Title: Microglia mediate contact-independent neuronal pruning via secreted Neuraminidase-3 associated with extracellular vesicles

Materials and Methods.

Cell culture

BV2 murine microglia (a kind gift from T. Wyss-Coray) were propagated in DMEM supplemented with 10% hiFBS. Microglia were maintained at a low passage number (< 10 since obtaining initial stocks) and split at or before ~ 70% confluency to avoid runaway inflammation as caused by dead or floating BV2 cells. If cells grew overconfluent, the culture was discarded. Typically, ~ 1e6 microglia were seeded in a T75 in 25 mL split every two days. Microglia were harvested using Gibco enzyme-free dissociation buffer, incubated for 5 min at room temperature, and lifted with gentle tapping and pipetting. Microglia were diluted in an equal volume of complete media, counted with a hemocytometer, pelleted by centrifugation (300 rcf, 5 min), and resuspended in complete media for subculturing.

HeLa cells (ATCC CCL-2) were propagated in DMEM supplemented with 10% hiFBS. HeLa cells were subcultured at a confluence below 80% to avoid overgrowth. Typically, HeLa cells were split 1:5 every two to three days. HeLa cells were lifted using trypsin and pelleted by centrifugation (300 rcf, 5 min), before resuspending in fresh media.

Preparation of microglia-conditioned media

Low passage and subconfluent BV2 microglia were harvested using Gibco enzyme-free dissociation media, incubated for 5 min at room temperature, and lifted with gentle tapping and pipetting. Microglia were diluted in an equal volume of complete media, counted with a hemocytometer, pelleted by centrifugation (300 rcf, 5 min), and resuspended in NB++ media (PN) for subculturing.

Cultures for conditioned media were seeded at 4e4 cells per cm^2 at 2e5 cells per mL. In some cases, microglia were stimulated with LPS (1 µg/mL). Cells were cultured at 37 °C in 5% CO_2 for 18 h. The media was then harvested with careful pipetting cleared of floating cells and large debris by centrifugation (500 rcf, 5 min). The clarified media was then transferred to a clean tube. In some cases, 5-N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) was added to 2 mM. An equal volume of conditioned media was then added to neuronal cultures.

Transfection of HeLa cells and conditioning of media

HeLa cells were transfected with plasmids encoding murine Neu3 (wt or the catalytically inactive mutant Y369F) using Transit2020 according to the manufacturer's protocol. Plasmids were custom ordered from Twist Biosciences encoding murine Neu3 (wt or Y369F) C-terminally tagged with a short linker (GSGGGSGGGGSG) followed by a 3xFLAG tag. Constructs were optimized for human codons and cloned into a pCMV vector from Twist. After 24 h, the cells were washed with OptiMEM and cultured in a low-volume of OptiMEM for an additional 24 h, at which point conditioned media was harvested and EV's were isolated.

Isolation and labeling of extracellular vesicles

Media was prepared as described above in 25 mL of media in a T125 cell culture flask. In some cases, bulk extracellular vesicles were isolated by concentrating media in a 100 kDa spin-filter. In other cases, exosomes were specifically isolated from conditioned media using Takara Capturem EV spin columns (Takara, 635723) according to the manufacturer's instructions.

For extracellular vesicle labeling experiments, BV-2 microglia were cultured in T25 flasks in 5 mL of Dulbecco's modified eagle medium (DMEM; Gibco) and activated overnight with LPS (1 µg/mL). Supernatant was isolated and incubated with PKH67 dye for 15 minutes at 37 °C following the PKH67GL-1KT kit (Sigma)[19]. Extracellular

50 vesicles were then isolated as described above and resuspended in Neurobasal media (Gibco) with 1%
51 GlutaMAX and 2% B-27 supplement. Primary hippocampal neurons were treated overnight with dyed EV
52 preparation and imaged following kit instructions.
53
54 **GW4869 treatment of microglia**
55 GW4869 (#D1692, Sigma) was dissolved in DMSO to make a stock solution of 0.2 mg/mL. For inhibition of
56 exosome generation, BV-2 microglia were treated for 3 μM GW4869 for 24 hours before 24-hour LPS
57 treatment. Culture supernatants were collected for exosome isolation and neuronal treatment.
58
59 **Periodate labeling of cell surface sialosides**
60 Cells were washed with HBSS solution (Gibco) and then incubated with 1 mM sodium (meta)periodate ($NaIO_4$;
61 Sigma) in DPBS (Sigma) for 30 minutes on ice. Cells were then washed twice with sodium acetate buffer (pH
62 4.7) and fixed for 10 minutes in a 1:1 acetate buffer:methanol solution on ice, then fixed for 10 min in pure
63 methanol. Cells were then washed with sodium acetate buffer and incubated with AlexaFluor 488
64 hydroxylamine dye (25 uM in sodium acetate buffer; Thermo) for 1 hour at 4 Celsius. Imaging was performed
65 on an Axio Observer Z-1 (Zeiss) equipped with a Spectra-X Light engine LED light (Lumencor), controlled with
66 Micro-Manager. Images were acquired with a Plan-Apochromat 20x/0.8 air objective (Zeiss) paired with image
67 capture from the ORCA-Flash4.0 sCMOS camera (sCMOS; Hamamatsu). Dye was excited with cyan LED
68 470/24 nm) and emission was collected after passing through the Zeiss 90 HE filter set (425/30 nm, 514/30
69 nm, 592/25 nm, 709/100 nm LP).
70
71 **Generation of CRISPR KO of mNeu3**
72 Plasmid constructs encoding Cas9 and a sgRNA were prepared in the lentiCRISPRv2 vector according to
73 published protocols.[39] Guides were selected based on the genome-wide guides described by Bassik and
74 coworkers.[40] Plasmid-bearing Stbl3 *E. coli* were grown in 50 mL cultures and DNA was extracted and
75 endotoxin purified by MiraPrep.[41]
76
77 LentiCRISPRv2 plasmids were packaged in lentiviruses produced from HEK293Ts cotransfected with
78 pGag/Pol, pRev, pTat, and pVSVG (gift from the Yi-Chang Liu and Jonathan Weismann). In brief, 1.5 μg of
79 LentiCRISPRv2 plasmid were combined with 0.1 μg of each packaging plasmid and Lipofectamine LTX
80 (Thermo Fisher, 15338100) in OptiMEM (Thermo Fisher, 31985062). Transfection complexes were added to
81 HEK293Ts at 70-80% confluency in a 6 well plate in 2 mL fresh media. The media was aspirated after 12 h
82 and bleached. After 48 h, the media was harvested and filtered through a 0.45 μm syringe filter to afford the
83 viral supernatants.
84
85 BV2 cells were resuspended in fresh viral-containing media with polybrene (8 μg/mL). Media was changed
86 after 24 h, and after 72 h antibiotic selection was started (2.5 μg/mL). After two weeks of selection, editing of
87 the target gene was validated by TIDE analysis.[42]
88
89 **Cytokine release assay**
90 Adherent NEU3 KO and WT BV-2 microglia were plated (100,00 cells/well in a 24-well plate) in Neurobasal
91 media (Gibco) with 1% GlutaMAX and 2% B-27 supplement one day prior to experiment. Media was treated
92 with LPS (1 μg/mL), LPS + DANA (2 μM), or left untreated during plating. Three technical replicates were
93 made per treatment. After 24 hours, cells were spun down at 500 rcf for 5 minutes to remove debris, and
94 supernatant was extracted for analysis. Cytokine levels were assessed using the BD Cytometric Bead Array
95 (CBA) Mouse Inflammation Kit. Flow cytometry was performed on a BD Accuri C6 Plus, and FlowJo software
96 was used to gate on single cells and live cells for analysis.
97 μL
98 **Generation of endogenous tagging of mNeu3 by homology-directed recombination**

