

Identification of candidate markers for the isolation of neuron-specific extracellular vesicles

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Supplementary Information

Associated SI Tables:

Supplementary Table S1: List of proteins detected by mass spectrometry in EVs isolated by MMR Slurry from pooled CSF and plasma samples

Supplementary Table S2: List of proteins detected by mass spectrometry in EVs isolated by MMR Slurry from 10 individual CSF and plasma samples

Supplementary Table S3: List of all proteins with transmembrane domains or GPI anchors

Supplementary Table S4: List of proteins that are brain and cell type-specific by bulk RNA-Seq and whether they have transmembrane domains

Supplementary Table S5: List of sorted proteins by SCimilarity Attribution Scores to quantify specificity from scRNA-Seq data for neurons, astrocytes, oligodendrocytes, and microglia and whether they have transmembrane domains

Supplementary Table S6: List of proteins that are cell type specific by both bulk RNA-Seq and scRNA-Seq

Supplementary Table S7: List of candidate EV markers that are transmembrane and specific to brain and neurons, astrocytes, oligodendrocytes, or microglia by bulk RNA-Seq with other attributes

Supplementary Table S8: List of candidate EV markers that have GPI anchors and are specific to brain and neurons, astrocytes, oligodendrocytes, or microglia by bulk RNA-Seq with other attributes

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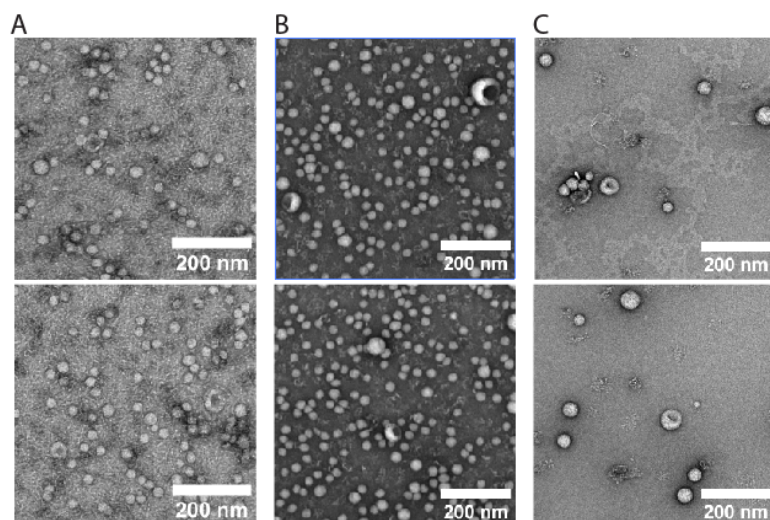


Figure S1: Electron microscopy of EVs isolated from plasma by SEC and MMR Slurry
Electron microscopy of two representative fields of EVs isolated from plasma by **A.** SEC **B.** SEC followed by MMR Slurry using CC 2.5 (μL slurry / $2.5 \mu\text{g}$ protein) or **C.** SEC followed by MMR Slurry using CC 0.3 (μL slurry / $0.3 \mu\text{g}$ protein).

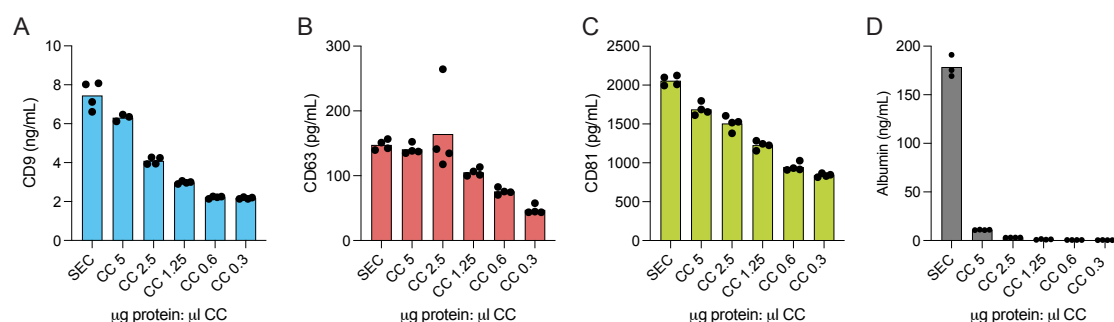


Figure S2: MMR Slurry isolation significantly reduces concentration of albumin relative to tetraspanins

Simoa measurement of **A.** CD9 **B.** CD63 **C.** CD81 **D.** and albumin measured after isolation of EVs from 0.5 mL plasma by SEC or SEC followed by MMR Slurry with increasing amounts of Capto Core beads. Two replicates were performed for each condition and Simoa measurements were taken in duplicate for each replicate.

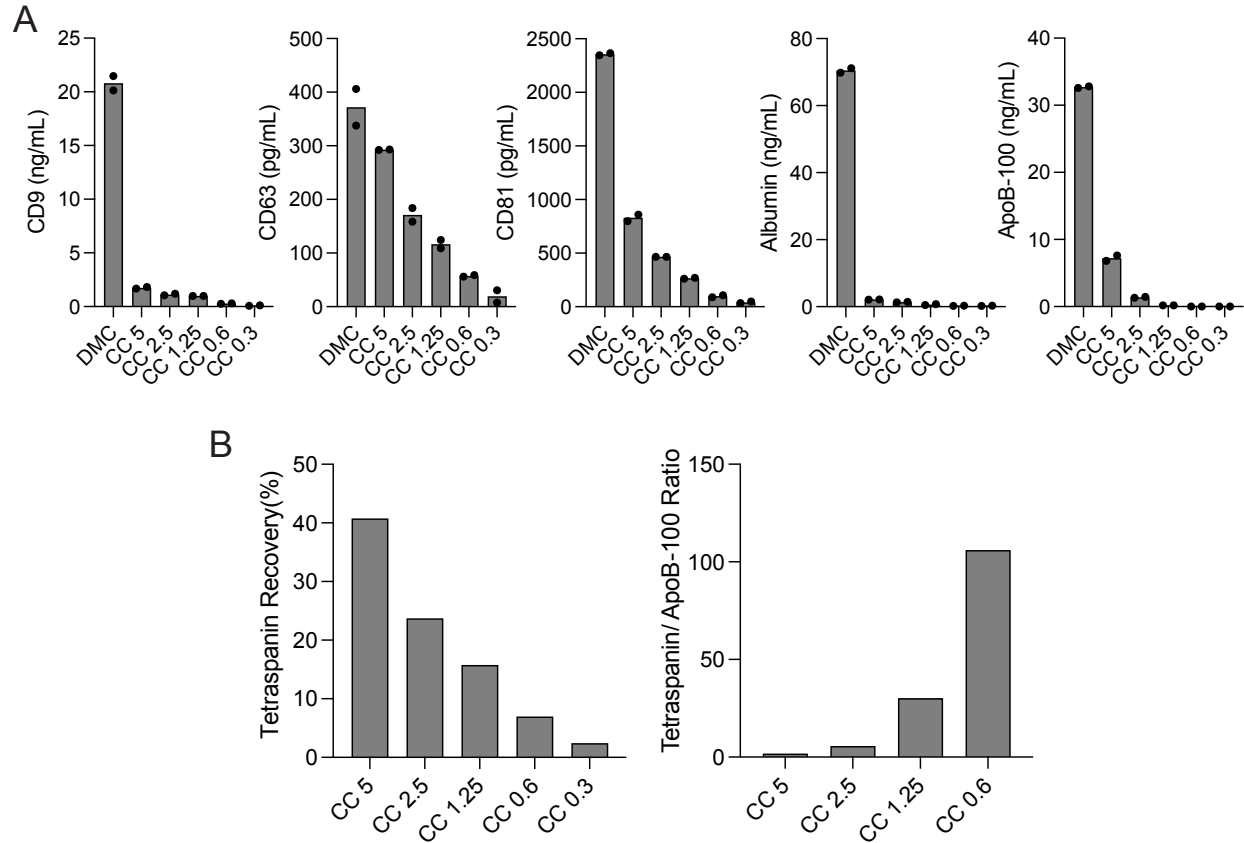


Figure S3: DMC followed by MMR Slurry significantly depletes lipoproteins from EVs

A. Simoa measurements of CD9, CD63, CD81, albumin, and ApoB-100 comparing DMC and DMC followed by MMR Slurry using the following conditions: CC 5, CC 2.5, CC 1.25, CC 0.6, and CC 0.3. **B.** Tetraspanin recovery is calculated as an average of the CD9, CD63, and CD81 concentrations in each condition relative to those concentrations in the DMC condition. Tetraspanin to ApoB-100 ratio is calculated by dividing relative tetraspanin yield by relative ApoB100 yield where each MMR Slurry condition is compared to DMC.

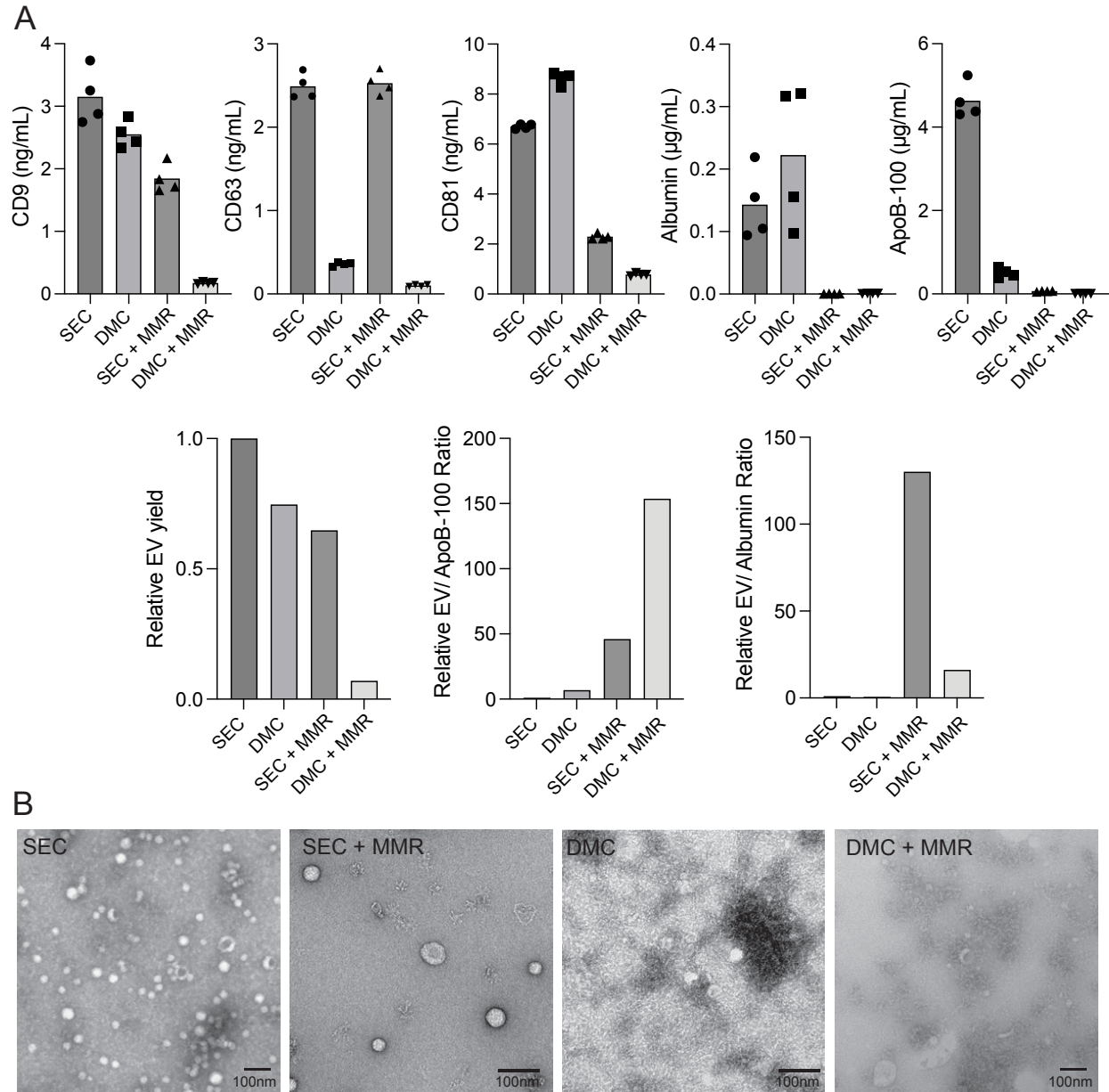


Figure S4: MMR slurry after SEC or DMC allows for additional enrichment of extracellular vesicles relative to lipoproteins and free proteins

Protein concentration measured by Simoa of **A.** CD9, CD63, CD81, albumin, and ApoB100 of 1 mL of plasma purified using SEC, DMC, SEC + MMR Slurry or DMC + MMR Slurry. For each MMR Slurry condition, the CC 0.6 ratio is used. To calculate Relative EV yield, CD9, CD63, and CD81 values for each condition are divided by the corresponding value for SEC and averaged. The relative EV yield of SEC is set to 1. These Relative EV yield values were then divided by relative yield of ApoB-100 and albumin to calculate the purity of each isolation method compared to SEC. **B.** Electron microscopy of representative fields of EVs from SEC, SEC + MMR Slurry, DMC, or DMC + MMR Slurry.

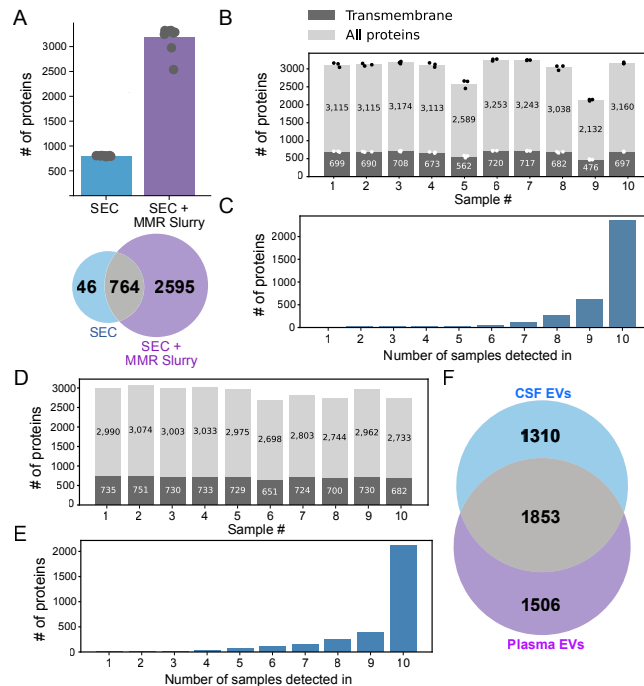


Figure S5: Proteomic analysis of EVs isolated by MMR Slurry from individual CSF and plasma samples

A. Number of unique proteins detected by mass spectrometry when SEC or SEC followed by MMR Slurry (using the CC 0.6 condition) were used to isolate EVs from plasma (Top). Overlap between SEC and SEC followed MMR Slurry (bottom). EVs were isolated from 10 different individual plasma samples and SEC was compared to SEC followed by MMR Slurry in each of the samples. **B.** Number of total proteins and transmembrane proteins that were detected by mass spectrometry from EVs isolated by SEC + MMR Slurry from 10 different individual plasma samples (same samples as in A). Mass spectrometry was run with three replicates for each sample. **C.** Number of unique proteins detected across EVs from the 10 individual plasma samples. **D.** Number of total proteins and transmembrane proteins that were detected by mass spectrometry from EVs isolated by MMR Slurry (using the CC 2.5 condition) from 10 different individual CSF samples. **E.** Number of unique proteins detected across EVs isolated by the MMR Slurry from 10 different individual CSF samples. **F.** Overlap between the EV proteins detected in the CSF and plasma samples.

