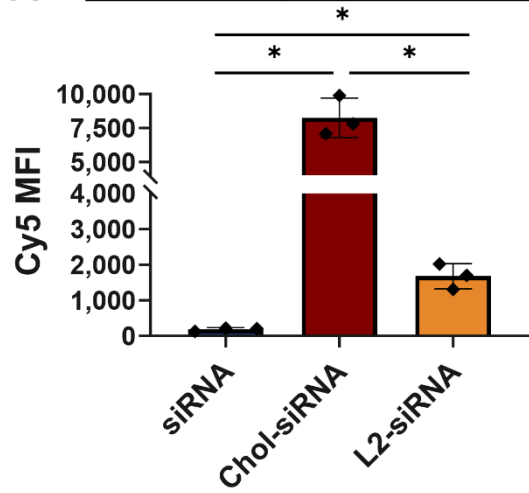
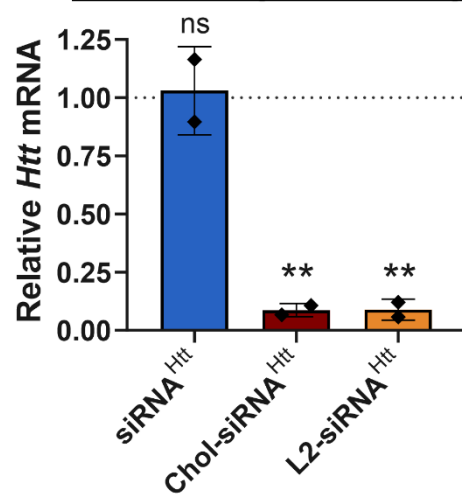