99 Endogenous tagging of murine Neu3 in BV2 cells was achieved using the Mendenhall-Myers system.[43] In brief,
100 a pFETCh donor plasmid (Addgene, 63934) containing homology arms for mNeu3 (see table of gene
101 fragments) was prepared along with PX458 plasmids containing one the only potential target cut site for
102 mNeu3, as outlined by the target selection described by Mendenhall and Myers. Plasmids were prepared from
103 50 mL cultures of Stellar *E. coli* and purified by MiraPrep.[41]
104
105 Low passage BV2 microglia were transfected by magnetofection (OZ Biosciences) according to the
106 manufacturer's protocols. After 48 h, microglia were treated with a low dose of antibiotic selection (G418,
107 200X). After two weeks of treatment, only the cells co-transfected with pFETCh donor and PX458 bearing the
108 sgRNA were alive and growing well in the presence of G418 (0.25 mg/mL). The polyclonal population was
109 grown out and the genomic DNA was isolated using a GeneJET Genome DNA Purification Kit (Thermo). PCR
110 was performed using primers +/- 750 bp of the insertion site and compared to PCR products from wt cells. A
111 clear 2.5 kbp band was observed in addition to a 1.5 kbp band of comparable intensity, indicating (mostly)
112 monoallelic insertion of the transfer gene.
113
114 **Extracellular vesicles bead capture and analysis by flow cytometry**
115 Immunocapture beads for murine extracellular vesicles were prepared by conjugating polyclonal anti-murine
116 CD63 (ThermoFisher, PA5-100713) to tosyl-functionalized M450 Dynabeads according to the manufacturer's
117 protocol for antibody conjugation. After antibody conjugation, the beads were blocked with excess BSA and
118 quenched in pH 8.5 TBS before use.
119
120 BV2-conditioned media was cleared by centrifugation (500 rcf, 5 min) and immunocaptured overnight with 2e6
121 anti-mCD63 beads/mL at 4 °C in a rotating Eppendorf. The beads were magnet captured and washed with
122 cold 0.1% BSA in PBS. EV-bead complexes were stained at 2e6 beads/mL with antibody solutions for 1 h at
123 room temperature protected from light. Antibodies were used at the following dilutions: anti-mCD9 clone KMC8
124 PE conjugate (ThermoFisher 12-0091-81, 1:50); anti-mCD81 clone Eat2 PE conjugate (BD Biosciences
125 559519, 1:50); anti-FLAG clone D6W5B AlexaFluor 647 conjugate (Cell Signaling Technologies 15009S, 1:50);
126 rat IgG2a isotype control PE conjugate (BD Biosciences 554689, 1:50); rabbit IgG isotype control AlexaFluor
127 647 conjugate (ThermoFisher 51-4616-82, 1:50). Beads were magnet captured, washed, resuspended at 1e6
128 beads/mL and analyzed by flow cytometry on an Accuri C6 flow cytometer. Data were analyzed in FlowJo.
129 Beads were gated on isotype-treated controls.
130
131 **Quantitative reverse transcriptase PCR analysis**
132 Expression levels of Neu1, Neu3, and Neu4 in murine BV2 microglia and primary murine hippocampal neurons
133 were evaluated by quantitative RT-PCR (qPCR). Total RNA was isolated by TRIzol reagent extraction
134 (ThermoFisher, 15596026) and Zymo RNA Clean and concentrator kits (Zymo, R1013) using manufacturer's
135 protocols. Libraries of cDNA were generated via EcoDry premix kit (Takara, 639542) using 2 µg total RNA.
136 Transcript levels were quantitated via qPCR using SybrGreen master mix (ThermoFisher, 4309155). Transcript
137 levels were normalized to transcript levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
138
139 **Dissociated hippocampal cultures**
140 All animal procedures were approved by Stanford University's Administrative Panel on Laboratory Animal Care
141 and conformed to the NIH Guide for Care and Use of Laboratory Animals and the Public Health Policy. Primary
142 hippocampal tissue was harvested from E16.5 C57BL/6 embryonic mice (Charles River) immediately after
143 sacrifice of the pregnant dam. The isolated hippocampi were dissociated using Papain Dissociation System
144 (Worthington Biochemical Corporation) and trituration with fire-polished Pasteur pipettes. The dissociated cells
145 were plated onto 12mm coverslips (Chemglass Life Sciences) pre-treated with Poly-D Lysine (PDL; 1 mg/mL,
146 Sigma-Aldrich) at a density of 6 × 104 cells per coverslip. The cells were maintained for 24 hours in Dulbecco's
147 modified eagle medium (DMEM; Gibco) supplemented with 4.5 g/L D-glucose, 10% FBS, 1% GlutaMAX, and