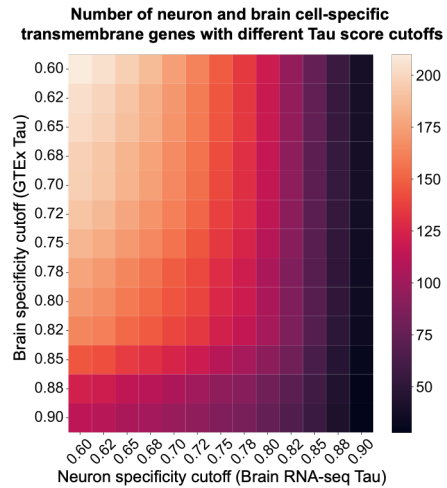


Figure S6: Heatmap of Tau Cutoffs for bulk RNA-Seq

Number of transmembrane proteins when different cutoffs are used of Tau specificity score for neurons within the brain (using Brain RNA-Seq) and the brain relative to other organs of the body (using GTEx).

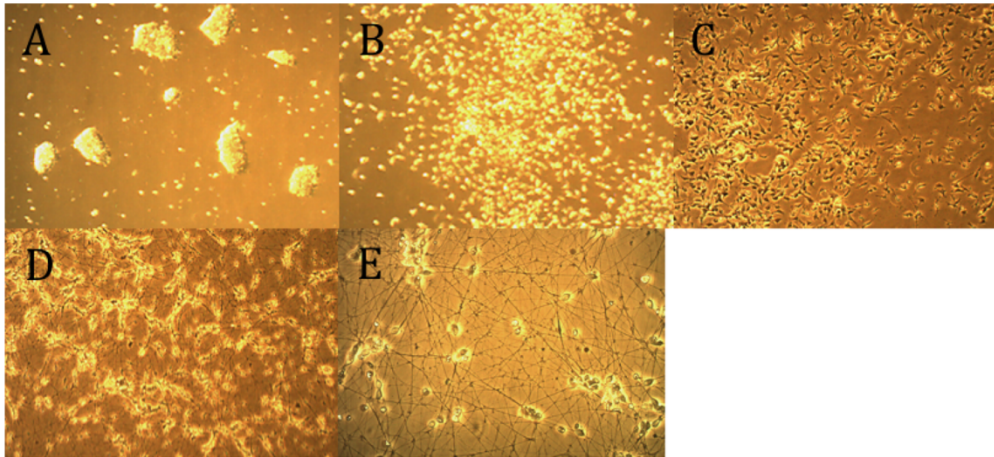


Figure S7: Differentiation of iNGN cells from iPS to neurons upon doxycycline addition

A. iPS iNGN cells before doxycycline addition (40x magnification). **B.** iPS iNGN cells 1 day after doxycycline addition (40x magnification). **C.** iPS iNGN cells 2 days after doxycycline addition (40x magnification). **D.** iPS iNGN cells 4 days after doxycycline addition (40x magnification) **E.** iNGN cells 5 days after doxycycline addition (200x magnification).

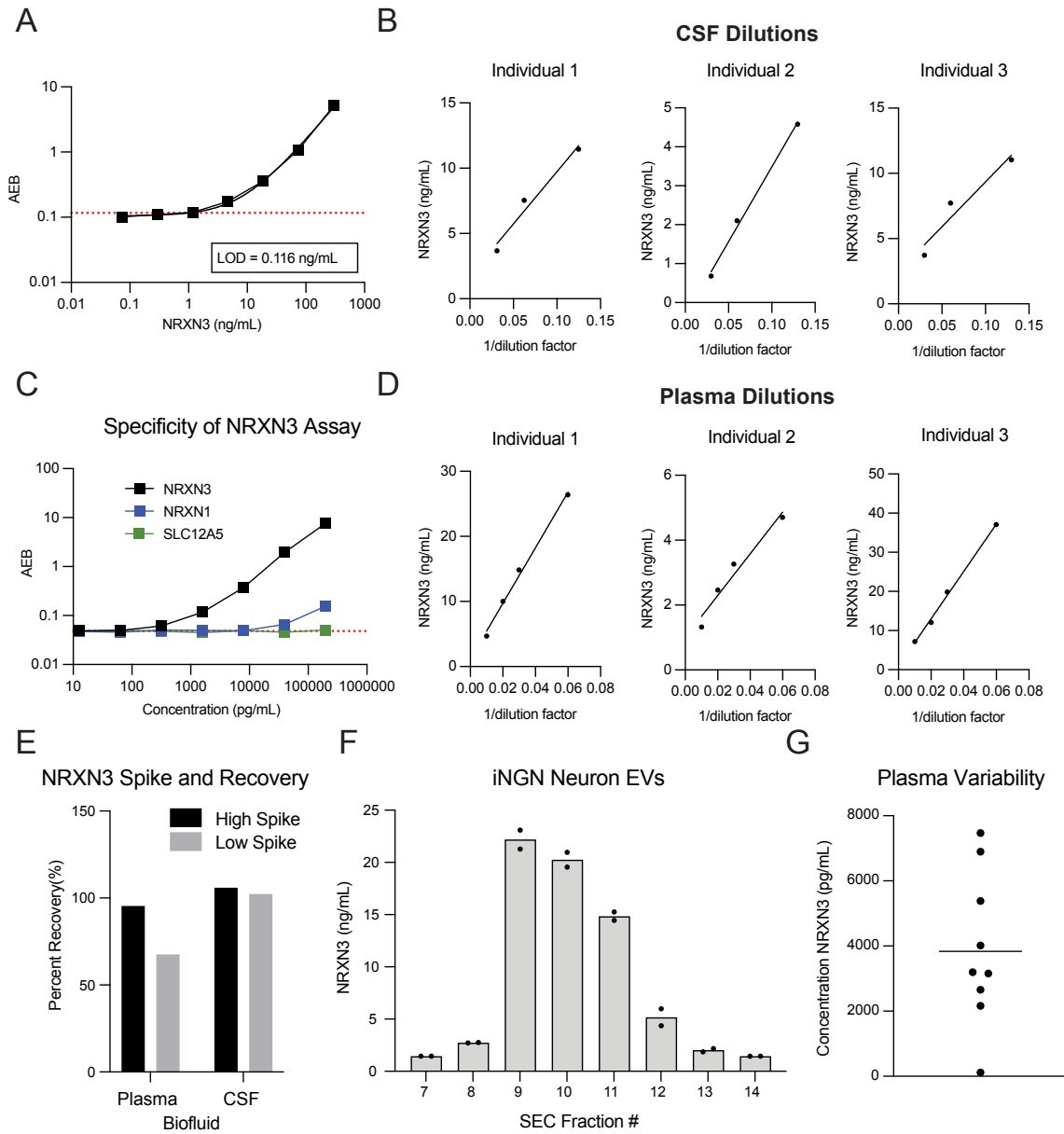


Figure S8: Development of Simoa assay for NRXN3

Simoa measurement of calibration curve with purified NRXN3 protein. Limit of detection (LOD) was determined to be 0.116 ng/mL. **B.** CSF dilutions from three individuals to determine dilution linearity of endogenous NRXN3 protein **C.** Calibration curve with NRXN3 (black), NRXN1 (blue) or SLC12A5 (green) purified protein standard to assess the specificity of the NRXN3 Simoa assay for NRXN3. **D.** Plasma dilutions from three individuals to determine dilution linearity of NRXN3 in human plasma. **E.** High (500ng) and low (50ng) spike of purified protein NRXN3 into plasma and CSF, measuring percent recovery relative to spike into diluent. **F.** Simoa measurement of SEC fractions from EVs enriched from the cell culture media of iNGN neurons by differential ultracentrifugation. **G.** Human pooled plasma variability of endogenous NRXN3 across n= 9 plasma pools. Simoa measurements were all performed in duplicate.

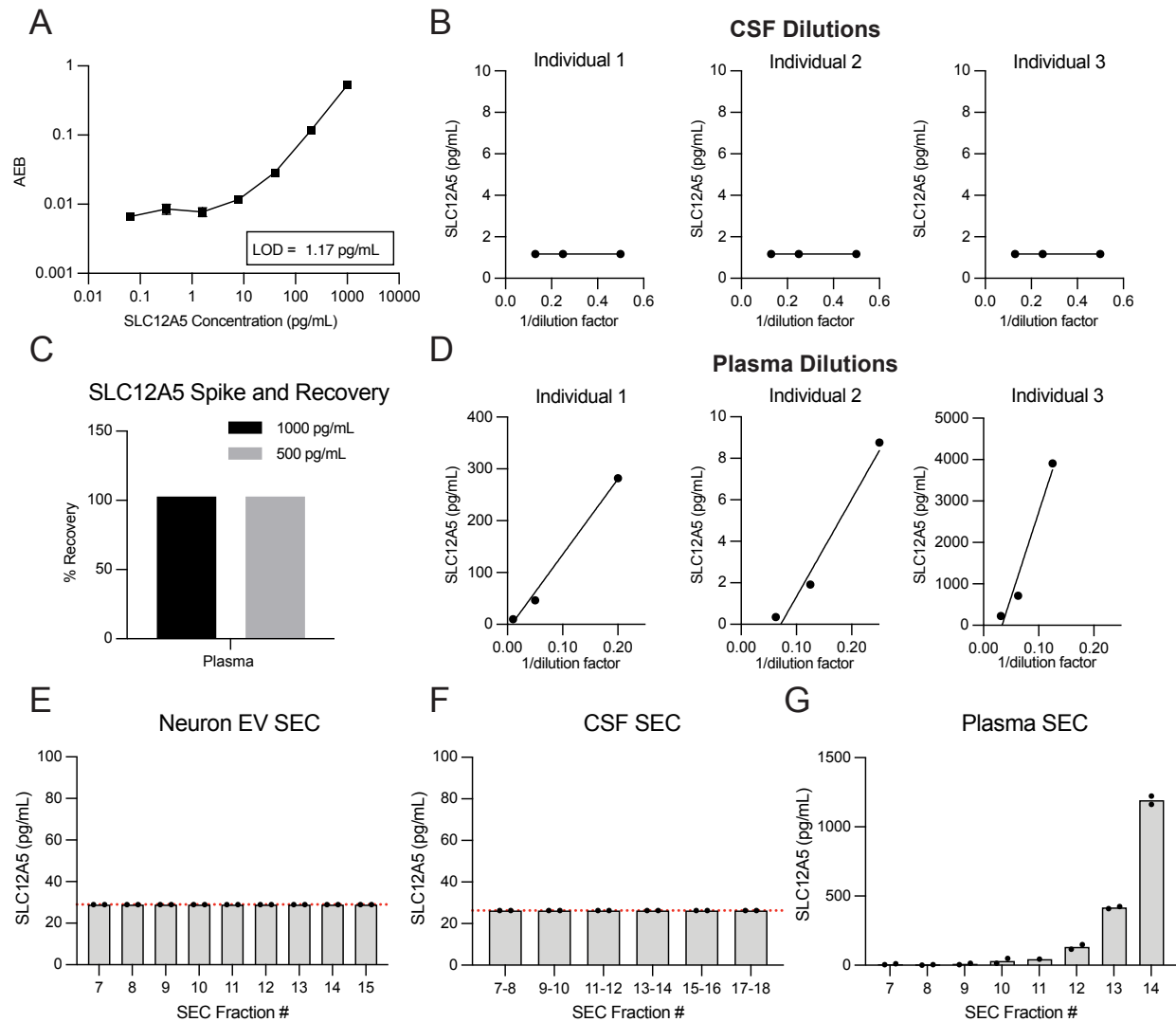


Figure S9: Development of Simoa assay for SLC12A5

A. Simoa measurement of calibration curve with purified SLC12A5 protein. Limit of detection (LOD) was determined to be 1.17 pg/mL. **B.** CSF dilutions from three individuals (all below LOD). **C.** High (1000pg) and low (500pg) spike of purified SLC12A5 protein into plasma measuring percent recovery. **D.** Plasma dilutions from three individuals. **E.** Simoa measurement of SEC fractions from EVs enriched from the cell culture media of iNGN neurons by differential ultracentrifugation (all measurements below LOD). **F.** Simoa measurement of SEC fractions from CSF (all measurements below LOD). **G.** Simoa measurement of SEC fractions from 1mL plasma. Simoa measurements were all performed in duplicate.

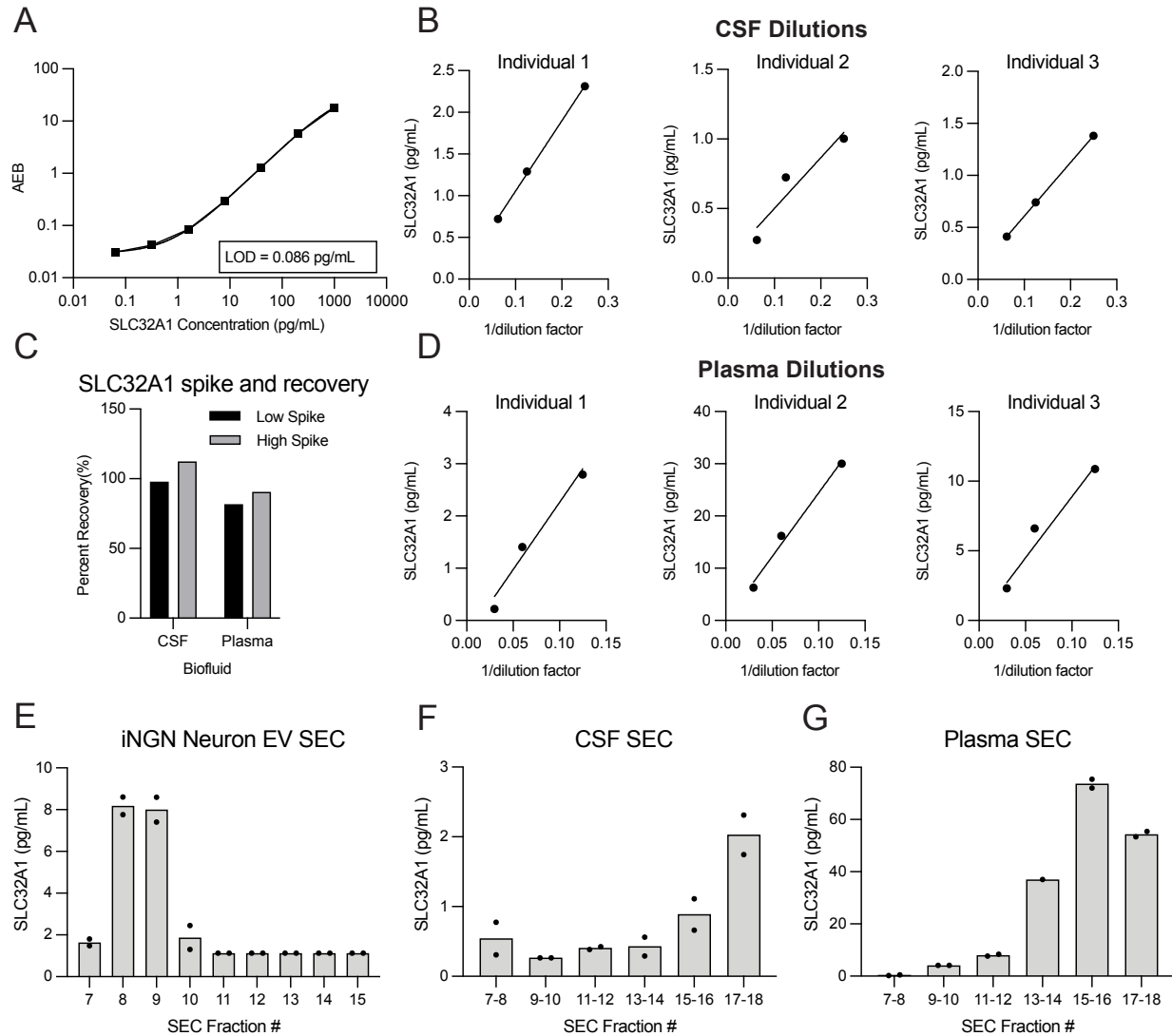


Figure S10: Development of Simoa assay for SLC32A1

SLC32A1 simoa assay validation using: **A.** Calibration curve with purified SLC32A1 protein. Limit of detection (LOD) was determined to be 0.086 pg/mL. **B.** CSF dilutions from three individuals to confirm dilution linearity of endogenous protein. **C.** High (500pg) and low (50pg) spike of purified SLC32A1 protein into plasma or CSF and measuring percent recovery compared to protein spike in diluent. **D.** Plasma dilutions from three individuals to confirm dilution linearity of endogenous SLC32A1. **E.** Simoa measurement of SEC fractions from EVs enriched from the cell culture media of iNGN neurons by differential ultracentrifugation. **F.** Simoa measurement of SEC fractions from plasma. **G.** Simoa measurement of SEC fractions from CSF. Simoa measurements were all performed in duplicate.