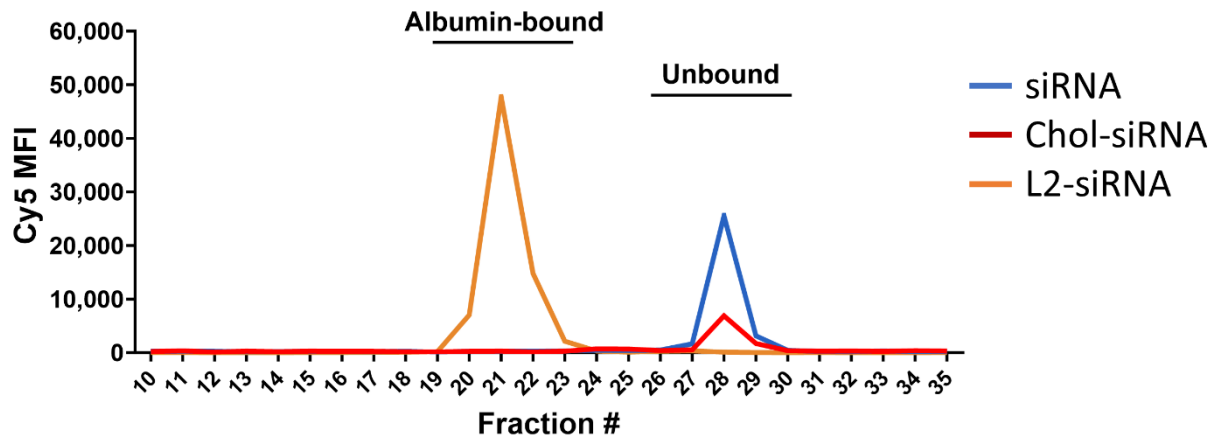
A Serum-free uptake in N2a cells



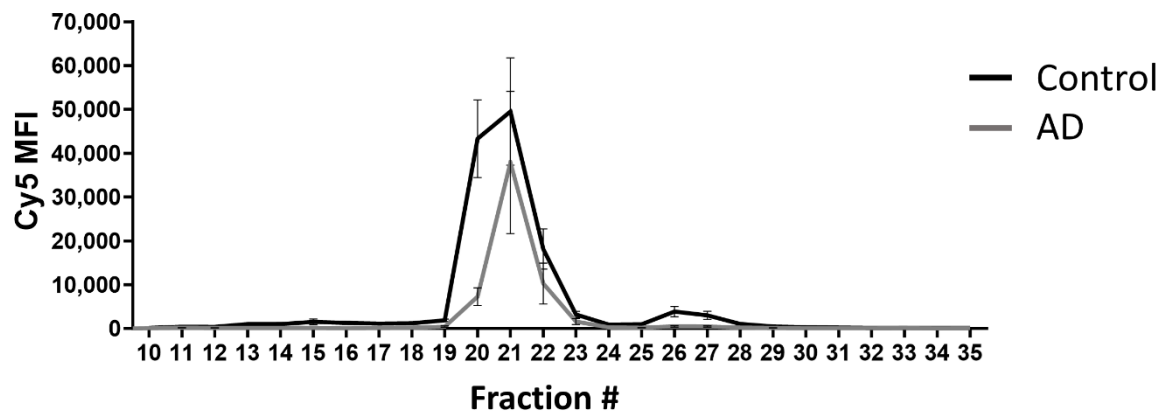
B Carrier-free gene silencing



C Protein binding in human CSF

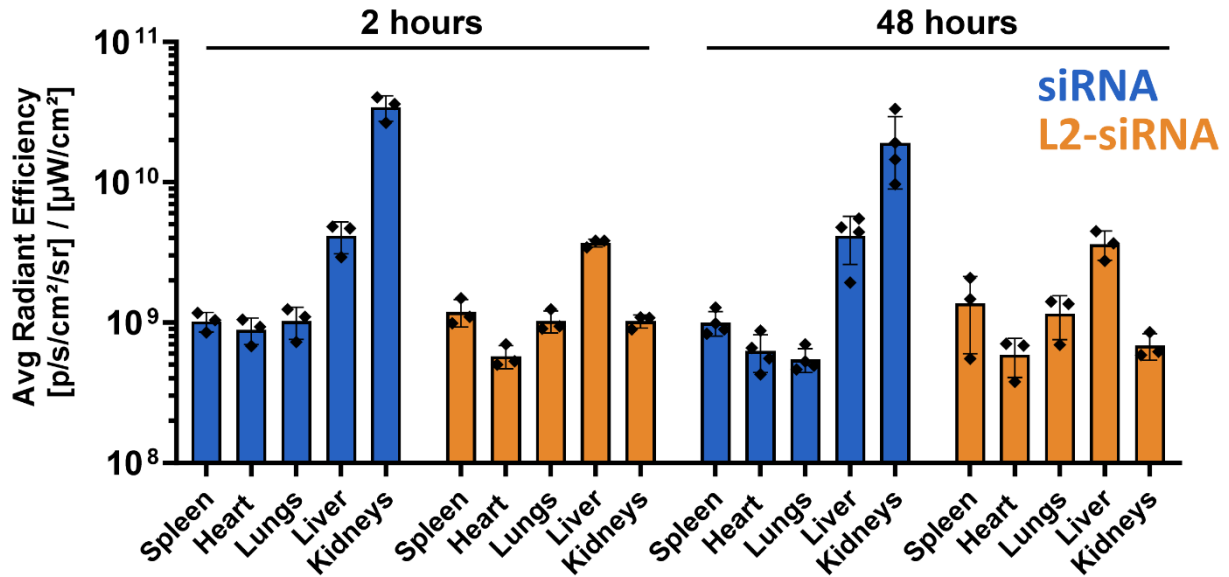
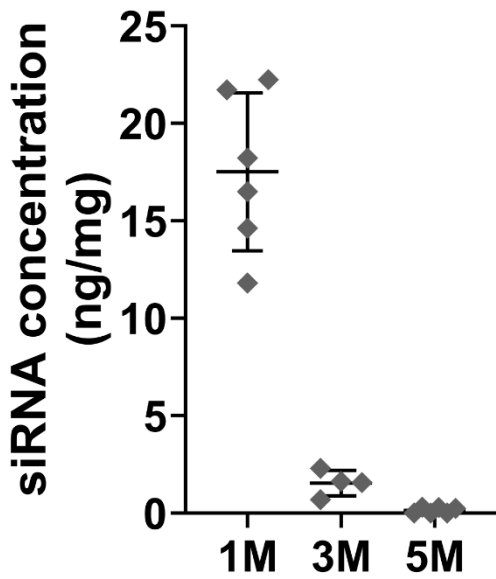
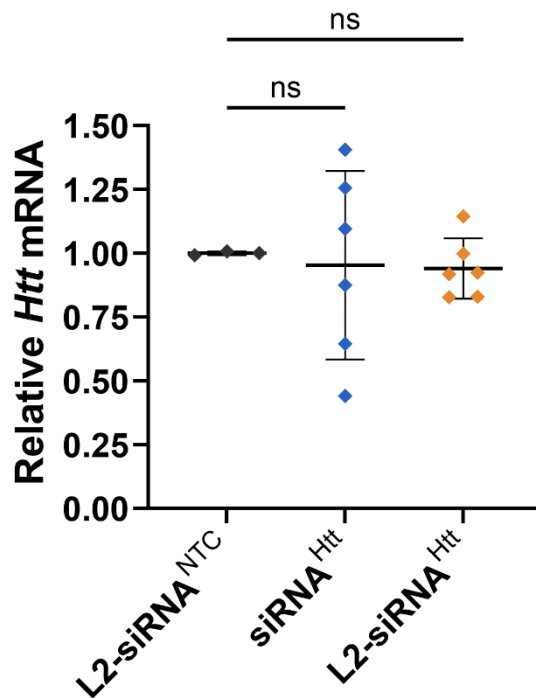