148 2% B-27 (Gibco), and then switched to Neurobasal media (Gibco) with 1% GlutaMAX and 2% B-27
149 supplement. Plates were incubated at 5% CO2, 37°C, and subsequent media changes took place every 7
150 days.
151
152 **Voltage imaging of neurons**
153 Neurons were incubated with an HBSS solution (Gibco) containing BeRST1 (1.0μM; Miller Lab, UC Berkeley)
154 for 20 minutes at 37°C prior to imaging. Functional imaging of hippocampal cells was performed on an Axio
155 Observer Z-1 (Zeiss) equipped with a Spectra-X Light engine LED light (Lumencor), controlled with Micro-
156 Manager. Images were acquired with a Plan-Apochromat 20x/0.8 air objective (Zeiss) paired with image
157 capture from the ORCA-Flash4.0 sCMOS camera (sCMOS; Hamamatsu) with 4x4 in-camera binning. We used
158 a sampling rate of 0.5 kHz over 5 or 10 seconds in order to resolve individual action potentials. To achieve
159 maximum sampling rate, a field of view (FOV) size of 512x100 pixels (665.6x130 μm) was used for
160 simultaneous recording of 5-15 neurons at a time. BeRST 1 was excited with a 631 nm light (LED; 631nm, 28
161 nm bandpass) with an LED power of 70%. Emission from BeRST 1 was collected with a 680/10 nm bandpass
162 emission filter after passing through a dichroic mirror (425/30 nm, 514/30 nm, 592/25 nm, 709/100 nm LP).
163
164 **Image analysis**
165 Analysis of voltage traces in primary neurons was performed using ImageJ and custom Python scripts. All
166 Python scripts used to analyze data in this study are available at github.com/rishi-kulkarni/SpykeMapper.
167 Regions of interest (ROIs) were drawn around cell bodies within a field of view and the average fluorescence
168 over time was extracted and inputted into an Excel workbook. The fluorescence time course data was then
169 analyzed using a custom Python script that performed subthreshold trace extraction and spike train generation.
170 Briefly, the subthreshold activity was identified using asymmetric least squares regression and subtracted from
171 raw time course data to generate a flattened trace containing a flat baseline and spiking activity. Spikes were
172 identified from the flattened trace using a threshold of +6 STDEV of all cellular fluorescence values within a
173 coverslip to generate a digitized spike train containing all-or-nothing firing data or Raster plot. Factor analysis
174 was performed using the FactoryAnalyzer module with no rotation. The shared variance values per network
175 were compared using Cohen's d.
176
177 **Preparation of extracellular vesicles for mass spectrometry**
178 Extracellular vesicles were enriched as described above in the section Isolation and labeling of extracellular
179 vesicles. Enriched EVs were labeled with membrane-impermeable sulfo-NHS-biotin (Thermo Fisher Scientific,
180 21217) per the manufacturer's protocol. In brief, EVs in pH 8.0 PBS were labeled for 30 min at room
181 temperature with sulfo-NHS-biotin (2 mM) and were quenched by the addition of 100 mM glycine. EVs
182 solutions were desalted using spin columns. EVs were lysed by the subsequent addition of 10X RIPA buffer.
183 Samples were prepared in triplicate, and triplicate control samples were treated equivalently, except sulfo-
184 NHS-biotin was excluded. All samples were enriched using streptavidin magnetic beads (Pierce, #88817) and
185 the DynaMag-2 Magnet (Invitrogen, #12321D) based on a protocol adopted from previous work.[44] Buffers were
186 made fresh, including ETDA buffer (150 mM NaCl, 100 mM Tris pH 8, 0.5 mM EDTA pH 8), urea buffer (2 <
187 urea, 10 mM Tris) and elution buffer (5% SDS in RIPA, 20 mM DTT, 2 mM biotin). 25 μL of streptavidin beads
188 were used per sample, and beads were washed with 50 μL of cold RIPA twice before being resuspended in 25
189 μL cold RIPA. Beads were added to each sample, which were continuously rotated overnight at 4 C. The next
190 day, the unbound fraction was removed from the beads and kept. Beads were washed with 100 μL RIPA,
191 which was added to the saved unbound fraction (labeled FT, flow through) for each sample. Next, beads were
192 washed twice with 200 μL RIPA, three times with EDTA buffer, three times with 1.5 M NaCl, three times with
193 0.1 M NaHCO3, and once with urea buffer. Proteins were eluted from the beads by boiling at 95 C with 60 μL
194 elution buffer for 10 min. The elution step was repeated, for a total of two elution steps, and elutes from each
195 step were combined for a given sample. Digestion was performed for eluted and FT proteins using a micro S-
196 trap protocol provided by the manufacturer (Protifi).[45] For FT samples, proteins were brought to 5% SDS and