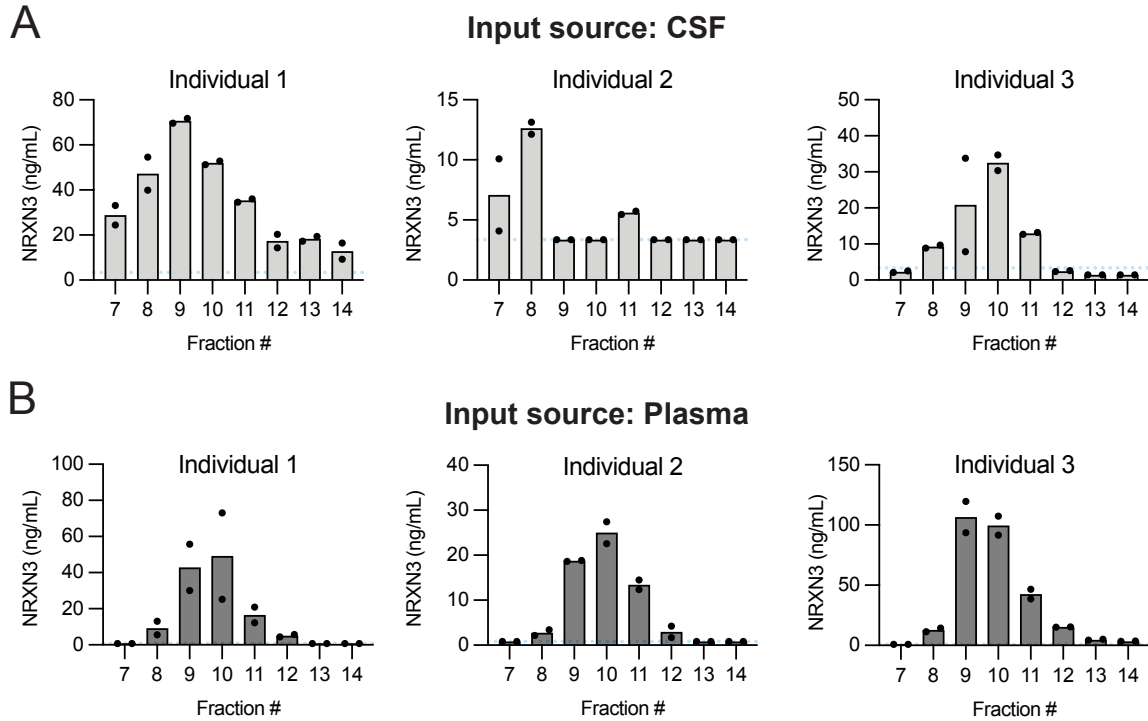


Figure S11: Simoa measurement of NRXN3 in SEC fractions across individual CSF and plasma

Simoa measurement of NRXN3 in SEC fractions 7-14 from three individual human 1mL CSF samples. Limit of detection shown in blue. B. Simoa measurements of NRXN3 in SEC fractions 7-14 from three individual 1 mL human plasma samples. Simoa measurements were performed in duplicate.

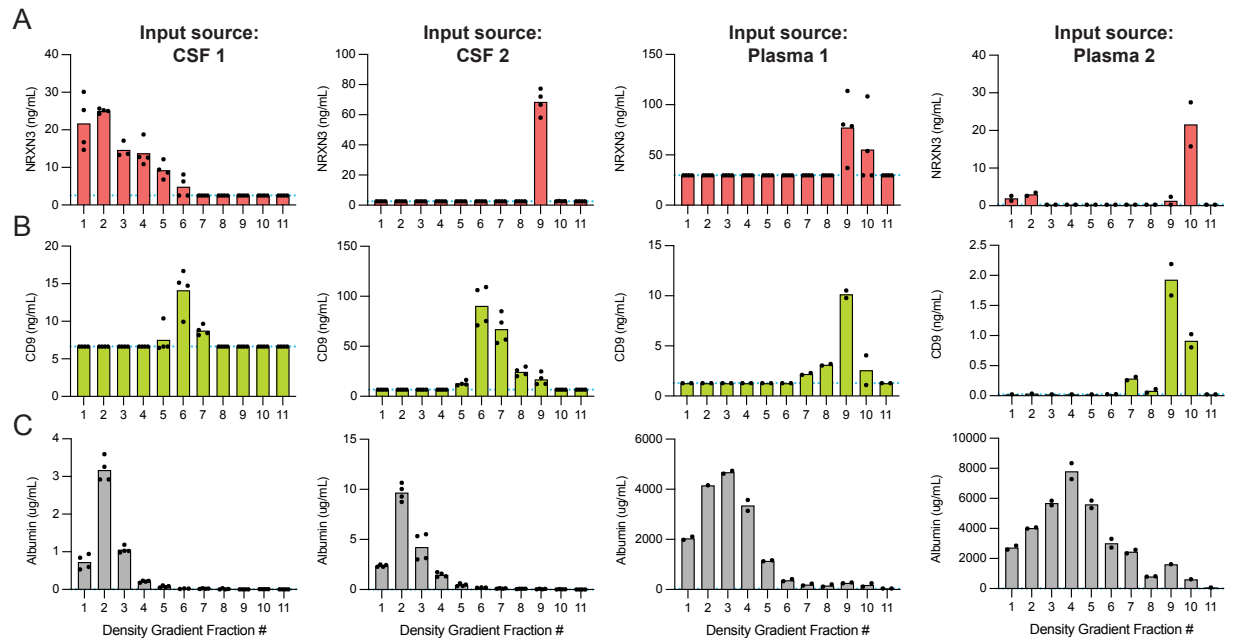


Figure S12: Simoa measurement of NRXN3 in pooled CSF and plasma fractionated by density gradient centrifugation

Density gradient centrifugation (DGC) fractions for two different pooled CSF samples (first and second column) and two different pooled plasma samples (third and fourth column) were analyzed by Simoa for: **A.** NRXN3 **B.** CD9 and **C.** albumin. Simoa measurements were all performed in duplicate. Dotted blue line represents limit of detection of Simoa assay.

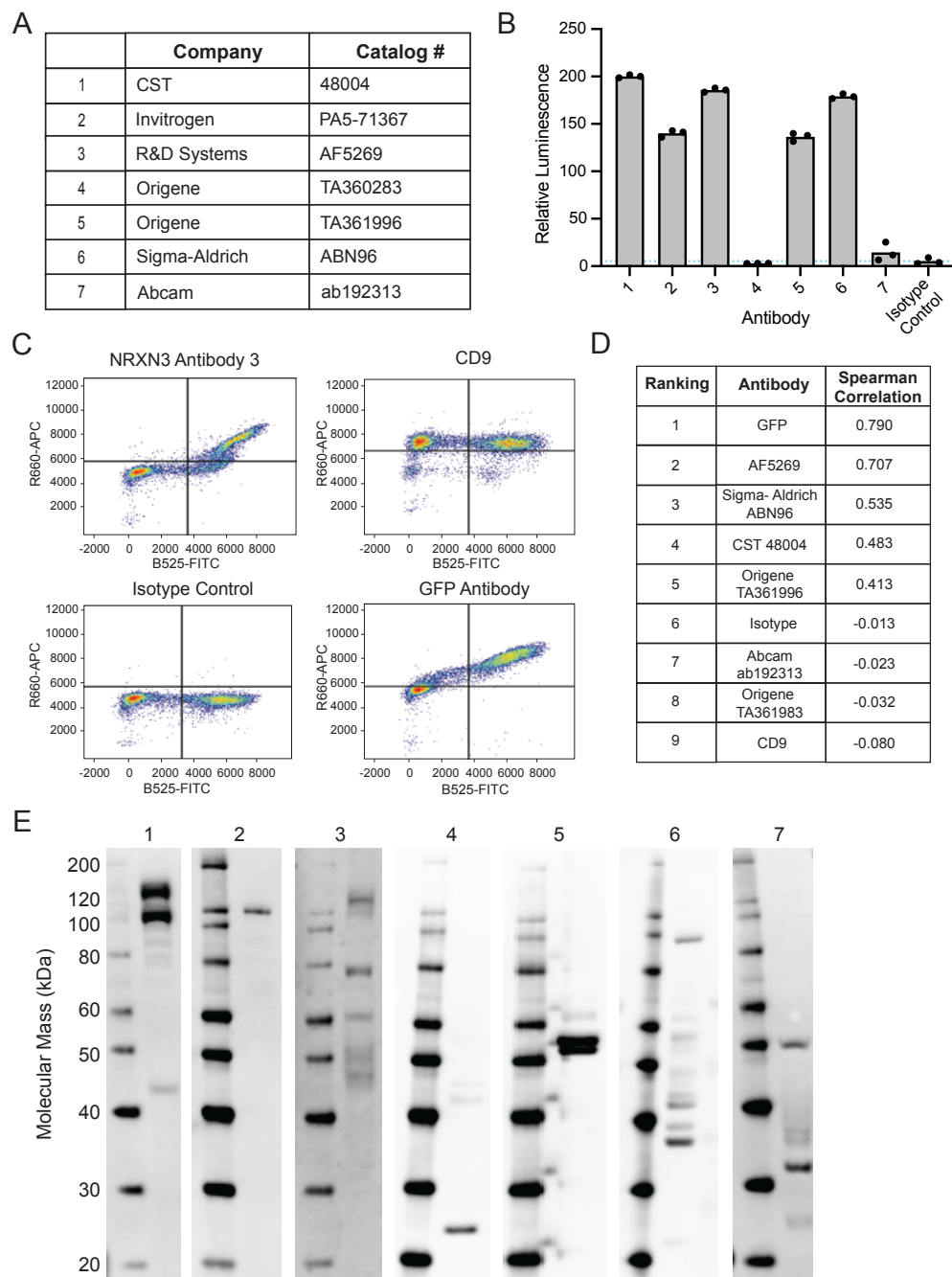


Figure S13: Comparison of commercially available NRXN3 antibodies

A. Table of commercially available NRXN3 antibodies that were using indirect ELISA, flow cytometry, and western blot. **B.** Indirect ELISA comparing 7 antibodies binding to recombinant NRXN3 protein standard and measured by chemiluminescence. Isotype control levels represented by dotted blue line. All conditions were measured in triplicate. **C.** Representative flow cytometry data of GFP-NRXN3 transfected cells stained by different NRXN3 antibodies followed by fluorescently-labeled secondary antibody. **D.** Table summarizing the flow cytometry data quantifying the correlation between different NRXN3 antibody staining and GFP-tagged NRXN3 levels. **E.** Western blots of human brain lysate using different NRXN3 antibodies.

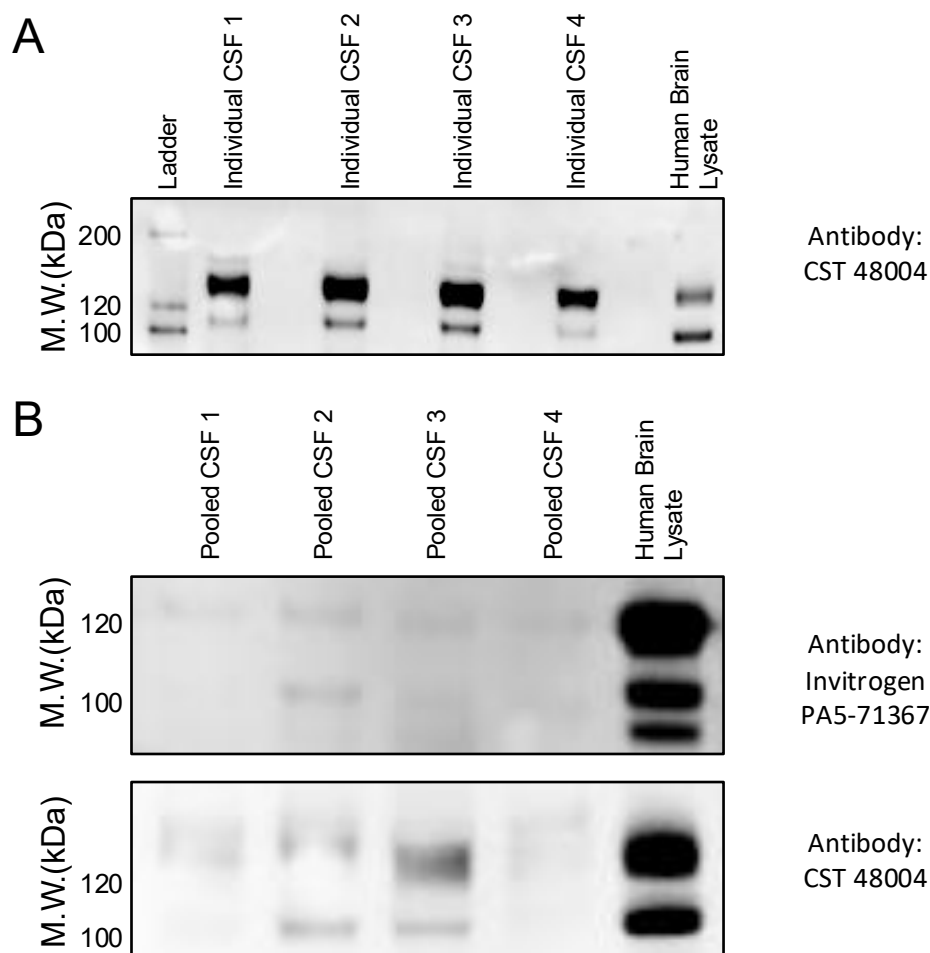


Figure S14: Western blot of NRXN3 in CSF isolated by MMR Slurry

A. Western blot of NRXN3 in four individual CSF samples after MMR slurry purification with 2.5 μ g protein: μ L resin. Human brain lysate is loaded as a positive control. **B.** Western blot of NRXN3 in four pooled CSF using 2 different NRXN3 antibodies (CST #48004, Invitrogen PA5-71367) to validate the correct band size of NRXN3.