D L2-siRNA albumin binding in AD and control CSF



Supplementary Figure 1: Physicochemical properties of siRNA conjugates

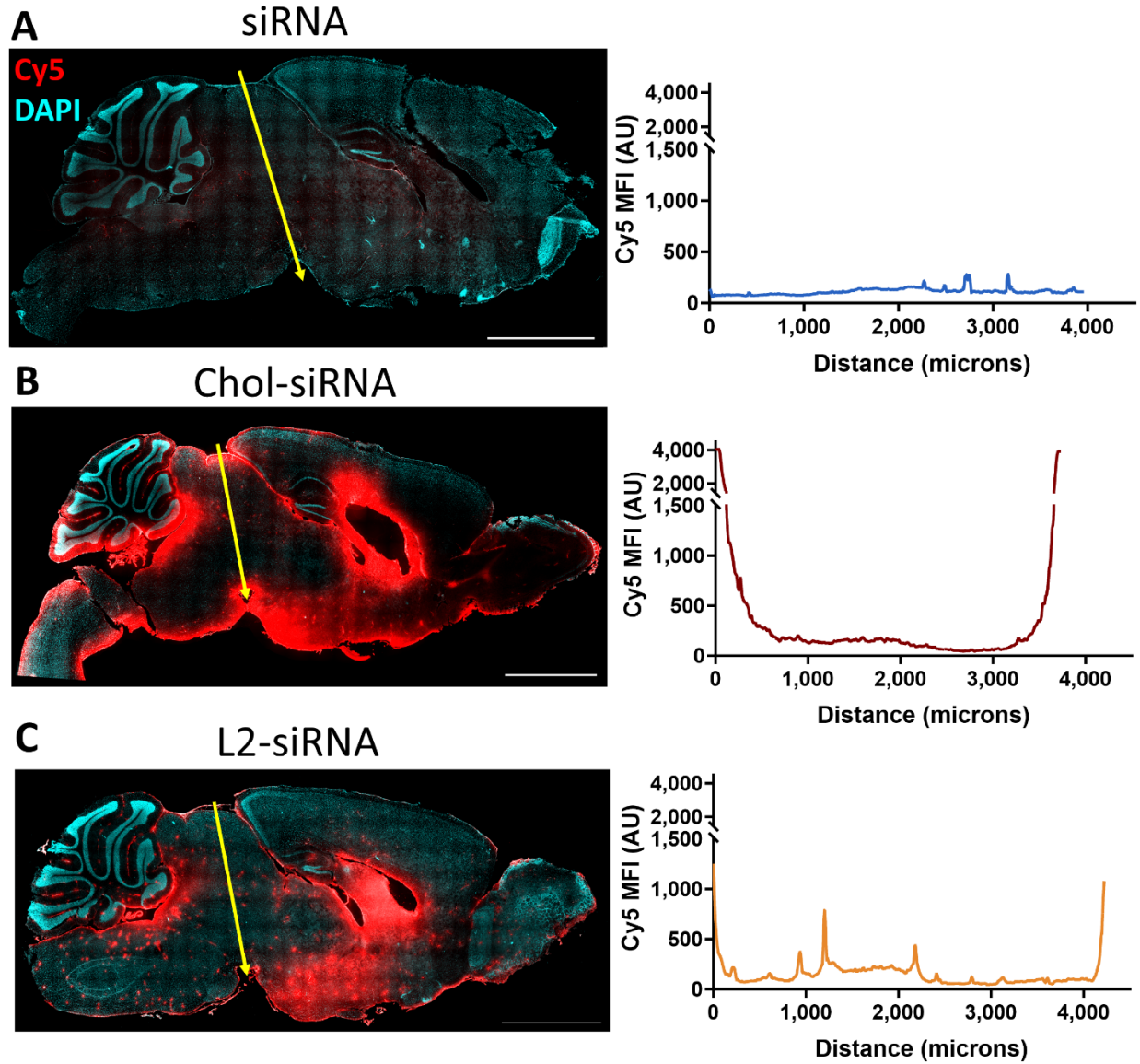
- A. Cell uptake was evaluated by flow cytometry in N2a cells after a 2-hour incubation with unconjugated siRNA, Chol-siRNA, or L2-siRNA (60 nM). Mean fluorescence intensity (MFI) is reported from three independent experiments, where the bars represent mean \pm SD. One-way ANOVA without assuming equal SD was performed with Dunnett's T3 correction for multiple comparisons (* p <0.05).
- B. Carrier-free *Htt* knockdown in N2a cells assessed by RT-qPCR after a 48-hour incubation with siRNA^{Htt}, Chol-siRNA^{Htt}, or L2-siRNA^{Htt} normalized to L2-siRNA^{NTC} (represented by dotted line at $Y=1.0$). Data reported from two independent experiments, each normalized to L2-siRNA^{NTC}, with bars representing mean \pm SD. One-way ANOVA compared to L2-siRNA^{NTC} with Bonferroni's multiple comparison correction (ns – not significant, ** p <0.01).
- C. Albumin-binding properties of siRNA conjugates in human CSF. Cy5-labeled siRNA, Chol-siRNA, or L2-siRNA (1 μ M) mixed with 300 μ l of human CSF was analyzed using fast protein liquid chromatography (FPLC). Only L2-siRNA elutes in known albumin-containing fractions. Same $N=1$ CSF sample used for each condition.
- D. L2-siRNA associates with albumin in CSF from patients with Alzheimer's Disease (AD) as well as those without neurodegeneration ($N=3$ control and AD samples). Error bars represent SD.

AIVIS organ biodistribution**B** L2-siRNA^{NTC} liver delivery**C** 3M liver gene silencing

Supplementary Figure 2: Clearance to peripheral organs

- A. Accumulation in peripheral organs assessed by In Vivo Imaging System (IVIS) 2 and 48 hours after ICV injection of Cy5-labeled siRNA or L2-siRNA (7.5-10 nmol). Average radiant efficiency of Cy5 fluorescence is reported. N=3-4 mice.
- B. L2-siRNA^{NTC} liver delivery 1, 3 and 5 months (M) after ICV injection (15 nmol) measured with the PNA assay. Values below the limit of detection were plotted as 0. N=4-6 mice.
- C. *Htt* mRNA expression levels in the liver as measured by RT-qPCR 3 months after ICV injection (15 nmol). Each point represents an individual mouse (N=3-6). Significance was calculated as a one-way ANOVA compared to L2-siRNA^{NTC} with Bonferroni's correction for multiple comparisons. Data presented as mean \pm SD in every graph (ns - not significant).

48-hour ICV biodistribution additional replicate

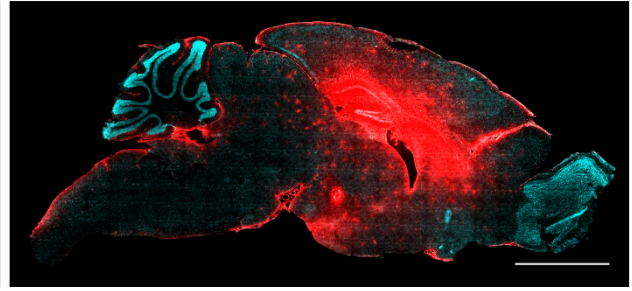
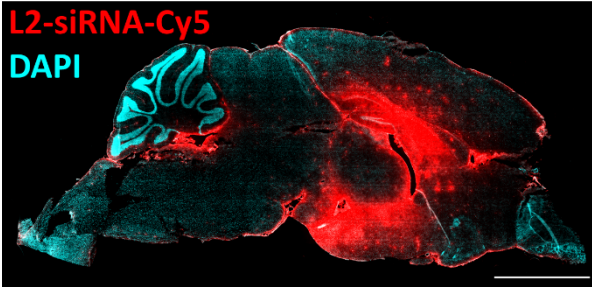