197 reduced with 10 mM DTT for 10 minutes at 95 C. Cysteines were alkylated using 40 mM iodoacetamide for 45
198 minutes each at room temperature in the dark. The lysate was then acidified with phosphoric acid, brought to
199 approximately 80-90% methanol with 100 mM TEAB in 90% methanol, and loaded onto the S-trap column.
200 Following washing with 100 mM TEAB in 90% methanol, trypsin (Promega) was added to the S-trap at a 20:1
201 protein:protease ratio for 90 minutes at 47 °C. Peptides were eluted in three steps that were collected in the
202 same tube for a given sample: 40 µL of 50 mM TEAB, 40 µL 0.2% FA in water, and 40 µL of 0.2% FA in 50%
203 ACN, all spun at 4,000 x g for 1 minute. Eluted peptides were dried via lyophilization.
204
205
206 **Preparation of cell-surface labeled neurons for mass spectrometry**
207 Samples were prepared following published cell surface capture protocols that label cell surface proteins
208 through sialic acids.[46,47] and lysate protein concentrations were quantitated by BCA (Pierce). Lysates were
209 digested using a mini S-trap protocol (Protifi), which is similar to the micro S-trap protocol above, but with
210 different volumes. Lysates were brought to 5% SDS and reduced with 5 mM DTT for 5 minutes at 95 C.
211 Cysteines were alkylated using 25 mM iodoacetamide for 45 minutes each at room temperature in the dark.
212 Lysates were then acidified with phosphoric acid, brought to approximately 80-90% methanol with 100 mM
213 TEAB in 90% methanol, and loaded onto the S-trap column. Following washing with 100 mM TEAB in 90%
214 methanol, trypsin (Promega) was added to the S-trap at a 20:1 protein:protease ratio for 90 minutes at 47 °C.
215 Peptides were eluted in three steps that were collected in the same tube for a given sample: 80 µL of 50 mM
216 TEAB, 80 µL 0.2% FA in water, and 80 µL of 0.2% FA in 50% ACN, all spun at 4,000 x g for 1 minute. Eluted
217 peptides were dried via lyophilization and dried peptides were then resuspended in 100 µL 100 mM Tris, pH 8
218 for enrichment via streptavidin beads (Pierce, #88817) and the DynaMag-2 Magnet (Invitrogen, #12321D).
219 Buffers were made fresh, including ETDA buffer (150 mM NaCl, 100 mM Tris pH 8, 0.5 mM EDTA pH 8). 50 µL
220 of streptavidin beads were used per sample, and beads were washed with 200 µL 100 mM Tris three times
221 before being resuspended in 400 µL 100 mM Tris. Beads were added to each sample (500 µL total per
222 sample), which were continuously rotated overnight at 4 C. The next day, the unbound fraction was removed
223 from the beads and kept. Beads were washed with 500 µL 100 mM Tris, which was added to the saved
224 unbound fraction (labeled FT, flow through) for each sample. Next, beads were washed five times with 500 µL
225 100 mM Tris, five times with 500 µL EDTA buffer, five times with500 µL 1.5 M NaCl, five times with 500 µL 0.1
226 M NaHCO3, once with 500 µL 80% (v/v) 2-isopropanol, twice with500 µL water, and three times with 500 µL
227 warm (60 °C) 100 mM Tris. Beads were then resuspended in 500 µL 100 mM Tris. Glycerol-free PNGaseF
228 (New England Biolabs, # P0705L) was diluted 2-fold, and 1 µL of diluted PNGaseF was added to each set of
229 beads. Beads with PNGaseF were incubated overnight, where PNGaseF enzymatic cleavgage release
230 formerly N-glycosylated peptides (i.e., de-glycopeptides). Eluted de-glycopeptides were acidified with 10% FA
231 before desalting with Strata-X reversed phase SPE cartridges (Phenomenex, #8B-S100-AAK) by conditioning
232 the cartridge with 1 mL ACN followed by 1 mL 0.2% formic acid (FA) in water. Acidified de-glycopeptides
233 loaded on to the cartridge, followed by a 1 mL wash with 0.2% FA in water. Peptides were eluted with 400 µL
234 of 0.2% FA in 80% ACN and dried via lyophilization.
235
236 **Mass spectrometry proteomics LC-MS/MS**
237 All samples were resuspended in 0.2% formic acid in water prior to LC-MS/MS analysis, where half of the
238 sample was injected for analysis (i.e., 5 µL or 10 µL total), and non-modified peptides and de-glycopeptides
239 (referred to collectively as peptides in this section) were analyzed with the same LC-MS/MS method. All
240 peptide mixtures were separated over a 25 cm EasySpray reversed phase LC column (75 µm inner diameter
241 packed with 2 µm, 100 Å, PepMap C18 particles, Thermo Fisher Scientific). The mobile phases (A: water with
242 0.2% formic acid and B: acetonitrile with 0.2% formic acid) were driven and controlled by a Dionex µLtimate
243 3000 RPLC nano system (Thermo Fisher Scientific). An integrated loading pump was used to load peptides
244 onto a trap column (Acclaim PepMap 100 C18, 5 um particles, 20 mm length, Thermo Fisher Scientific) at 5
245 µL/min, which was put in line with the analytical column 5.5 minutes into the gradient. The gradient increased

246 from 0% to 5% B between 6 and 6.5 minutes, followed by an increase from 5% to 22% B from 6.5 to 66.5
247 minutes, an increase from 22% to 90% B from 66.5 to 71 minutes, isocratic flow at 90% B from 71 to 75
248 minutes, and a re-equilibration at 0% B for 15 minutes for a total analysis time of 90 minutes per injection.
249 Eluted peptides were analyzed on an Orbitrap Fusion Tribrid MS system (Thermo Fisher Scientific). Precursors
250 were ionized using an EASY-Spray ionization source (Thermo Fisher Scientific) source held at +2.2 kV
251 compared to ground, and the column was held at 40 °C. The inlet capillary temperature was held at 275 °C.
252 Survey scans of peptide precursors were collected in the Orbitrap from 350-1350 Th with an AGC target of
253 1,000,000, a maximum injection time of 50 ms, and a resolution of 60,000 at 200 m/z. Monoisotopic precursor
254 selection was enabled for peptide isotopic distributions, precursors of z = 2-5 were selected for data-dependent
255 MS/MS scans for 2 second of cycle time, and dynamic exclusion was set to 30 seconds with a ±10 ppm
256 window set around the precursor monoisotope. An isolation window of 1 Th was used to select precursor ions
257 with the quadrupole. MS/MS scans were collected using HCD at 30 normalized collision energy (nce) with an
258 AGC target of 100,000 (200%) and a maximum injection time of 54 ms. Mass analysis was performed in the
259 Orbitrap with a resolution of 30,000 with a first mass set at 120 Th.
260
261 **Proteomics data analysis**
262 Peptides from EV labeled data were searched with Morhpeus search algorithm[48] housed in the MetaMorpheus
263 software environment (version 0.0.312)[49] using the entire mouse proteome downloaded from Uniprot[50]
264 (reviewed, 17030 entries). Cleavage specificity was set to fully tryptic with 2 missed cleavage allowed and a
265 minimum length of 7 residues, oxidation of methionine was set as a variable modification, and
266 carbamidomethylation of cysteine was set as a fixed modification. Precursor and product ion tolerances were
267 set to 5 ppm and 20 ppm, respectively. Output was set to filter at a q-value of 0.01, and FlashLFQ[51] with
268 matching between runs was enabled. All other parameters were set as default. For quantitative comparisons,
269 protein intensity values were log2 transformed prior to further analysis, proteins with greater than three missing
270 values (i.e., half) per condition were removed, and missing values were imputed from a normal distribution with
271 width 0.3 and downshift value of 1.8 (i.e., default values) using the Perseus software suite[6]. De-glycopeptides
272 were searched with the Andromeda search engine[52] in MaxQuant.[53] Cleavage specificity was set to fully tryptic
273 with 2 missed cleavage allowed and a minimum length of 7 residues, oxidation of methionine and deamidation
274 of asparagine were set as a variable modification, and carbamidomethylation of cysteine was set as a fixed
275 modification. Defaults were used for the remaining settings, including PSM and protein FDR thresholds of 0.01;
276 and 20 ppm, 4.5 ppm, and 20 ppm for first search MS1 tolerance, main search MS1 tolerance, and MS2
277 product ion tolerance, respectively. The Deamidation Sites table was used for further data analysis, randomly
278 chosen spectra were spot-checked for identification accuracy using IPSA to ensure data quality, and
279 deamidated peptides were marked as either "True" or "False" for whether they contained the N-glycosylation
280 sequon, N-X-S/T, where X is any residue but proline. This Deamidation Sites table file was then uploaded into
281 Perseus and further filtered to remove 1) potential contaminants and reverse hits, 2) peptides that did not
282 contain the N-glycosylation sequon, 3) sites with less than 0.5 localization probability, and 4) identifications that
283 had more than three missing values. Missing values for the remaining identifications were imputed from a
284 normal distribution with width 0.3 and downshift value of 1.8 (i.e., default values).
285
286
287 **Statistical analysis**
288 All statistical hypothesis tests were performed using either a hierarchical permutation test[54] (20,000 resamples
289 for n=3 treatments, 70,000 for n=4, 200,000 for n>4) or a Welch's t-test. Code is available at github.com/rishi-
290 kulkarni/hierarch.
291
292 **DNA oligonucleotides**