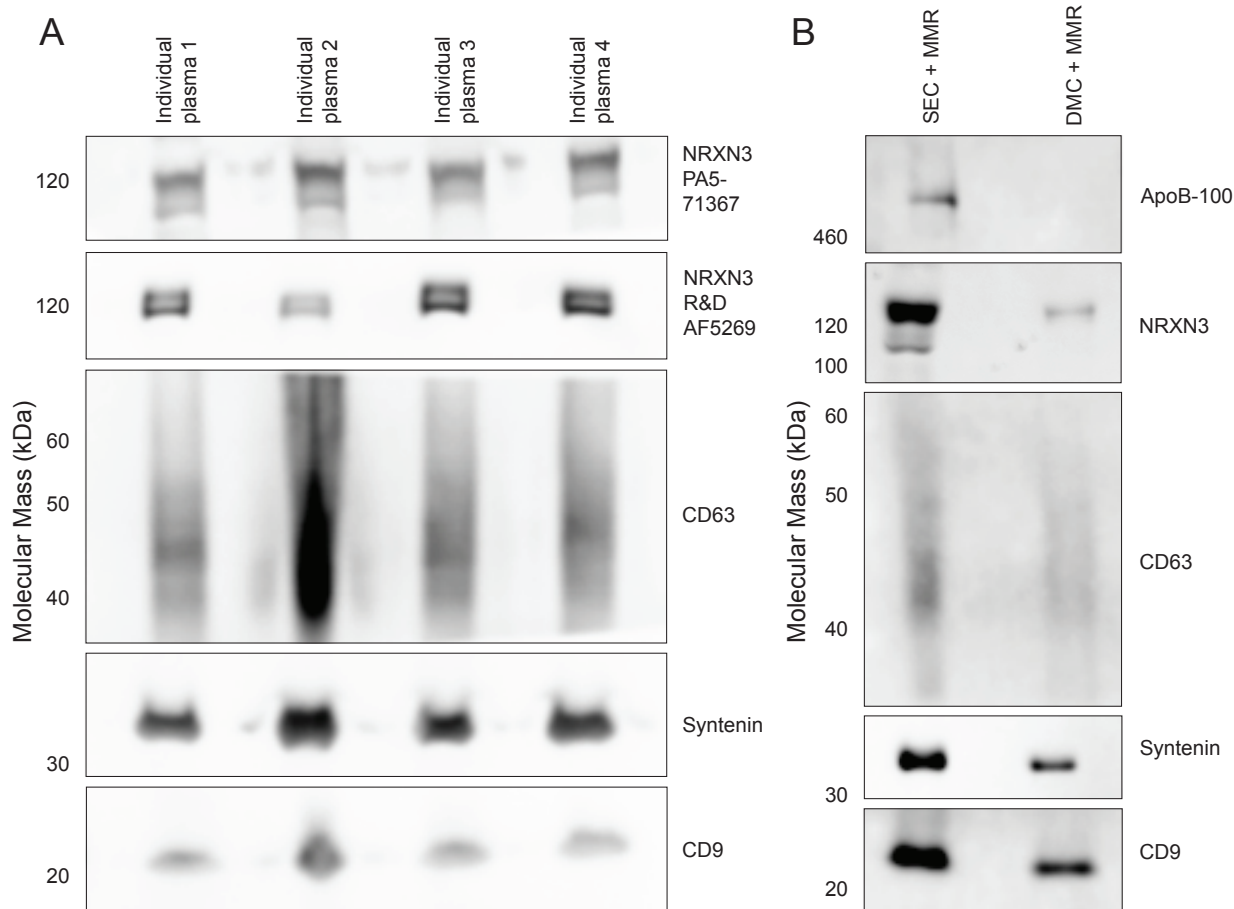


Figure S15: Western blot of NRXN3 and other proteins in plasma isolated by MMR Slurry
A. Western blot of NRXN3 in four individual plasma samples after MMR slurry purification with 2.5 μg protein: μL resin. Comparison of two commercially available antibodies and validation of EV marker presence with CD63, Syntenin, and CD9. **B.** Western blot of plasma purified by SEC + MMR or DMC+ MMR with 2.5 μg protein: μL resin for each MMR condition. Top blot shows ApoB-100 depletion to undetectable levels with DMC+MMR. NRXN3 is still present following both purification methods as are EV markers CD63, Syntenin, and CD9.

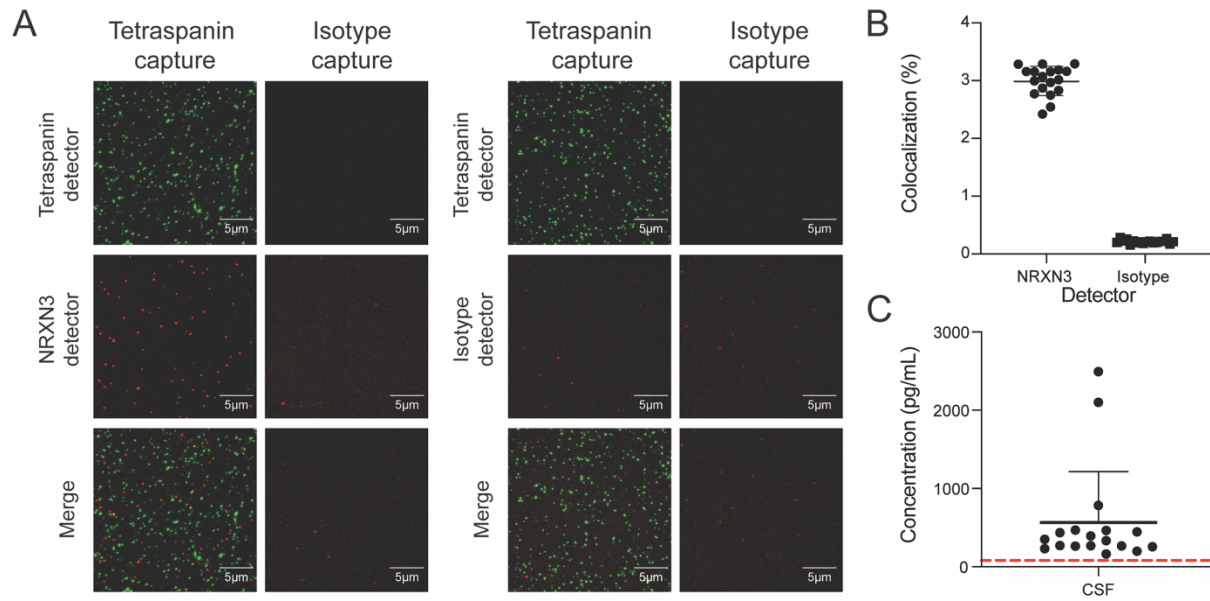


Figure S16: Imaging of NRXN3 on EVs from CSF

EVs isolated by SEC from 18 individual CSF samples were added to coverslips coated with anti-tetraspanin (combination of CD9, CD63, and CD81) or isotype control antibodies and then incubated with either fluorescently labeled (CF647) anti-NRXN3 or isotype control antibodies. For co-localization analysis, a second set of fluorescently-labeled (CF568) anti-tetraspanin (combination of CD9, CD63, and CD81) detector antibodies were added. **A.** Representative fields from all combinations of capture and detector antibodies. The bottom row is a composite of the two channels imaged. **B.** Co-localization of tetraspanin detector with NRXN3 or isotype control detector antibodies across 18 samples. **C.** NRXN3 measurement by Simoa from same set of CSF EV samples. Simoa measurements were all performed in duplicate. Error bars represent standard deviation of the mean. Red dotted line indicates limit of detection of assay.

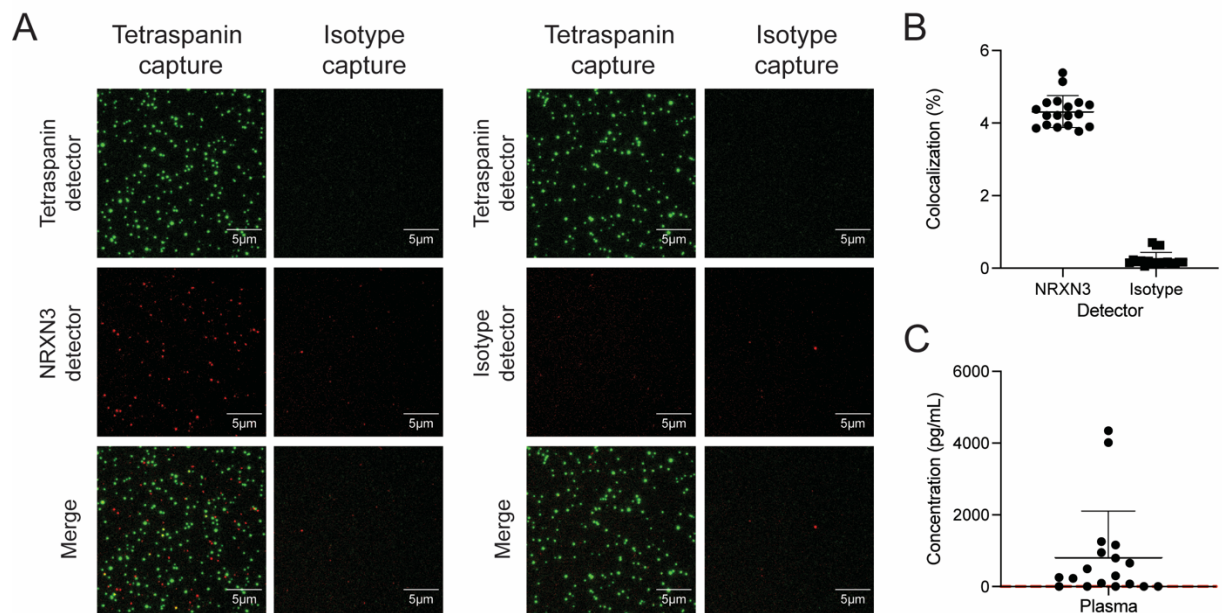


Figure S17: Imaging of NRXN3 on EVs from plasma

EVs isolated by SEC from 18 individual plasma samples were added to coverslips coated with anti-tetraspanin (combination of CD9, CD63, and CD81) or isotype control antibodies and then incubated with either fluorescently labeled (CF647) anti-NRXN3 or isotype control antibodies. For co-localization analysis, a second set of fluorescently-labeled (CF568) anti-tetraspanin (combination of CD9, CD63, and CD81) detector antibodies were added. **A.** Representative fields from all combinations of capture and detector antibodies. The bottom row is a composite of the two channels imaged. **B.** Co-localization of tetraspanin detector with NRXN3 or isotype control detector antibodies across 18 samples. **C.** NRXN3 measurement by Simoa from same set of plasma EV samples. Simoa measurements were all performed in duplicate. Error bars represent standard deviation of the mean. Red dotted line indicates limit of detection of assay.

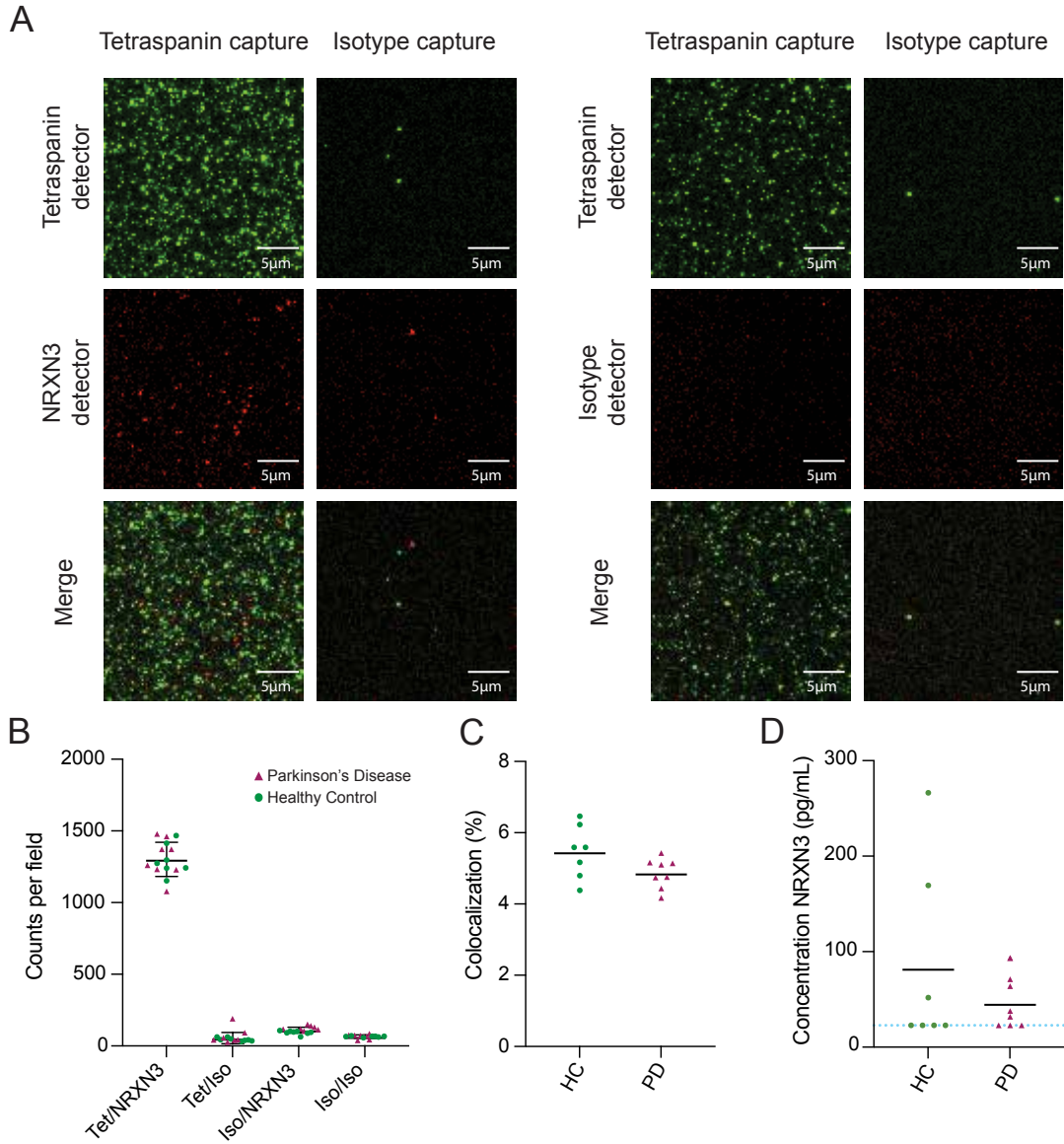


Figure S18: Imaging of NRXN3 on EVs from Parkinson's Disease patients and controls

EVs isolated by SEC from 15 individual plasma samples (8 from Parkinson's Disease patients and 7 from healthy controls) were added to coverslips coated with anti-tetraspanin (combination of CD9, CD63, and CD81) or isotype control antibodies and then incubated with either fluorescently labeled (CF647) anti-NRXN3 or isotype control antibodies. For co-localization analysis, a second set of fluorescently-labeled (CF568) anti-tetraspanin (combination of CD9, CD63, and CD81) detector antibodies were added. **A.** Representative fields from all combinations of capture and detector antibodies. The bottom row is a composite of the two channels imaged. **B.** Counts per field of different capture/detector antibody combinations. Parkinson's disease (PD) plasma is indicated in purple and healthy control (HC) is indicated in green. All conditions were measured in duplicate and then averaged. **C.** Co-localization of tetraspanin detector with NRXN3 or isotype control detector antibodies across samples. **D.** NRXN3 measurement by Simoa from same set of plasma EV samples. Simoa measurements were all performed in duplicate. Dotted line indicates limit of detection of assay.