Supplementary Figure 3: Additional replicates of biodistribution after ICV delivery

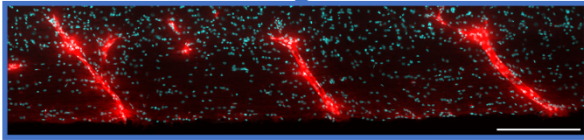
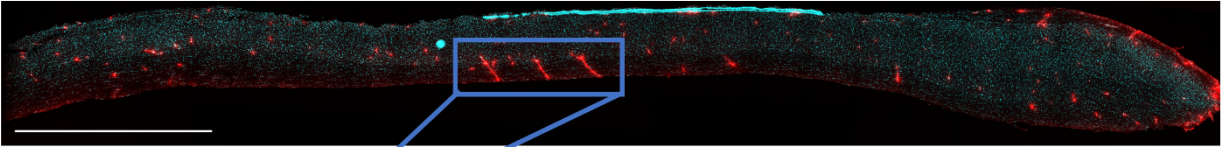
Delivery throughout CNS 48 hours after ICV delivery. Cy5 mean fluorescence intensity (MFI) plotted along distance indicated by yellow line. Additional example shown for siRNA (A), Chol-siRNA (B), and L2-siRNA (C). Section thickness = 30 μ m, scale bar = 2.5 mm.

A 2 μ l injection volume

10 μ l injection volume

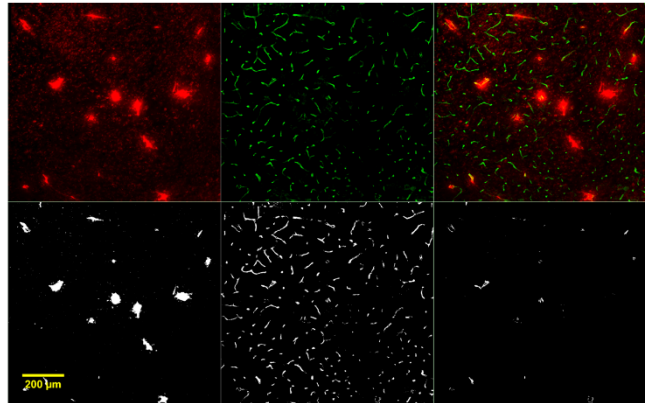


B spinal cord (2 μ l)

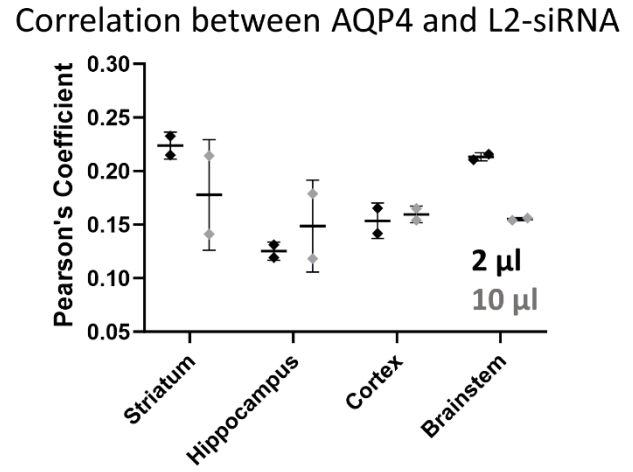
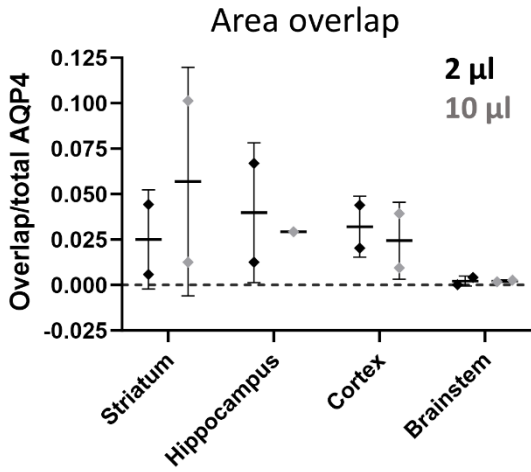


