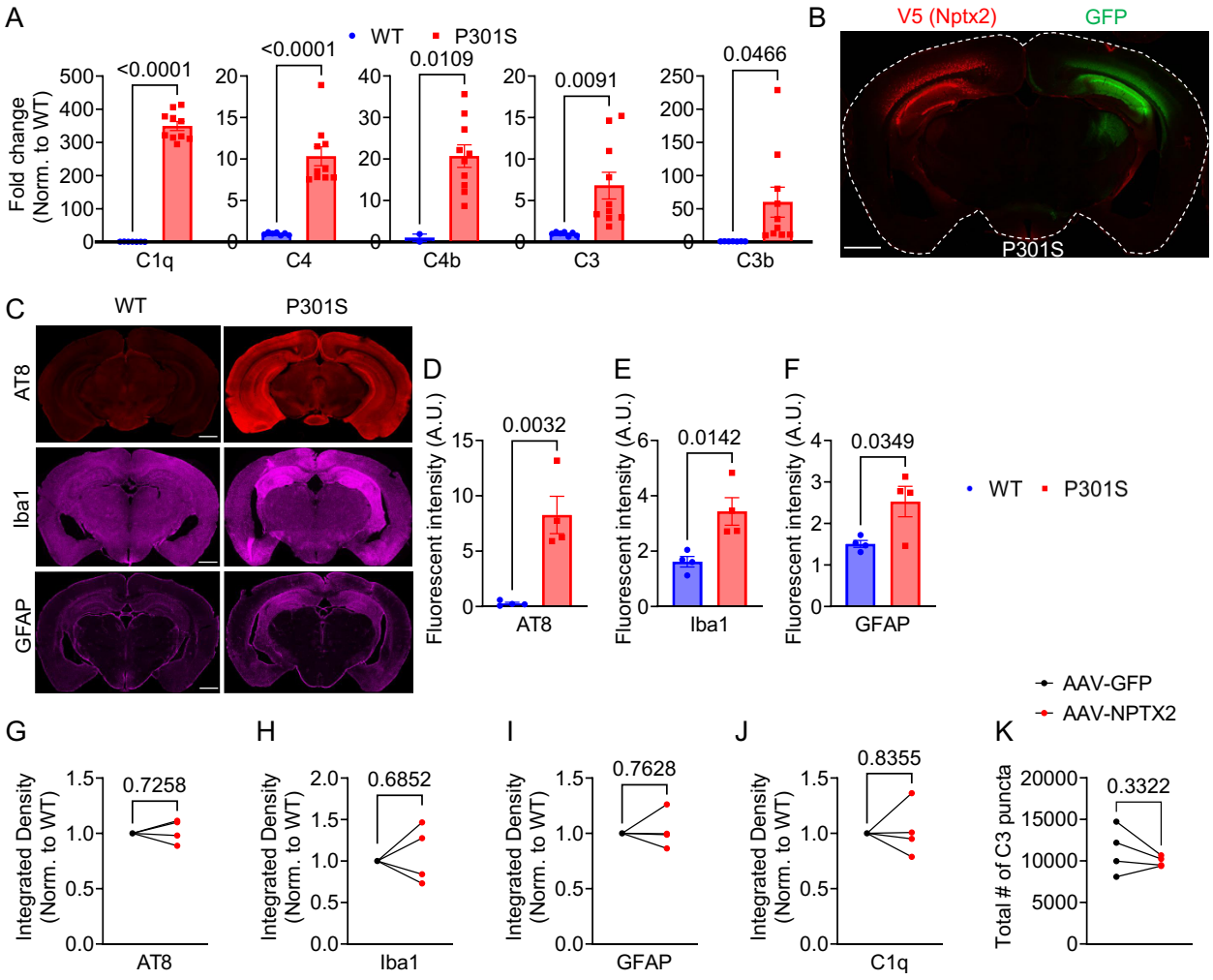

Fig. S7. Concentration of complement proteins and histopathological analysis of brains from WT and P301S brains.