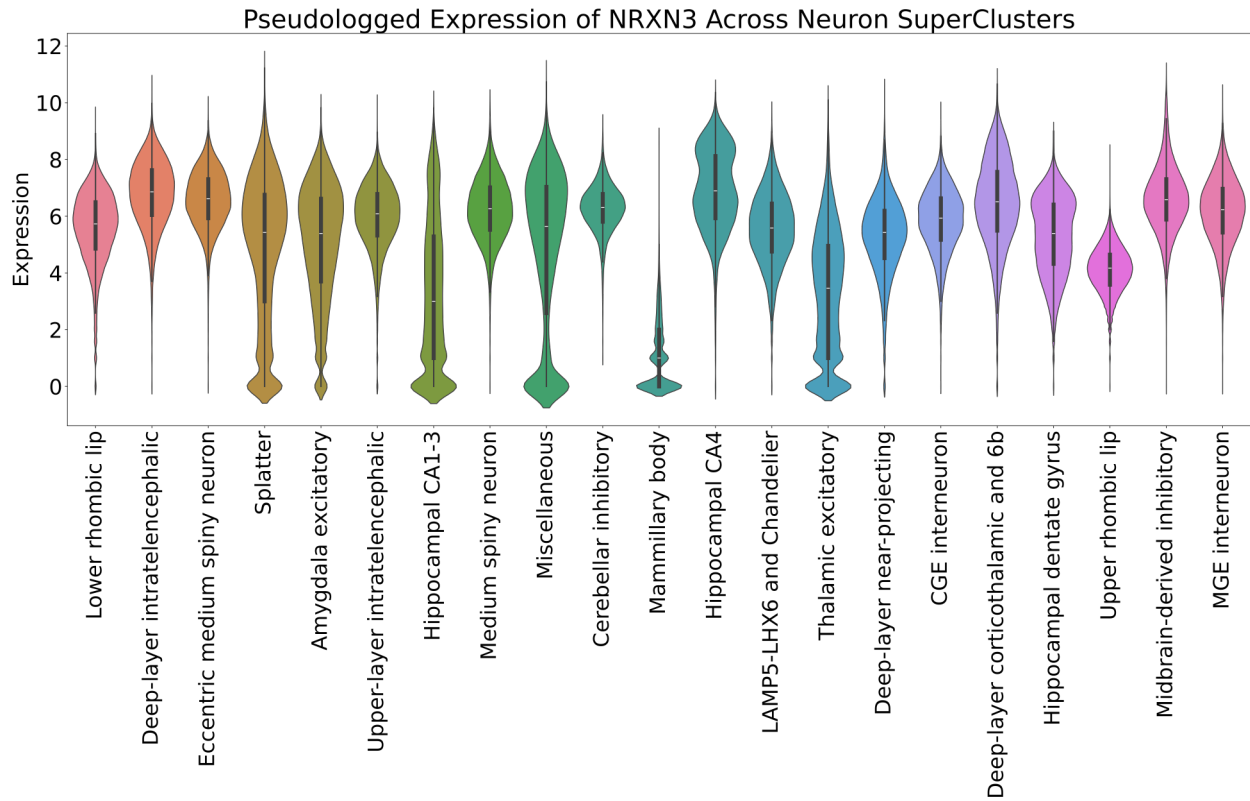


Figure S19: Expression of NRXN3 across neuronal subtypes in the human brain

NRXN3 expression in single cells in the human brain across neuron cell subtypes annotated in Siletti *et al*, 2023. Expression is plotted as $\log_2(\text{UMI Counts}+1)$.

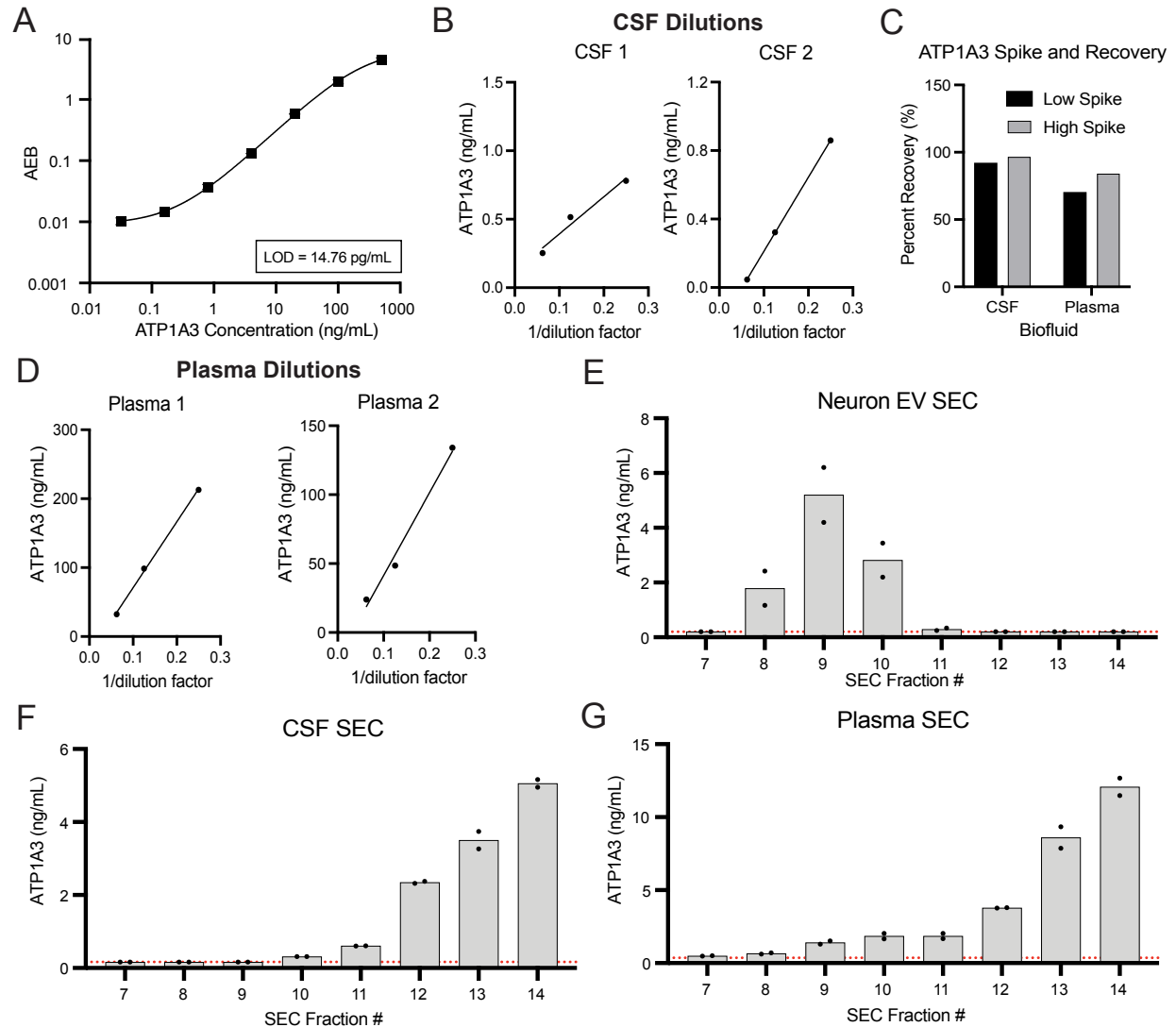


Figure S20: Development of Simoa assay for ATP1A3

A. Simoa measurement of calibration curve with purified ATP1A3 protein. Limit of detection (LOD) was determined to be 14.76 pg/mL. **B.** CSF dilutions from two individuals. **C.** High (50ng) and low (10ng) spike of purified ATP1A3 protein into plasma and CSF measuring percent recovery. **D.** Plasma dilutions from two individuals. **E.** Simoa measurement of SEC fractions from EVs enriched from the cell culture media of iNGN neurons by differential ultracentrifugation. **F.** Simoa measurement of SEC fractions from CSF. **G.** Simoa measurement of SEC fractions from 1mL plasma. Simoa measurements were all performed in duplicate.

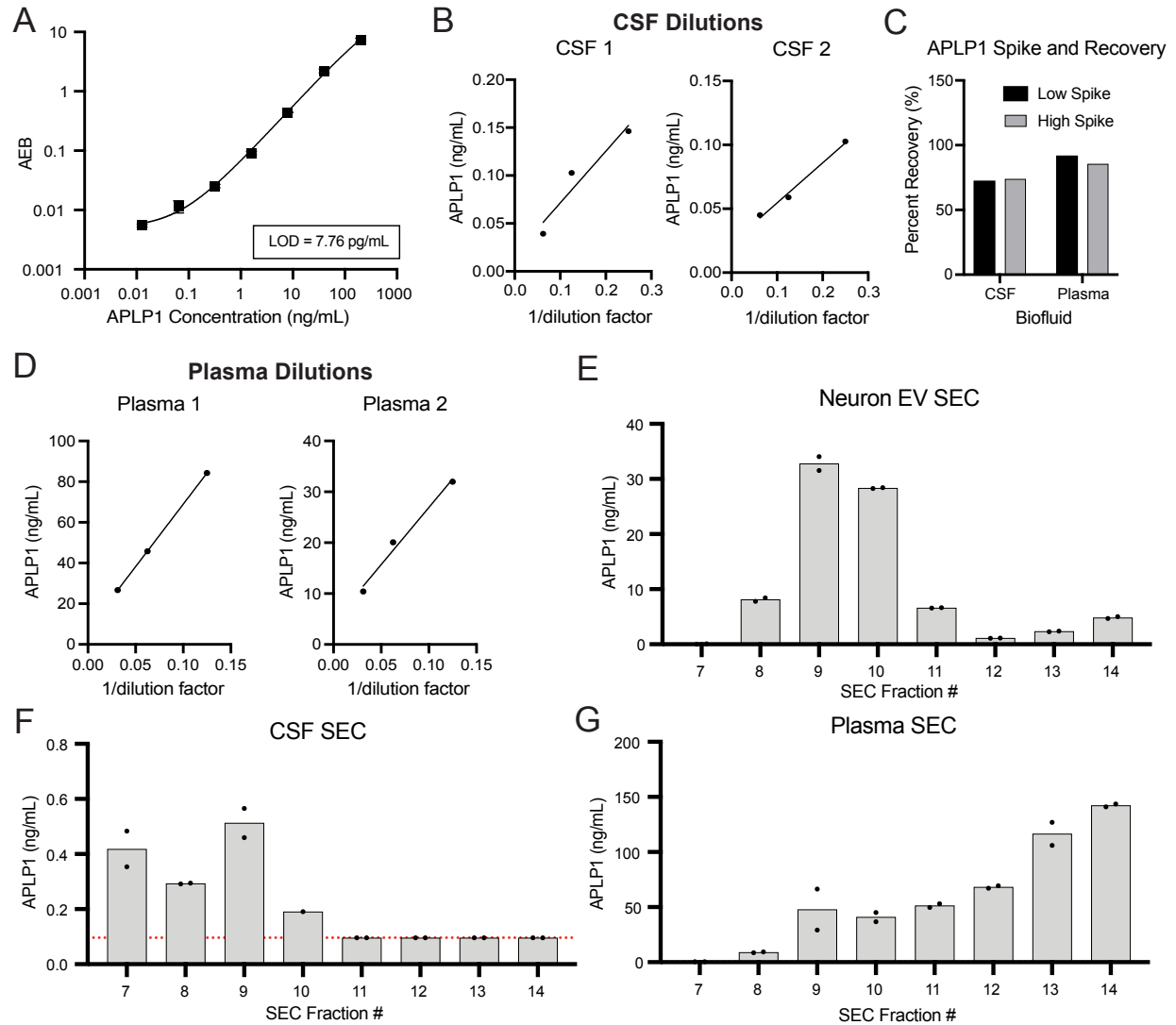


Figure S21: Development of Simoa assay for APLP1

A. Simoa measurement of calibration curve with purified APLP1 protein. Limit of detection (LOD) was determined to be 7.76 pg/mL. **B.** CSF dilutions from two pooled samples. **C.** High (50ng) and low (10ng) spike of purified APLP1 protein into plasma and CSF measuring percent recovery. **D.** Plasma dilutions from two individuals. **E.** Simoa measurement of SEC fractions from EVs enriched from the cell culture media of iNGN neurons by differential ultracentrifugation. **F.** Simoa measurement of SEC fractions from CSF. **G.** Simoa measurement of SEC fractions from 1mL plasma. Simoa measurements were all performed in duplicate.