C Approach to perivascular quantification

L2-siRNA-Cy5 AQP4 Overlap



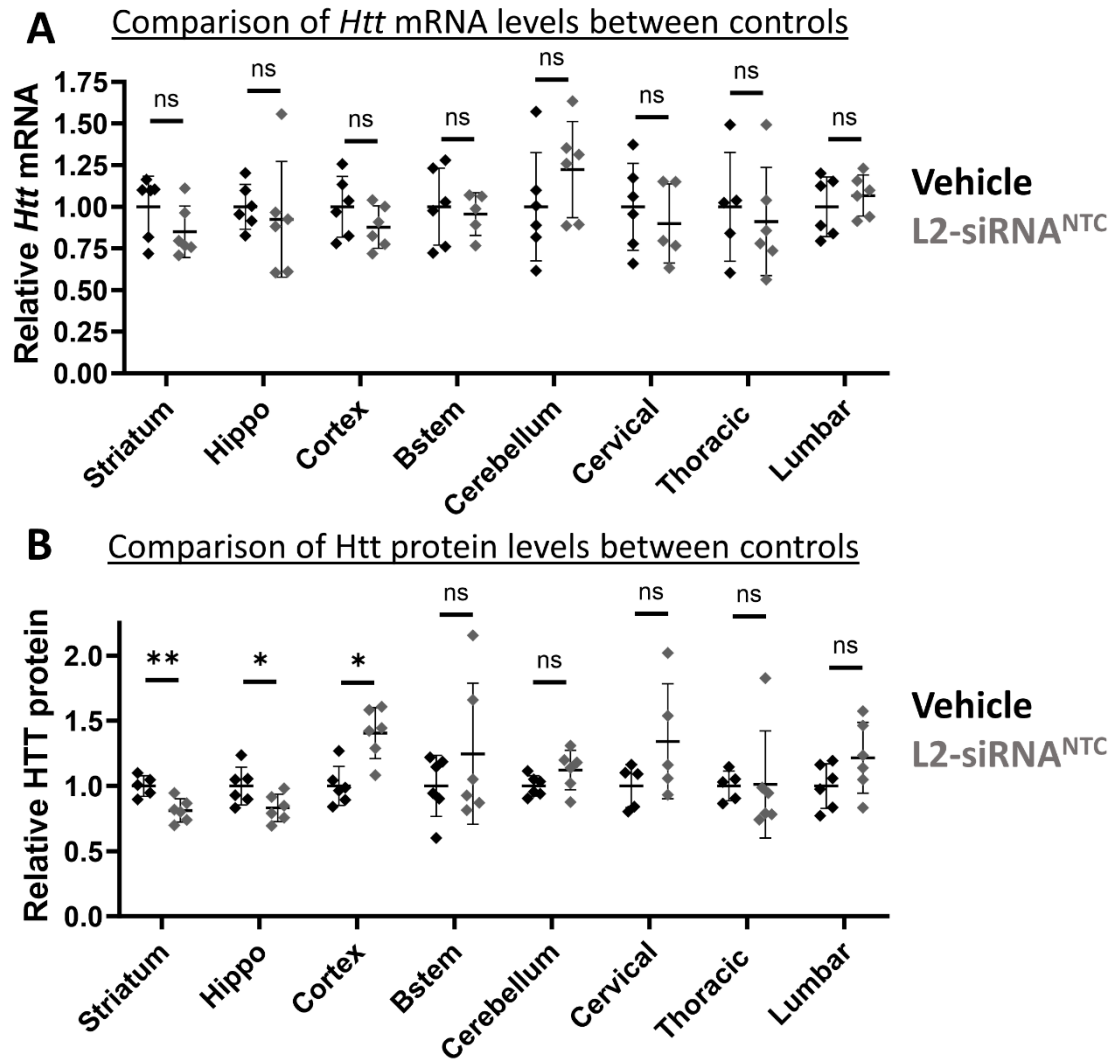
D Quantification of perivascular delivery



Supplementary Figure 4: L2-siRNA delivery to perivascular spaces is observed with low injection volumes

A, B. To test whether perivascular delivery is an artifact of injection volume, mice were administered an equivalent ICV dose of L2-siRNA (2 nmol), in either 2 μ l or 10 μ l by adjusting the initial compound concentration appropriately. After 48 hours, biodistribution was examined in the parenchyma (A) and spinal cord (B), with L2-siRNA shown in red, counterstained with DAPI in cyan (30 μ m sections, scale bar = 2.5 mm).

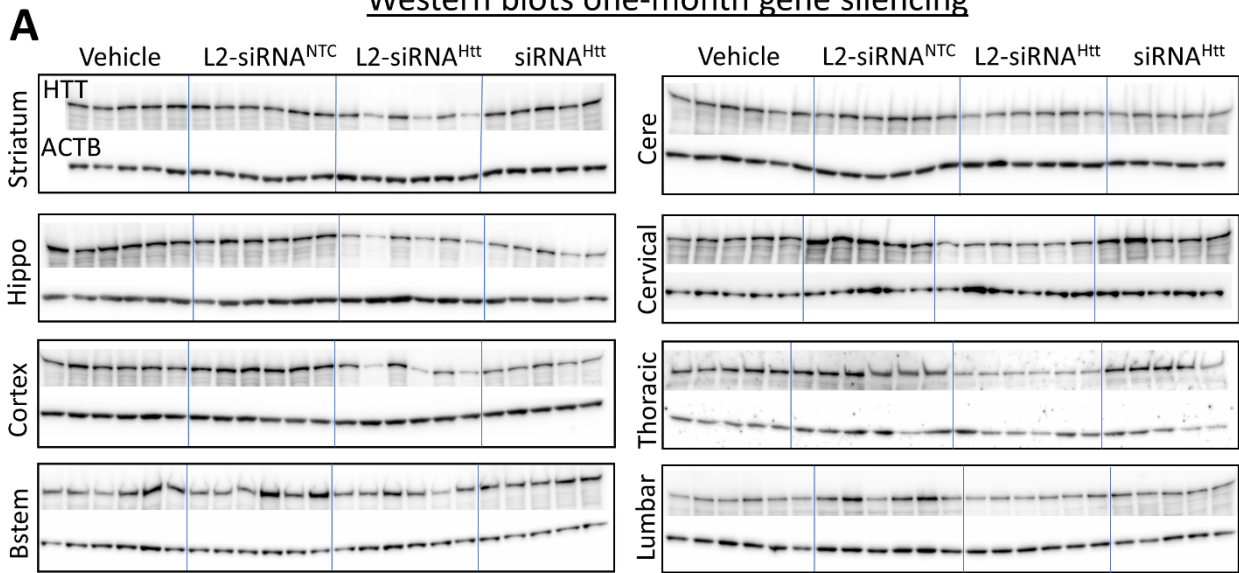
C, D. Quantification of L2-siRNA delivery to perivascular spaces computed as the colocalization of AQP4, demarcating the outer boundary of the PVS, with Cy5-tagged L2-siRNA. Area overlap is determined by the Mander's coefficient, which reflects the fraction of AQP4 staining that is Cy5 positive. The overall association between the signals was determined using Pearson's correlation with predefined regions of interest for different brain regions. Scale bar = 200 μ m. N=2 mice per treatment, mean \pm SD.