Usage	DNA Sequence
mNeu3 KO sgRNA (FWD)	CACCGGAGAGGTGCCAGATTGTGTG

mNeu3 KO sgRNA (REV)	AAACCACACAATCTGGCACCTCTCC
murine safe-targeting sgRNA (FWD)	CACCGGAAATCCTTACCTAAGACAA
murine safe-targeting sgRNA (REV)	AAACTTGTCTTAGGTAAGGATTTCC
mNeu3 KO PCR (FWD)	GGGCCTTCAAGATTCTGTCCATTT
mNeu3 KO PCR (REV)	GTGCCATGTGACTCCAAAGTCATC
mNeu3 KO SEQ	TTTCAATGTCCTATATTGTTTGAAAAAGAACTG
mNeu3 HDR sgRNA (FWD)	CACCGCGACTAAAGCCAAATCAAGA
mNeu3 HDR sgRNA (REV)	AAACTCTTGATTTGGCTTTAGTCGC
mNeu3 HDR PCR (FWD)	TCCCCGACCTGCAGCCCAGCT
mNeu3 HDR PCR (REV)	TGGAGAGGACTTTCCAAG
GAPDH qPCR (FWD)	CCCATCACCATCTTCCAGGAGC
GAPDH qPCR (REV)	CCAGTGAGCTTCCCGTTCAGC
mNEU1 qPCR (FWD)	TTCATCGCCATGAGGAGGTCCA
mNEU1 qPCR (REV)	AAAGGGAATGCCGCTCACTCCA
mNEU3 qPCR (FWD)	CTCAGTCAGAGATGAGGATGCT
mNEU3 qPCR (REV)	GTGAGACATAGTAGGCATAGGC
mNEU4 qPCR (FWD)	AGGAGAACGGTGCTCTTCCAGA
mNEU4 qPCR (REV)	GTTCTTGCCAGTGGCGATTTGC

293

294 **DNA gene sequences and gBlocks**

Gene	**DNA Sequence**
NEU3 HOM1	TCCCCGACCTGCAGCCCAGCTCTACACTCGGGAAGGCTGATCATCCCC GCCTATGCCTACTATGTCTCACGTTGGTTTCTCTGCTTTGCGTGTTCAGTCAAG CCCCATTCCCTGATGATCTACAGTGATGACTTTGGAGTCACATGGCACCATGGC AAGTTCATTGAGCCCCAGGTGACAGGGGAGTGCCAAGTGGCCGAAGTGGCTG GGACGGCTGGTAACCCTGTGCTCTACTGCAGTGCCCGAACACCAAGCCGATTT CGAGCAGAGGCTTTTAGTACTGATAGTGGTGGCTGCTTTCAGAAGCCAACCCT GAACCCACAACTCCATGAGCCTCGAACCGGCTGCCAAGGTAGTGTAGTGAGCT TCCGGCCTTTGAAGATGCCAAATACCTATCAAGACTCAATTGGCAAAGGTGCTC CCGCTACTCAGAAGTGCCCTCTGCTGGACAGTCCTCTGGAGGTGGAGAAAGGA GCTGAAACACCATCAGCAACATGGCTCTTGTACTCACATCCAACTAGCAAGAGG AAGAGGATTAACCTAGGCATCTACTACAACCGGAACCCCTTGGAGGTGAACTG CTGGTCCCGCCCGTGGATCTTGAACCGTGGGCCCAGTGGCTACTCTGATCTGG CTGTTGTGGAAGAACAGGACTTGGTGGCGTGTTTGTTTGAGTGTGGGGAGAAG AATGAGTATGAGCGGATTGACTTCTGTCTGTTTTCAGACCATGAGGTCCTGAGC TGTGAAGACTGTACCAGCCCTAGTAGCGACGGGAGCGGAGGAGGTTCCGG
NEU3 HOM2	AGTTCTTCTGATTCGAACATCGATGAGTGAGGCCCAGCTTCCCACAGAA AGGAATGGCAGCTACAGCCAGGGTAACAGAGGTCTCTGATGTCTAGAGAAAAC TCTAAAAACTAATAATCTGCTCCTTGAATTTTTTCACTTTTCCCTTCAATGAGCAT GGTGAAAATTGTGCCATATCTTACATAACGAGGCTCTTGAACTGGGAGTTTGAA TCTCTTCTCTTCCCATTAAAAGGAGAGGCCATGTGCTCGCTTCGCGTTCGACAA AGCCTGGATTCTGATCTTGAGTGGAAGCCACAGGCTTGTCTTTTCCAATGGTTC ACTGCTCACCTGAGTATTAGGTGATGTGTAGGTGCCTTGGCCAGAAGAAAGAT CTGTGTTGTTGTATTTTTTTAAATTTATTTATTTACTATATGTAAGTACACTGCAG CTGTCTTCAGACACACCAGAAGAGGGCGTCAGATCTCATTAGAGATGGTTGTG AGCCACCATGTGGTTGCTGGGATTTGAACTCAGGACCTTCAGAAGAGCAGTCA