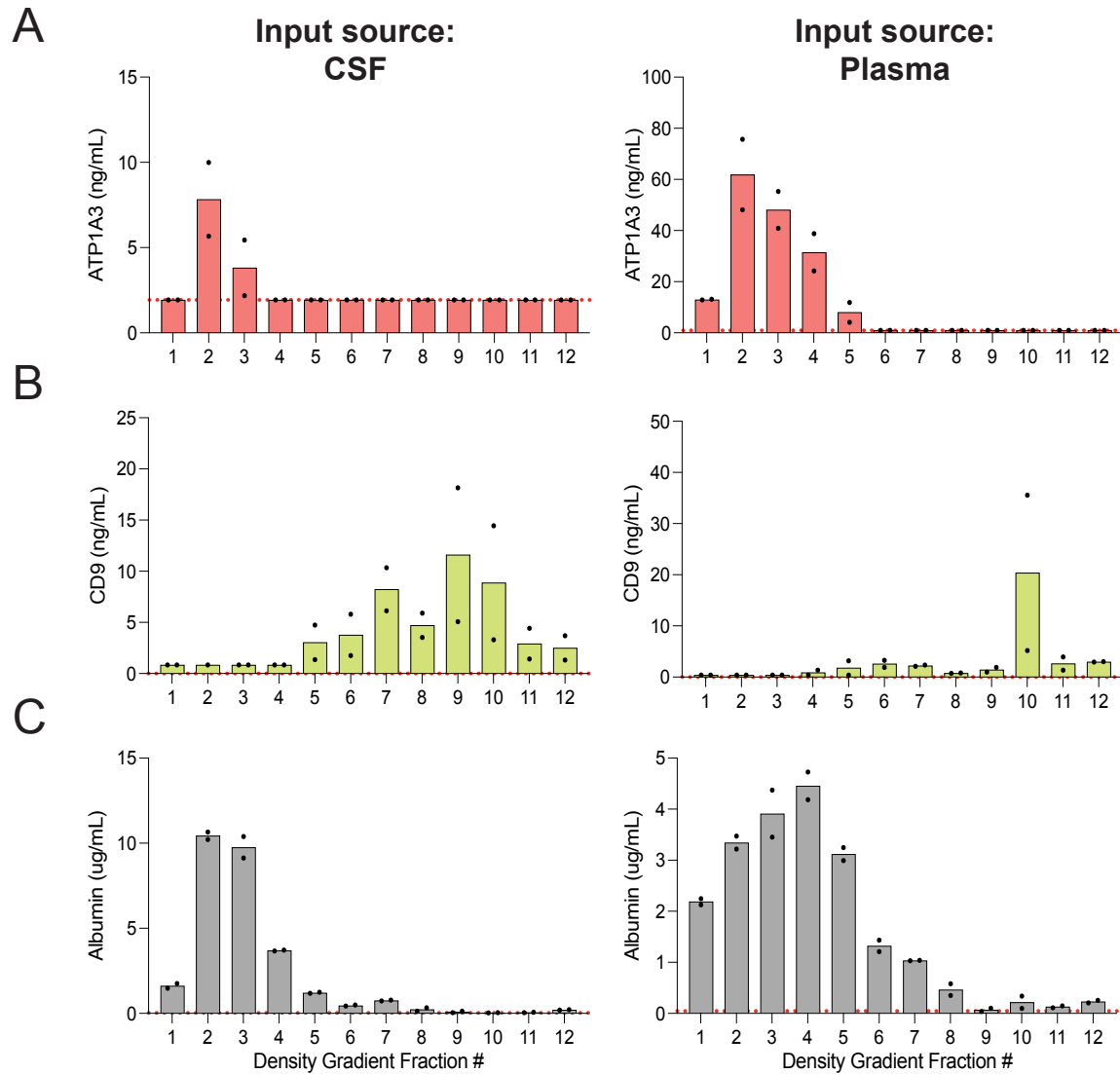


Figure S22: Simoa measurement of ATP1A3 in pooled CSF and plasma fractionated by density gradient centrifugation

Density gradient centrifugation (DGC) fractions for an individual CSF sample (first column) and a pooled plasma sample (second column) were analyzed by Simoa for: **A.** ATP1A3 **B.** CD9 and **C.** albumin. Simoa measurements were all performed in duplicate. Dotted red line represents limit of detection of Simoa assay.

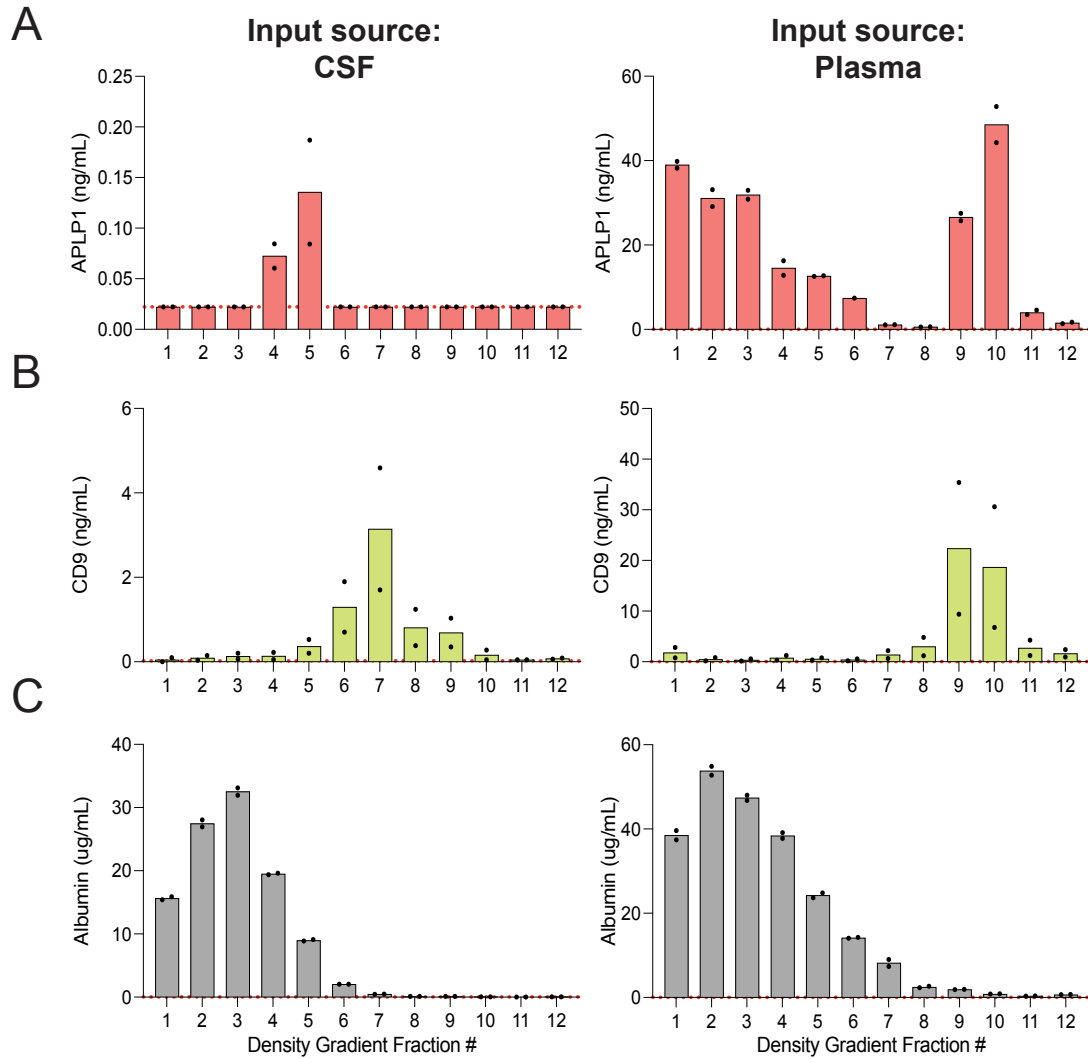


Figure S23: Simoa measurement of APLP1 in pooled CSF and plasma fractionated by density gradient centrifugation

Density gradient centrifugation (DGC) fractions for a pooled CSF sample (first column) and a pooled plasma sample (second column) were analyzed by Simoa for: **A.** APLP1 **B.** CD9 and **C.** albumin. Simoa measurements were all performed in duplicate. Dotted red line represents limit of detection of Simoa assay.

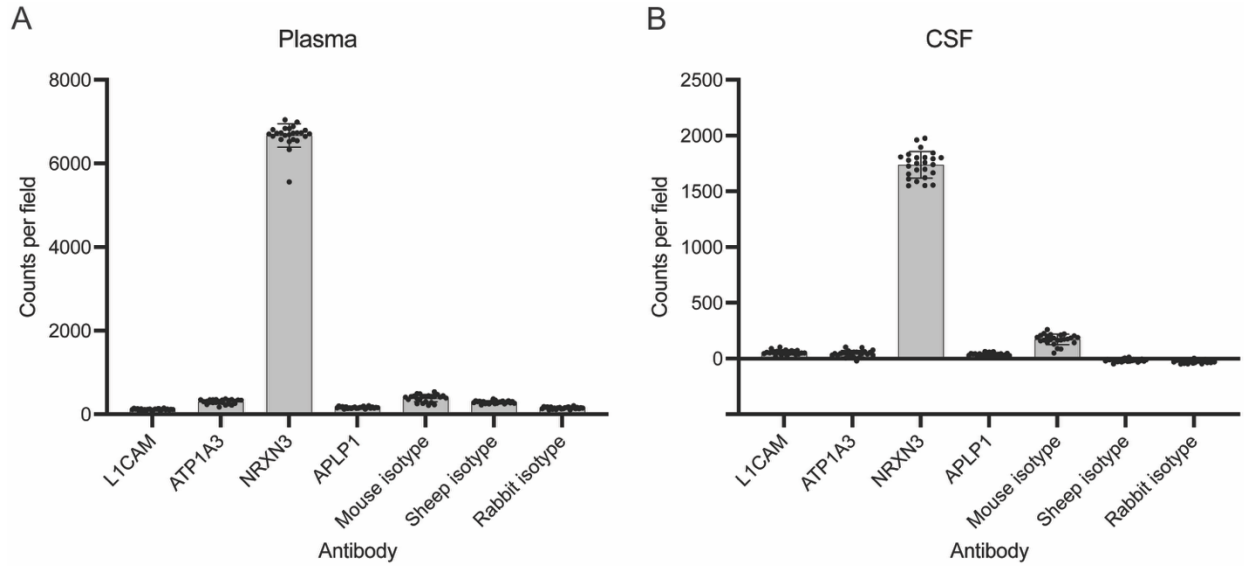


Figure S24: Imaging of EVs from CSF and plasma for candidate marker comparison

Imaging of EVs isolated from **A.** pooled plasma or **B.** pooled CSF. EVs isolated from each biofluid were captured on a glass surface coated with antibodies against CD9, CD63, and CD81. Fluorescently-conjugated detector antibodies against L1CAM, ATP1A3, APLP1, and NRXN3 or their corresponding (mouse, rabbit, sheep) isotype controls were then added to the EVs and imaged. To account for non-specific binding for each capture/detector antibody combination, signal for every sample was corrected by subtracting the non-specific signal obtained with a no sample control. Error bars represent standard deviation from the mean.

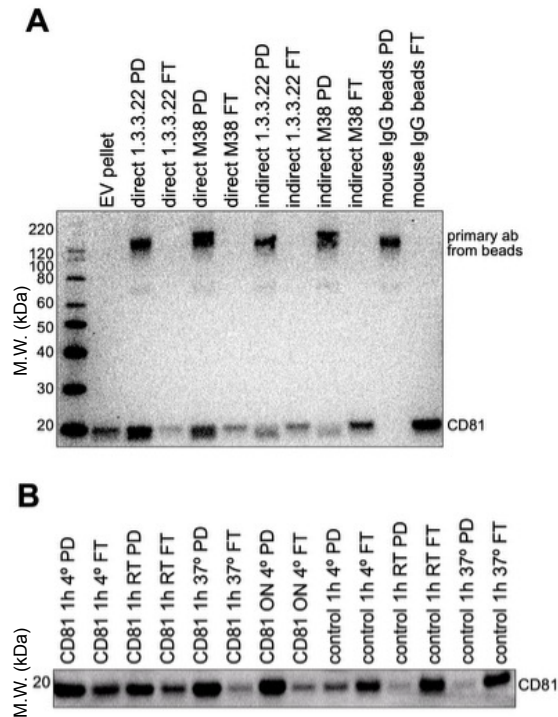


Figure S25: Optimization of CD81 immuno-isolation parameters using EVs from K562 cells

A. Western blot of CD81 after EVs isolated by differential ultracentrifugation were immuno-isolated using CD81 antibody. Direct (primary antibody conjugated to beads and then added to sample) vs. indirect (primary antibody is added to sample first and then added to beads) pulldown methods are compared for CD81 immuno-isolation using two different antibodies (clones M38 and 1.3.3.22). Note: band at 150 kDa is primary antibody coming off the beads. **B.** Western blot against CD81 after EVs were incubated with beads for either 1 hour or overnight (ON), at different temperatures (4°C, room temperature, or 37°C) using beads conjugated to either CD81 or a control non-specific antibody (against mCherry).

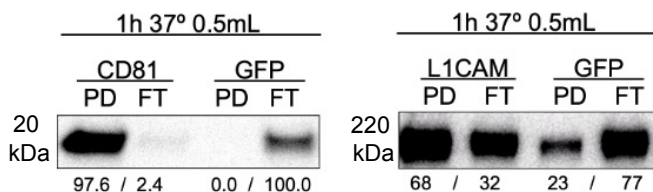


Figure S26: Optimal immuno-isolation conditions vary for different antibodies

Immuno-isolation for 1 hour at 37°C in 0.5 mL volume was performed on EVs isolated by differential ultracentrifugation from the conditioned media of K562 cells using CD81 (or control GFP antibody) and neuron EVs using L1CAM (or control GFP antibody) and corresponding Western blot was performed against CD81 or L1CAM. Intensities of bands on Western blot were quantified using ImageJ as percentage of band relative to sum of pulldown (PD) + flow-through (FT) for that condition.

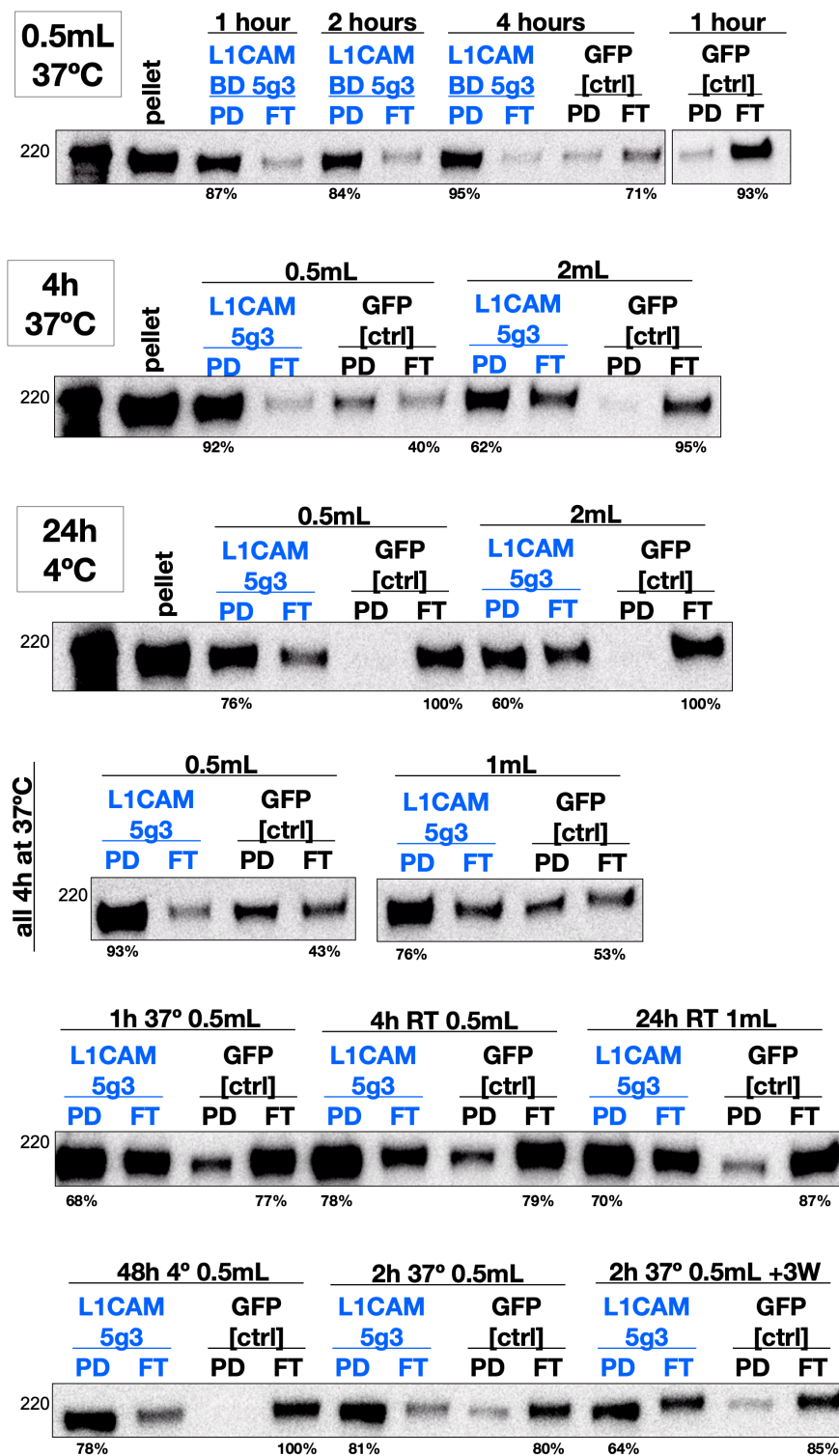


Figure S27: Optimization of conditions for iNGN neuron EV immuno-isolation

Western blot against L1CAM after iNGN neuron EVs were incubated with antibody conjugated beads (against L1CAM or GFP) for different times (1, 2, 4, or 24 hours), in different volumes (0.5 mL or 2 mL) at different temperatures (4°C or 37°C) and with different numbers of wash steps (+3W). Intensity of bands on Western blot was quantified using ImageJ as percentage of band relative to sum of pull-down (PD) + flow-through (FT) for that condition.