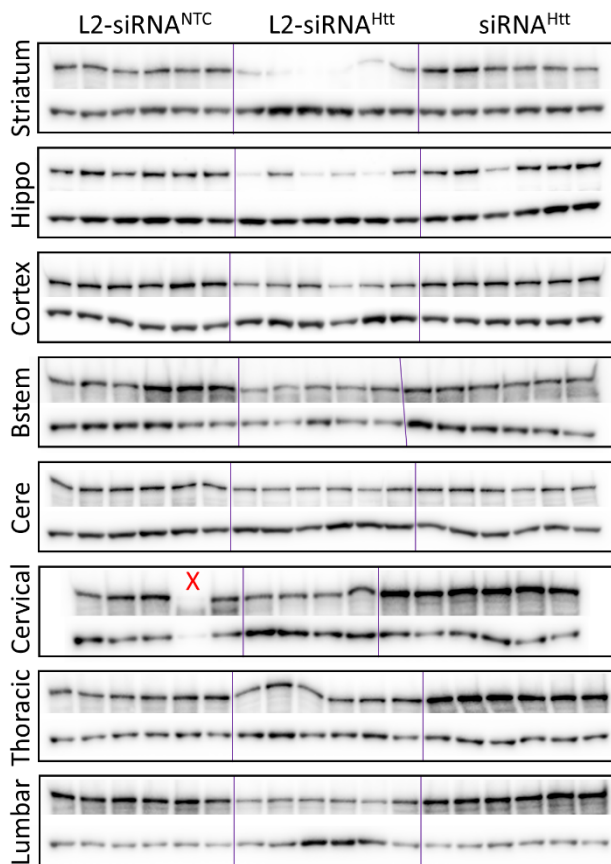
Supplementary Figure 5: Comparison of Htt expression between negative controls

Knockdown of Htt was assessed one month after ICV injection of vehicle (0.9% NaCl) or L2-siRNA^{NTC} (15 nmol) at the mRNA level by RT-qPCR (A) and protein level by western blot (B). Each data point represents one mouse (N=6), is normalized to vehicle, and represented as mean \pm SD. Statistics were computed as unpaired, two-tailed t-tests (ns – not significant, * p <0.05, ** p <0.01). Hippo = hippocampus, Bstem = brainstem.