	GTGCTCTTAACTACTGAGCCATCTCTCAAGCCCCGCATTGCTGTATTTTTAATAA GAAAAATGCCCTTATCCTTCCAATAATGCCTGGAGCTGTACAAATTCTCTGTCTT AGAAGACTTGAGAAAGCAGAACTGTAAGGTCAGATGCTTTCTCCAGCCTTGATG CTGTGTTCCACCTTCCCTTCCTCATCCAGAAAACAGTTACTAGGGAGAAAATGA GAAACCCATGCCAGCTGCCTTGGAAAGTCCTCTCCA
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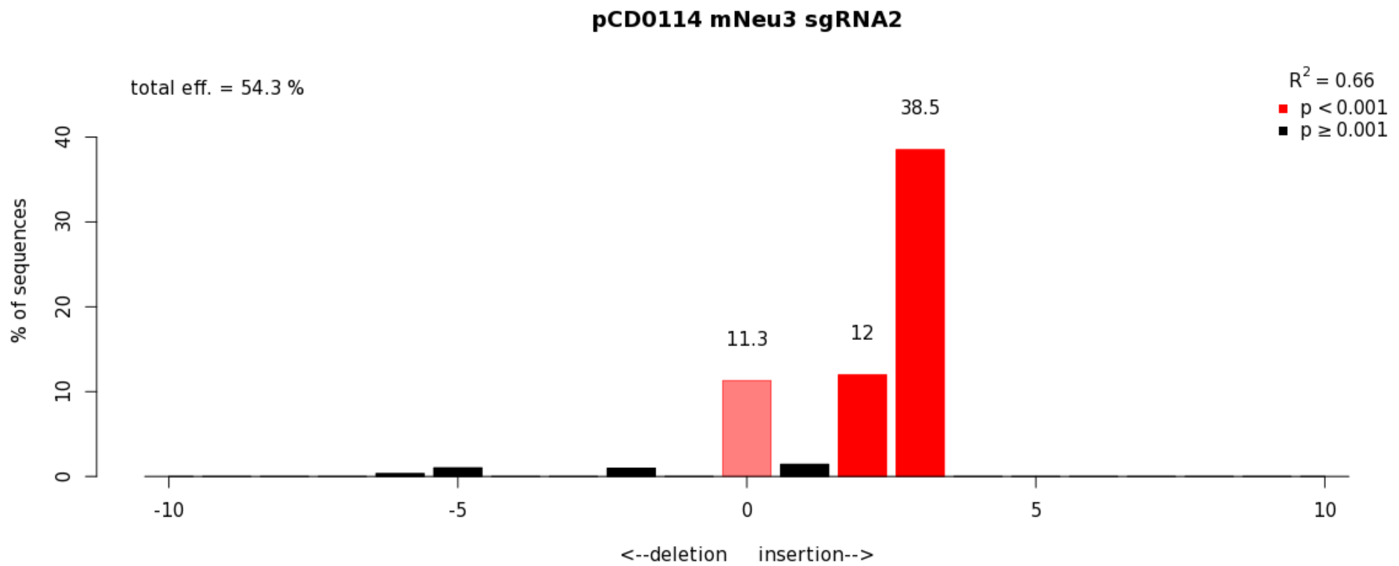
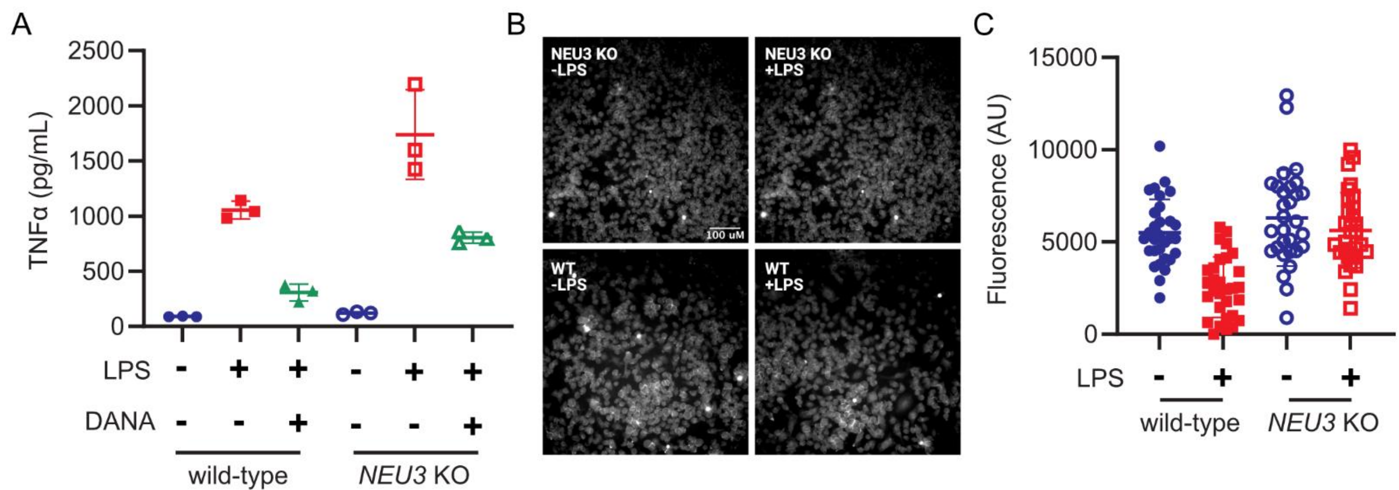