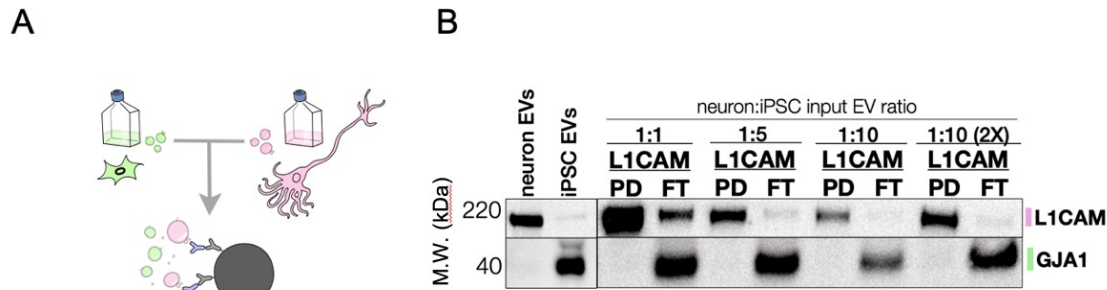


Figure S28: iNGN neuron EV immuno-isolation from iPS/iNGN EV mixture

A. Mixing EVs isolated by differential ultracentrifugation from the conditioned media of iPS cells and iPS-derived iNGN neurons allows for assessment of pulldown efficiency and purity in a more complex mixture model. L1CAM was used as we could not detect NRXN3 by Western blotting in EVs from isolated from the media of iPS-derived iNGN neurons. **B.** Western blot of L1CAM and GJA1 after L1CAM immuno-isolation demonstrates that the immuno-isolation efficiently and specifically isolates L1CAM+ neuron-derived EVs from pooled iNGN neuron EVs and iPS EVs in varying ratios (normalized relative to cell number). An iPS specific marker, GJA1, is analyzed to ensure iPS EVs are not non-specifically bound to L1CAM beads. L1CAM was found on iNGN neuron EVs by mass spectrometry and we have previously found L1CAM to be present on iNGN EV, unlike on plasma and CSF EVs, by SEC (Norman *et al*, 2021).

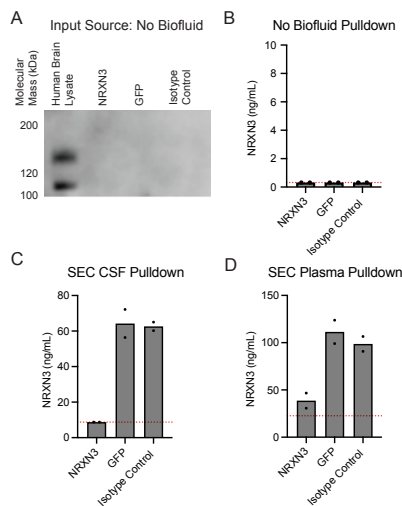
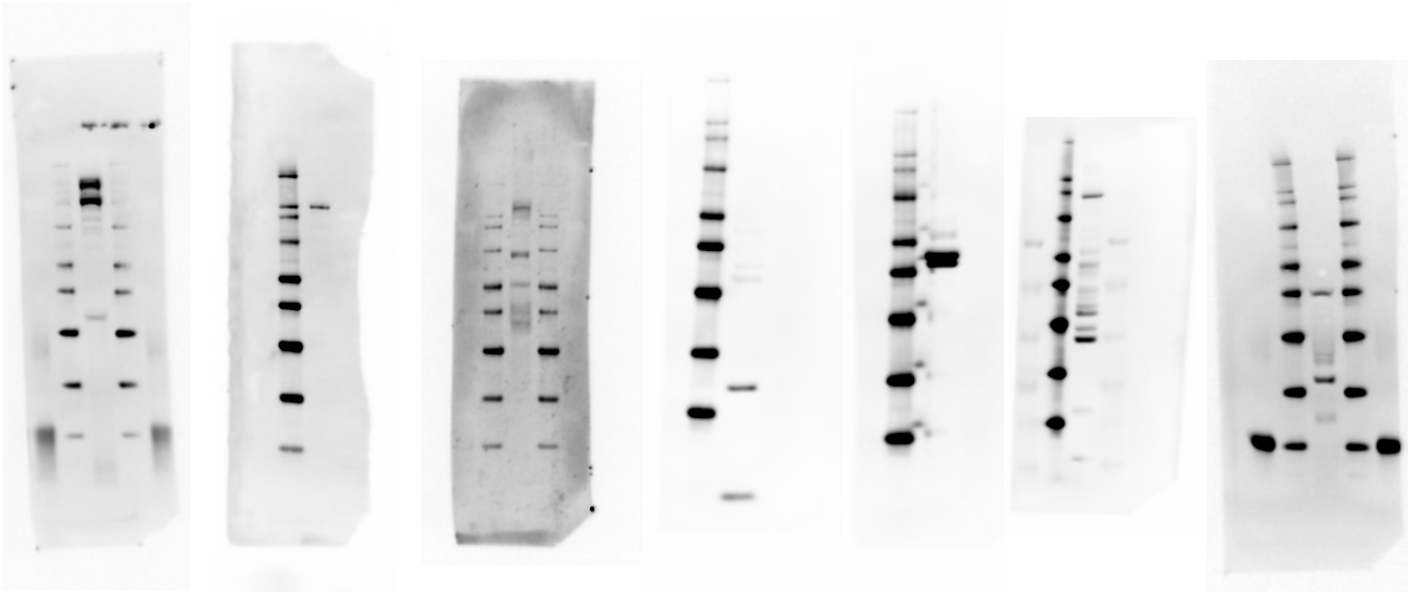


Figure S29: Immuno-depletion of NRXN3 from human CSF and plasma after SEC

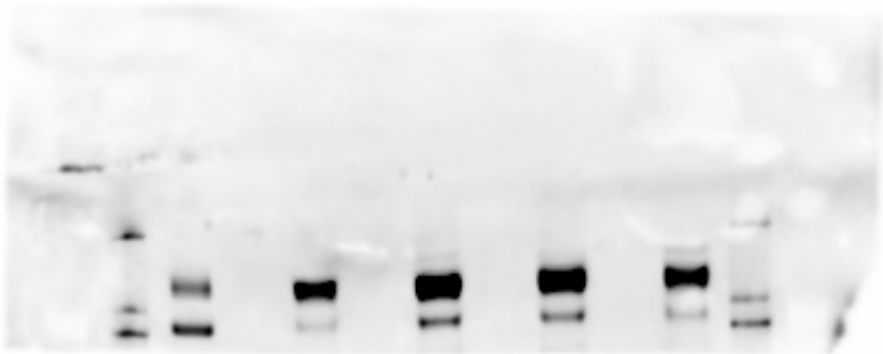
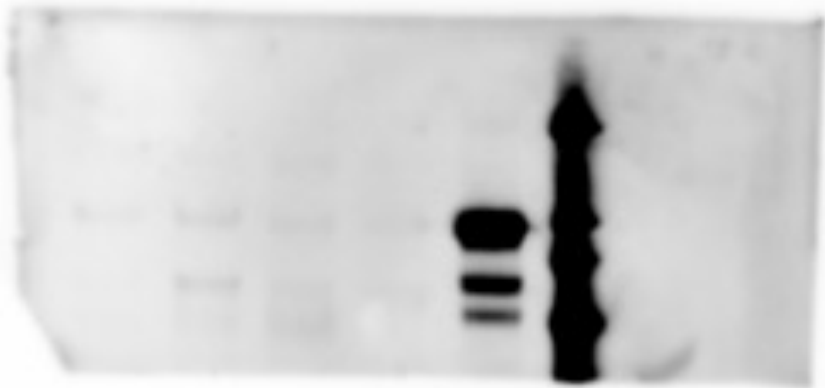
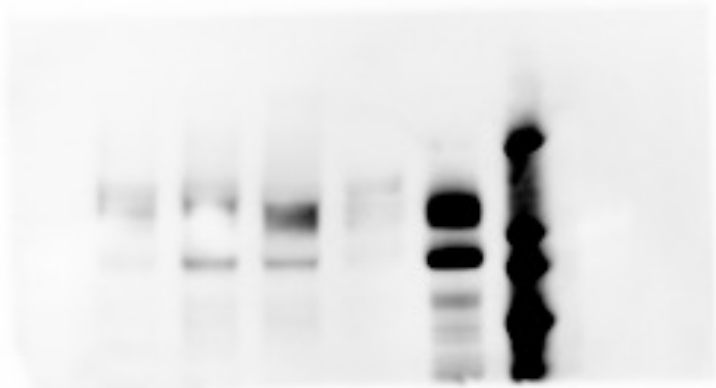
A. Western blot of NRXN3 after incubation of beads without biofluid as a negative control to show no cross-reactivity between the Western primary/secondary antibody and the pulldown antibody. **B.** Simoa measurement of NRXN3 after incubation of immunoprecipitation beads without biofluid as a negative control to show no cross-reactivity between beads with pulldown antibody and Simoa assay components. **C.** Simoa measurement of NRXN3 concentration after incubation of NRXN3 beads or control GFP/ isotype control beads with CSF fractions 7-10 collected from SEC. **D.** Simoa measurement of NRXN3 from plasma fractions 7-10 collected after SEC. Limit of detection for the assay shown in red. Simoa measurements were all performed in duplicate.