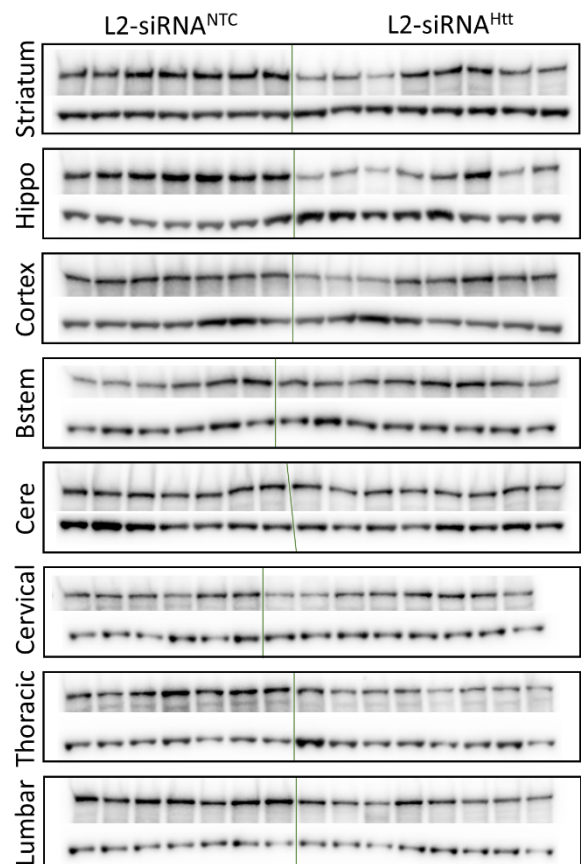
Western blots one-month gene silencing



B Three-month HTT western blots

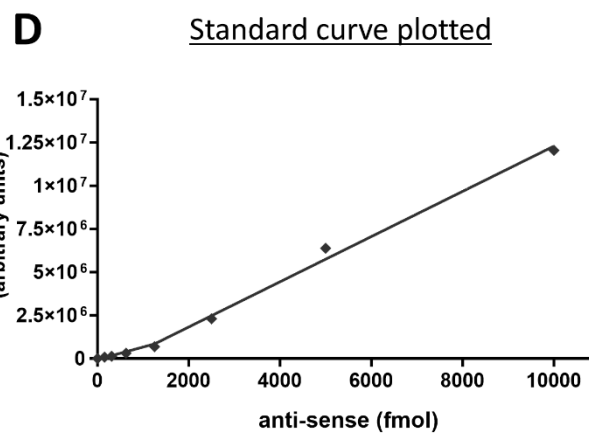
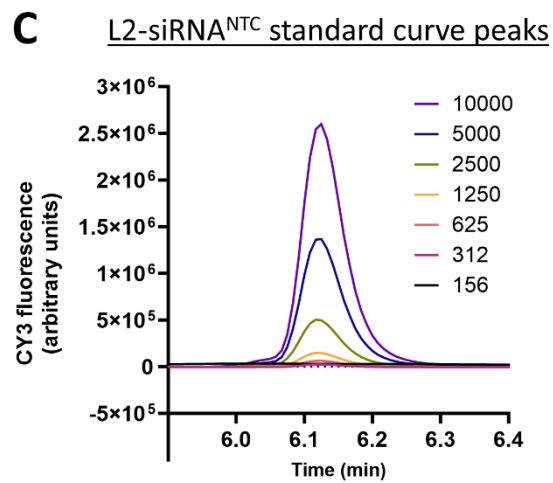
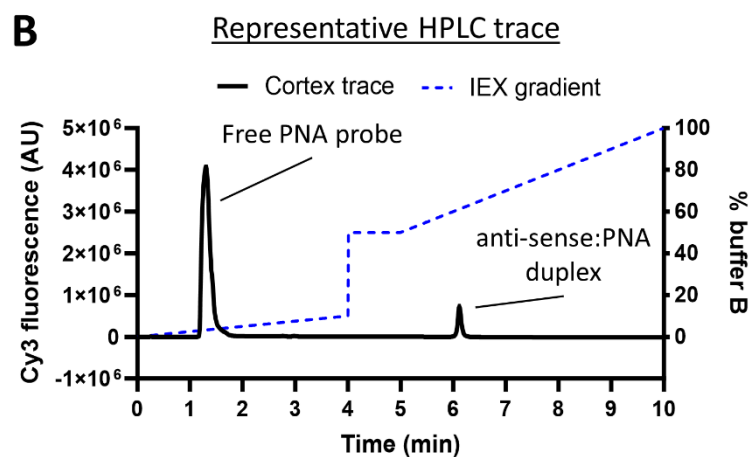
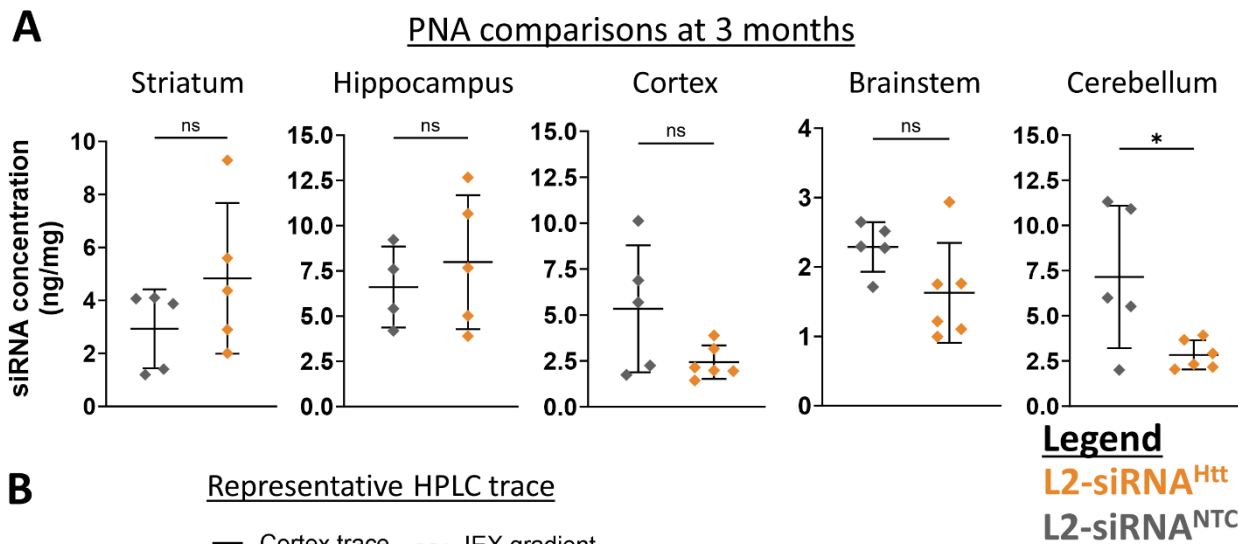


C Five-month HTT western blots



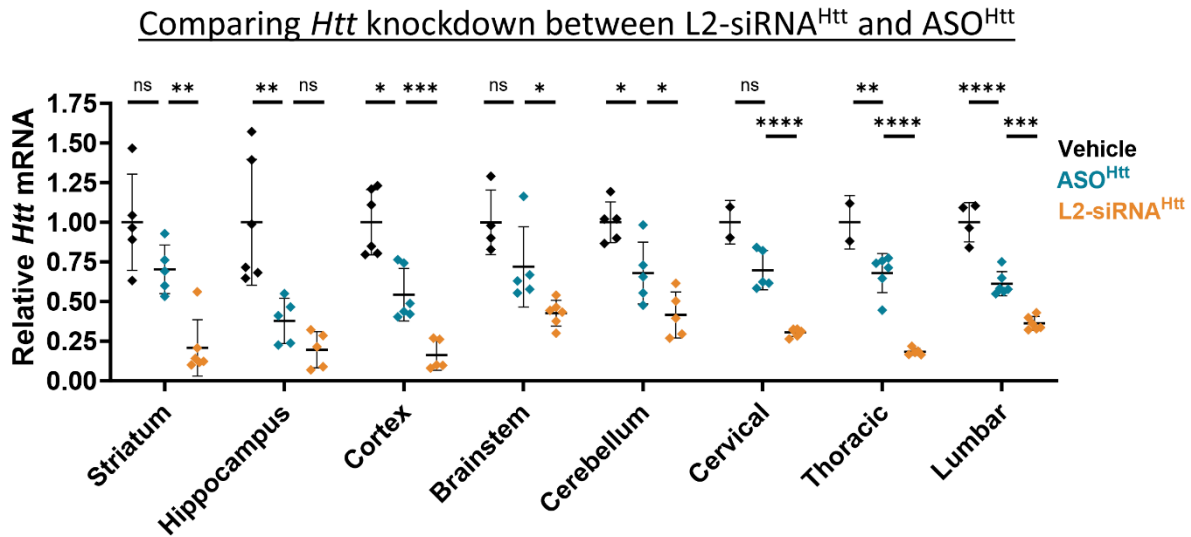
Supplementary Figure 6: Raw western blots to assess HTT protein silencing

Western blots associated with protein knockdown quantified in Figure 2C. For all boxes, the top bands are HTT and the bottom bands are the housekeeping gene beta-actin (ACTB). Each band represents an individual mouse (i.e. biological replicate). Blots are shown for each region and timepoint. Red X marks a sample with degraded protein that was excluded from analysis.