296

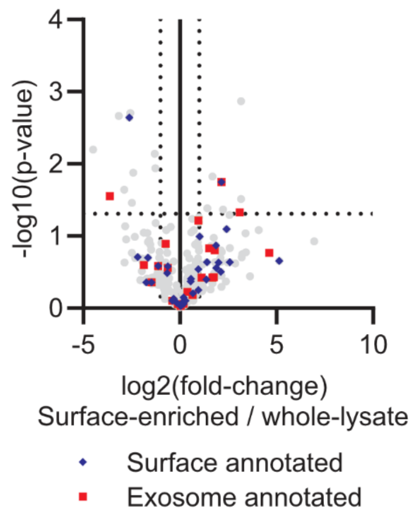
297 **Figure S1. Tracking of Indels by Decomposition (TIDE) analysis of *NEU3* KO BV-2 microglia.** To confirm
298 knock-out of the *NEU3* gene in murine BV-2 microglia, CRISPR-Cas9 edited cells were subjected to two weeks
299 of selection before genomic DNA was harvested and the cut region was amplified and sequenced. TIDE [ref]
300 deconvolutes the possible changes from CRISPR-based editing to provide gene-level editing quantiation.

301



302

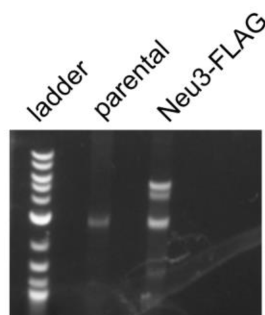
303 **Figure S2. Neu3 desialylates microglia in cis but is not necessary for inflammatory activity. (A,B)** Wild-
304 type or *NEU3* knock-out (*NEU3* KO) BV-2 microglia were stimulated with LPS for 24 h, with or without deoxy-
305 2,3-anhydroneuraminic acid (DANA). **(A)** Quantification of TNF-α secretion by LPS-treated WT and NEU3 KO
306 microglia by flow cytometry reveals that NEU3 KO microglia are capable of activating in response to LPS (WT:
307 -LPS vs. +LPS, p=0.002; -LPS vs. +LPS+DANA, p=0.039; +LPS vs. +LPS+DANA, p=0.0003) (NEU3 KO: -LPS
308 vs. +LPS, p=0.02; -LPS vs. +LPS+DANA, p=0.001; +LPS vs. +LPS+DANA, p=0.055). n=3 wells/condition.
309 Hypothesis tests performed with Welch's *t*-test. **(B, C)** Periodate labeling of activated vs. resting WT and NEU3
310 KO microglia (WT, p=0.0383; NEU3 KO, p=0.5073). n=3 wells/condition, 10 microglia/well. All hypothesis tests
311 performed with hierarchical permutation test.

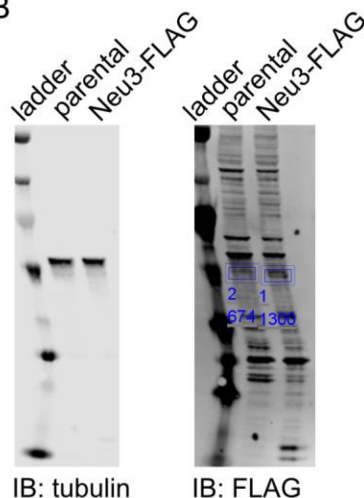


312

313 **Figure S3. Surface or whole-lysate proteomics of enriched extracellular vesicles does not identify**
314 **Neu3.** Extracellular vesicles from BV-2 microglia were enriched and either lysed (whole-lysate) or subjected to
315 cell surface biotinylation using sulfo-NHS-biotin, followed by lysis and biotin/streptavidin enrichment as
316 described in the materials and methods. Data were quantitated by label-free quantitation. Known surface
317 proteins (blue diamonds) and exosomal proteins (red squares), based on Uniprot GOCC annotation, are
318 labeled.

319



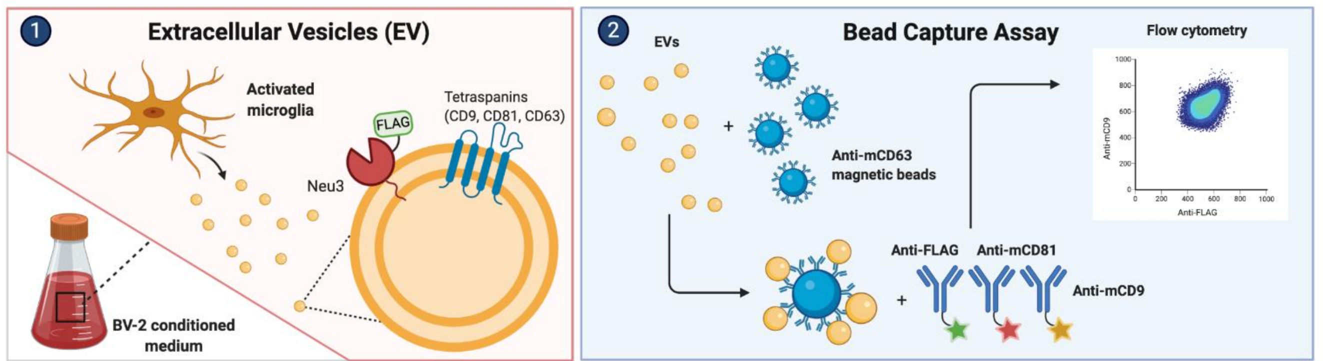


320

321 **Figure S4. Homology-directed recombination (HDR) produces endogenous Neu3 with a 3xFLAG tag.**
322 **(A,B)** BV-2 microglia were co-transfected with a CRISPR-Cas9 based cutting plasmid and a donor plasmid for
323 HDR to insert the coding sequence for a 3xFLAG tag at the C-terminus of the endogenous *NEU3* gene. (A)
324 PCR of the NEU3 gene shows increased amplicon size in a polyclonal population of transfectants, congruent
325 with tag incorporation. **(B)** Western-blot of whole-cell lysates from parental BV-2 or HDR-transfectants shows a
326 noticeable and quantifiable band in the transfectants at the expected molecular weight of mNeu3.