SI uncropped Western blots

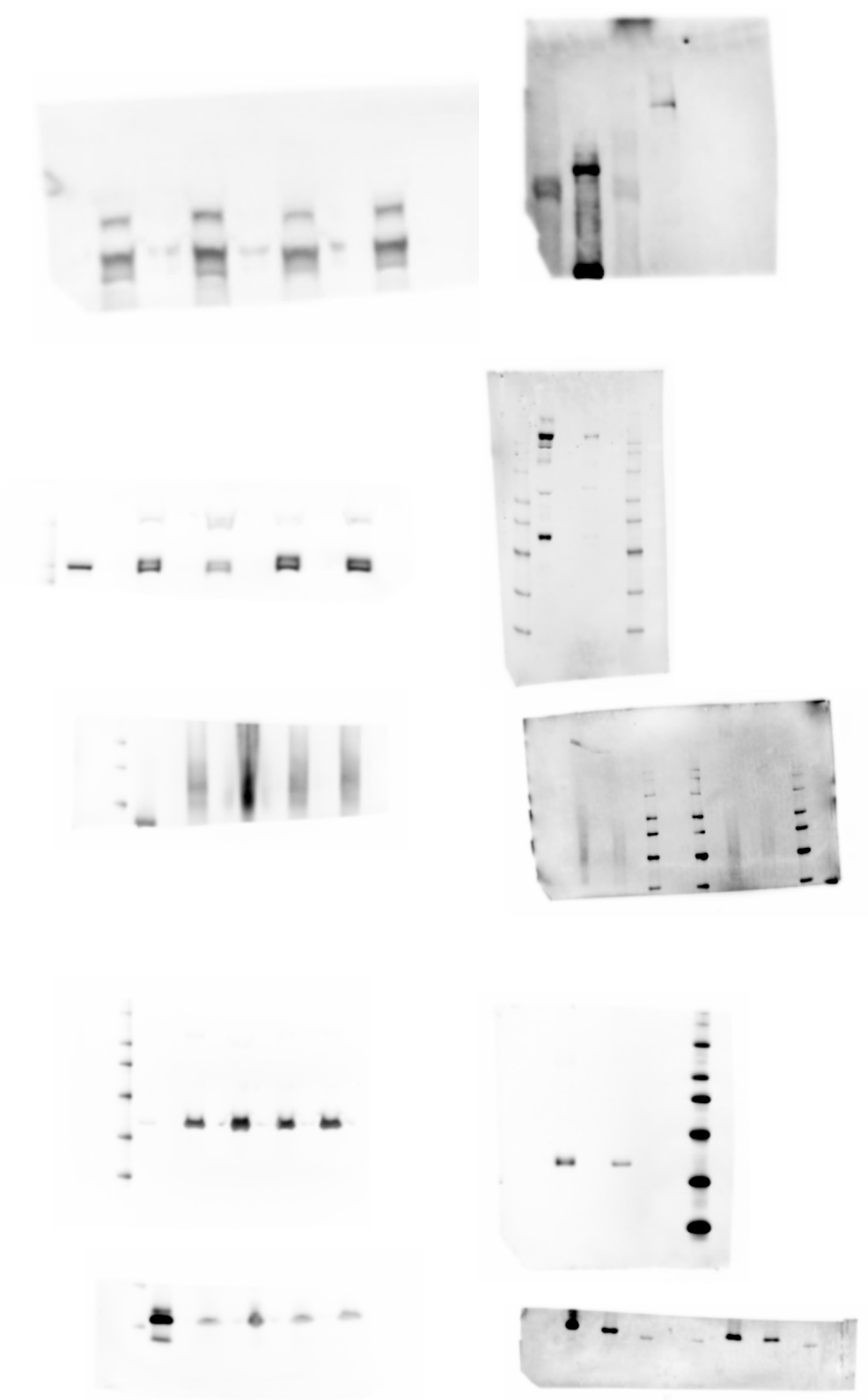
SI Figure S13: Western blots



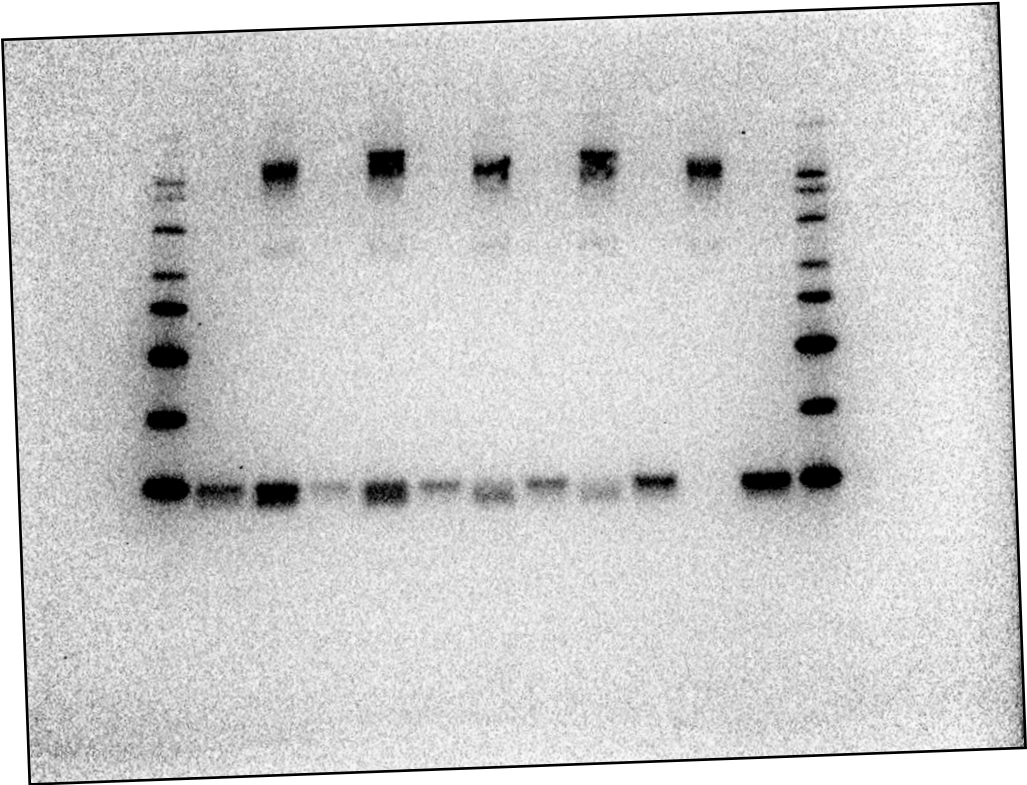
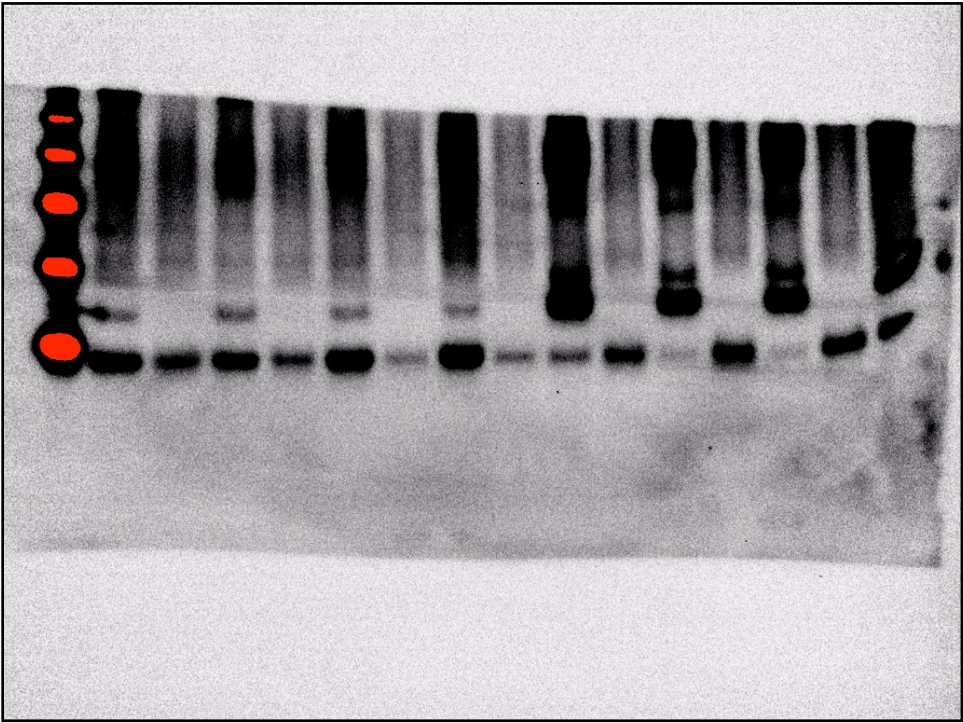
SI Figure S14: Western blots



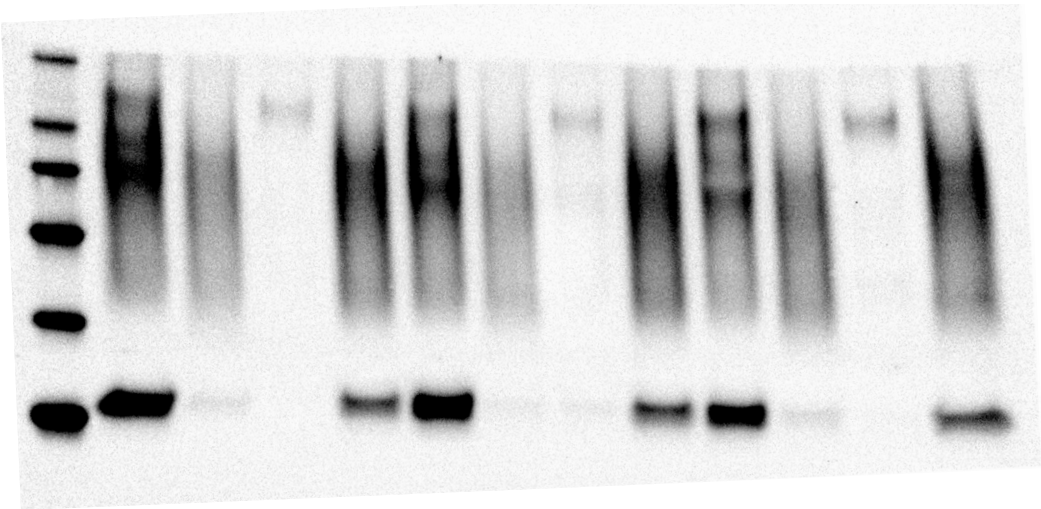
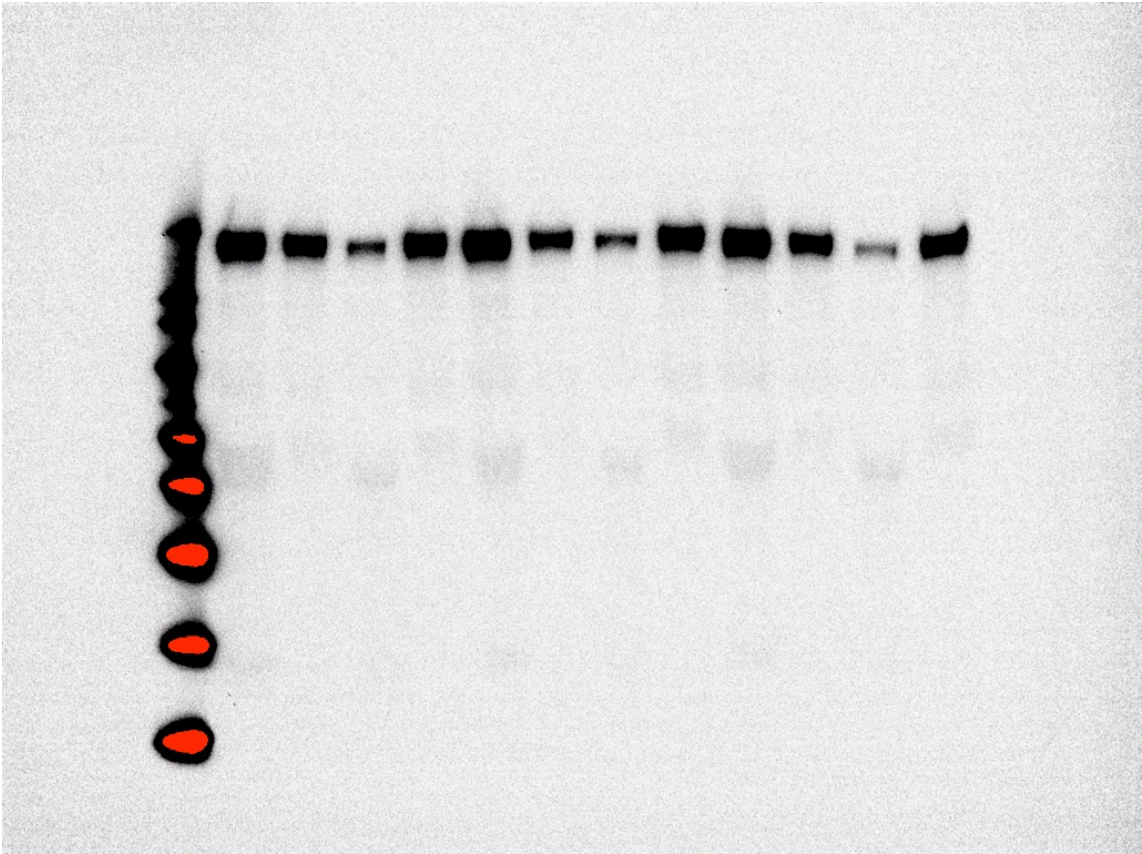
SI Figure S15: Western blots



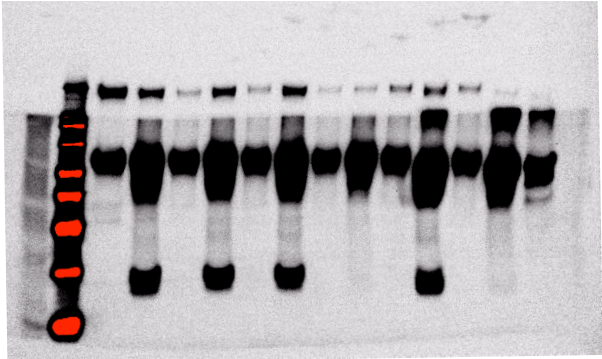
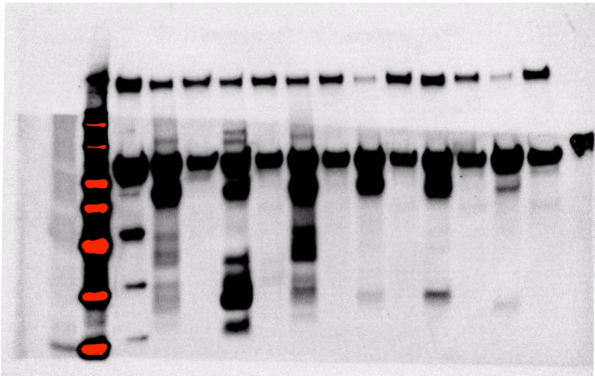
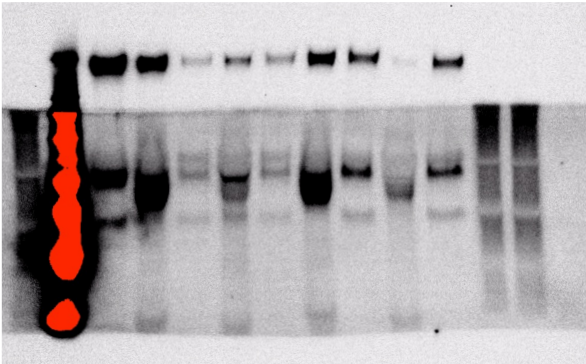
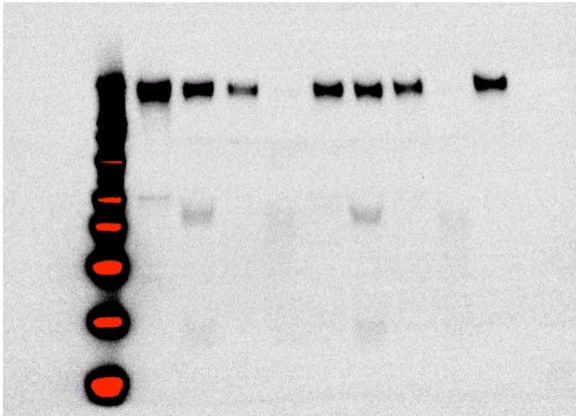
SI Figure S25: Western blots



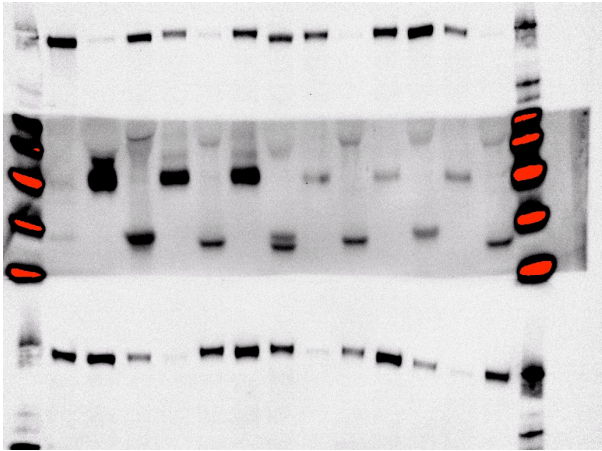
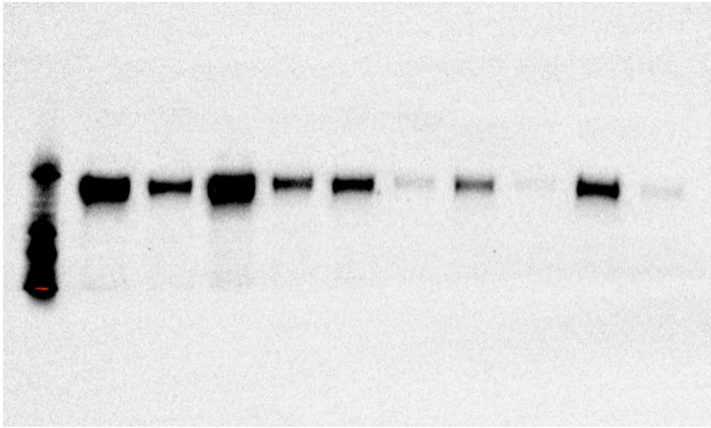
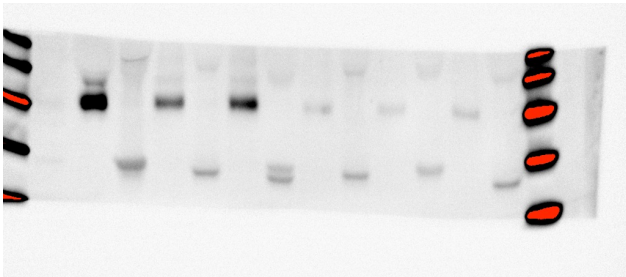
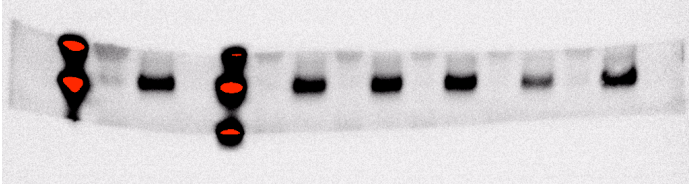
SI Figure S26: Western blots



SI Figure S27: Western blots



SI Figure S28: Western blots



SI Figure S293: Western blots