(**A**) Shown is the fold change of C1q, C4, C4b, C3 and C3b protein abundance in hippocampus from AAV-GFP injected P301S (n = 10) versus WT mice (n = 7). Note that for C4b, WT n = 2 as the other samples were below the limit of detection. p values were determined by unpaired *t* test. (**B**) Shown is a representative confocal image of GFP and Nptx2 fluorescence (detected by immunostaining the V5-tag) in P301S brains injected with AAV-Nptx2 and AAV-GFP. Scale bar is 1000 µm. (**C**) Shown are representative images of WT and P301S brains immunostained for phospho-Tau (AT8), Iba1 and GFAP. Scale bars are 1000 µm. (**D to F**) Immunofluorescence intensity of AT8 (D), Iba1 (E), and GFAP (F) was measured in 9-month-old WT and P301S brains. p values were determined by unpaired *t* test. WT n = 4. P301S n = 4. (**G to J**) Immunofluorescence intensity of AT8 (G), Iba1 (H), GFAP (I), and C1q (J) was measured in AAV-GFP and AAV-Nptx2 injected hippocampi of P301S mice. p values were determined by paired *t* test. n = 4. (**K**) The number of C3 structures was determined in AAV-GFP and AAV-Nptx2 injected hippocampi of P301S mice. p value was determined by paired *t* test. n = 4. Data are presented as mean ± SEM. Each dot represents data from individual samples.

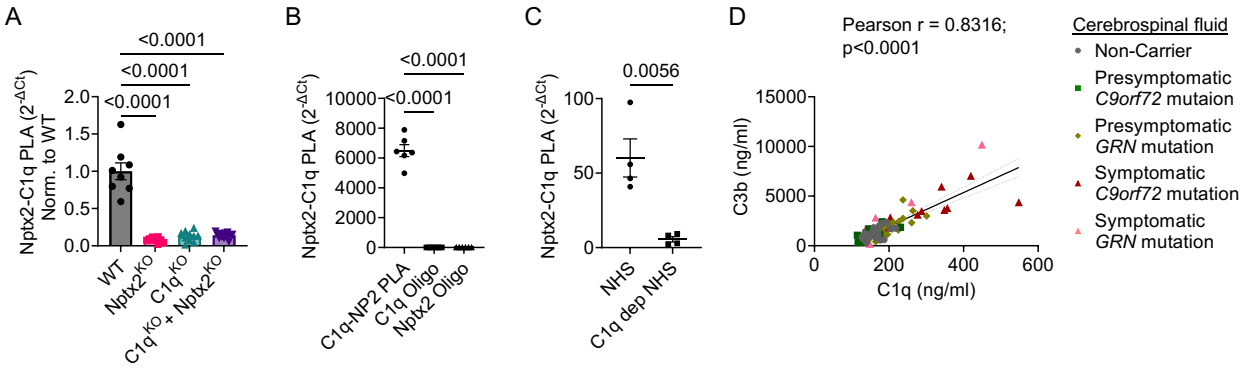

Fig. S8. Validations of the NPTX2-C1q PLA and correlation of C1q with C3 in CSF samples.

(**A**) NPTX2-C1q complex abundance in brain lysates from WT, Nptx2^{KO} and C1q^{KO} was measured by PLA. Nptx2^{KO} + C1q^{KO} represent mixed Nptx2^{KO} and C1q^{KO} lysates. Quantitative qPCR data are represented using $2^{-\Delta Ct}$. p values were determined by one-way ANOVA test followed by Šidák's post hoc test. n = 8 for WT, n = 10 for Nptx2^{KO}, n = 10 for C1q^{KO}, n = 13 for Nptx2^{KO} + C1q^{KO} mixed lysates. (**B**) NPTX2-C1q complex abundance in WT mouse brain lysates was detected in presence of mixed oligo-labeled anti-C1q and anti-Nptx2 antibodies or in presence of individual anti-C1q or anti-Nptx2 antibodies. Quantitative qPCR data are represented using $2^{-\Delta Ct}$. p values were determined by one way ANOVA test. n = 6 samples per condition. (**C**) NPTX2-C1q complex abundance was measured in normal human serum (NHS) and C1q-depleted NHS. Quantitative qPCR data are represented using $2^{-\Delta Ct}$. p value was determined by unpaired *t* test. n = 4 for NHS, n = 4 for C1q-depleted (dep) NHS. (**D**) Shown is the correlation between C3b and C1q concentrations in CSF samples. P value was determined by two-tailed *t* test. Data are presented as mean ± SEM. Each dot represents data from one individual. Non-carriers, n = 22; presymptomatic *GRN* mutation, n = 25; presymptomatic *C9orf72*, n = 15; symptomatic *GRN* mutation, n = 7; symptomatic *C9orf72*, n = 14.

Data file S1. Raw, individual-level data for experiments where n<20.