327

A



B

CD81-capture

FLAG

Q1 0 | Q2 0.073753 | Q4 0.36262 | Q3 99.5636

Q1 0 | Q2 0.58954 | Q4 0.33751 | Q3 99.0729

CD63

CD9-capture

FLAG

Q1 0 | Q2 0.18505 | Q4 0.40935 | Q3 99.4056

Q1 0 | Q2 0.58954 | Q4 0.33751 | Q3 99.0729

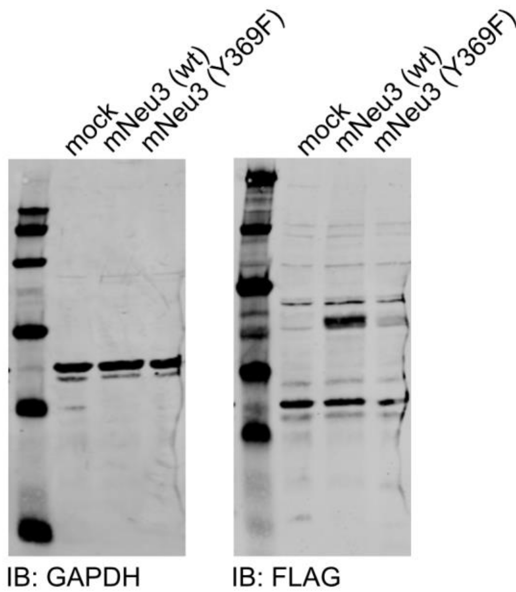
CD63

resting

LPS-stimulated

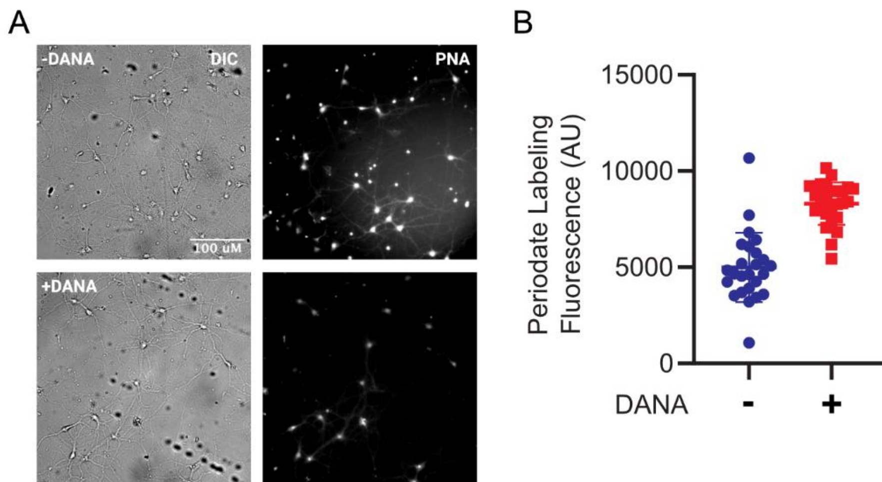
328

329 **Figure S5. LPS-stimulation increases a population of Neu3+ extracellular vesicles in BV-2 microglia.**
330 After exposure of BV-2 microglia with endogenously FLAG-tagged Neu3 to vehicle or LPS, EVs were captured
331 on anti-mCD81 or anti-mCD9 coupled beads, labeled with fluorophore-coupled anti-FLAG or anti-mCD63, and
332 analyzed by flow cytometry. **(A)** Representative experimental scheme. **(B)** Representative pseudocolor dot
333 plots from bead-capture experiments in **Figure 2A**.



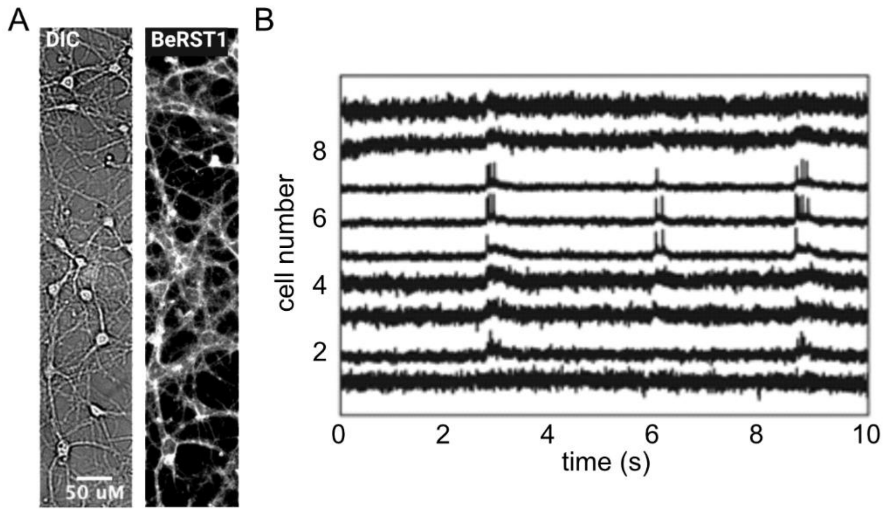
334

335 **Figure S6. HeLa cells overexpress FLAG-tagged mNeu3 upon transient transfection.** HeLa cells were
336 transfected with plasmid encoding either wild-type mNeu3 or a loss-of-function mutant (Y396F) with a C-
337 terminal FLAG tag. Whole cells were lysed and lysates were separated by SDS-PAGE, transferred to
338 nitrocellulose, and probed by Western blot with IR-dye conjugated anti-FLAG or anti-GAPDH antibodies.



339

340 **Figure S7. Neu3 on HeLa-derived extracellular vesicles is sufficient to desialylate neurons in culture.**
341 Primary mouse hippocampal neurons were treated with conditioned media from NEU3-overexpressing HeLa
342 cells in the presence or absence of deoxy-2,3-anhydroneuraminic acid (DANA). Cell surface sialic acid levels
343 were visualized by periodate labeling. Representative images **(A)** and quantification of fluorescence **(B)** reveal
344 that Neu3 from HeLa-derived extracellular vesicles significantly reduce surface sialic acids (p=0.028) in a
345 sialidase-inhibitor dependent manner. n=3 coverslips/condition, 25 cells/condition.



346

347 **Figure S8. Neu3 on HeLa-derived extracellular vesicles does not produce significant change in firing**
348 **rate of neurons.** Representative images **(A)** and spike traces **(B)** from **Figure 3E** and **3F**.

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350
351
352
353