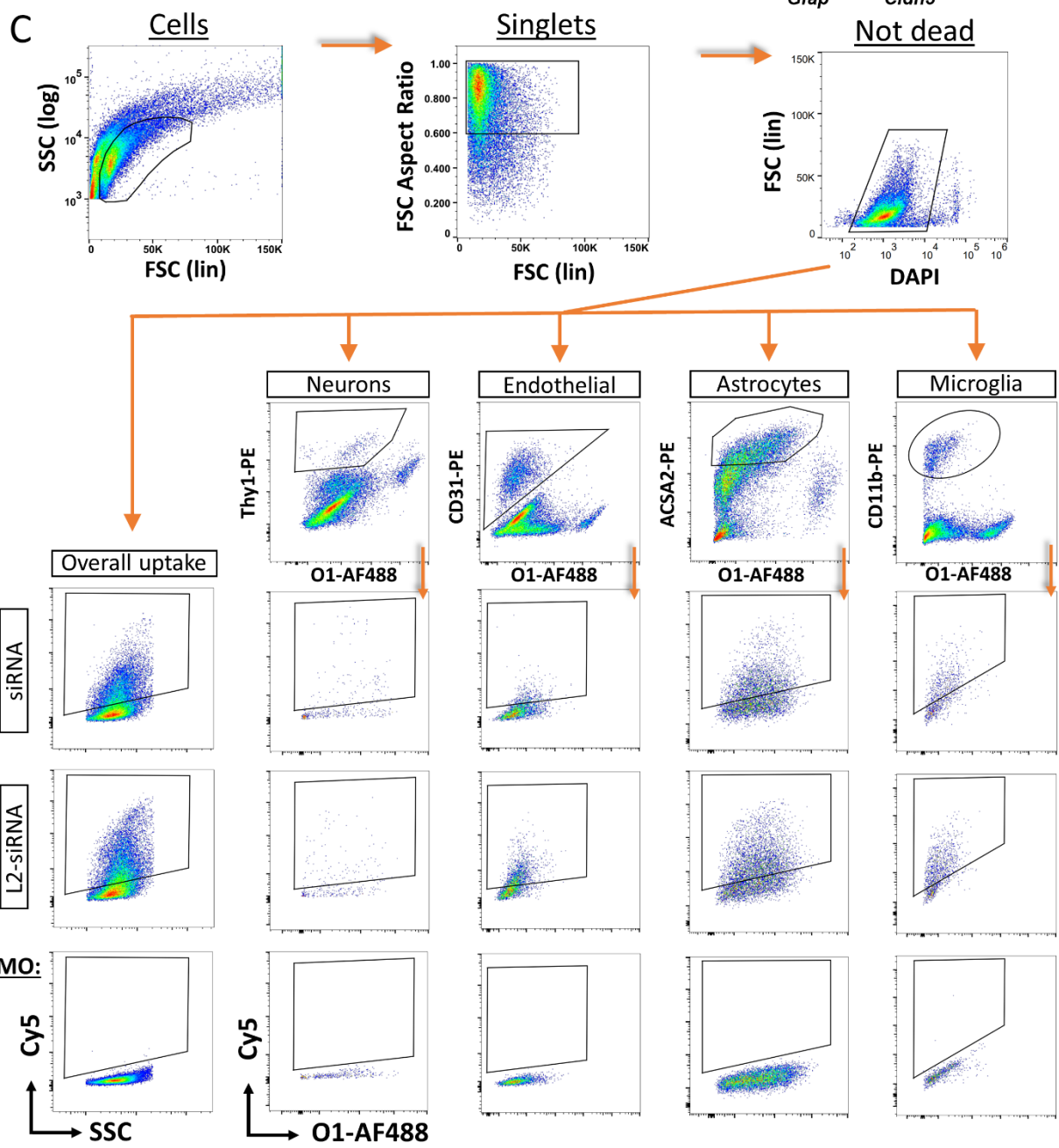
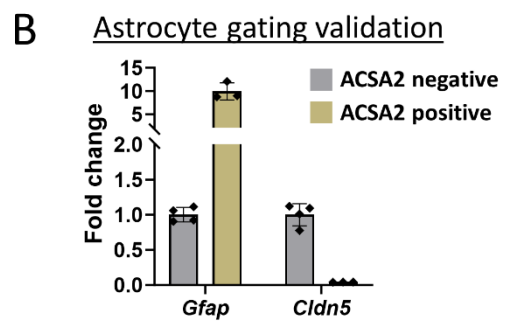
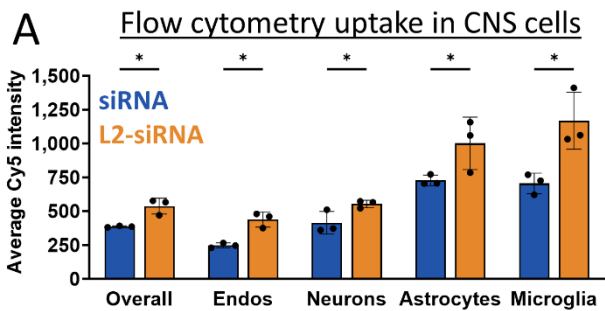
Supplementary Figure 7: PNA assay supporting information

- A. Comparing siRNA delivery between L2-siRNA^{NTC} and L2-siRNA^{Htt} 3 months after ICV injection (15 nmol). Units are nanograms (ng) of anti-sense strand per milligram (mg) of tissue. Mean \pm SD from N=5-6 biological replicates, two-tailed unpaired t-tests for each region (ns – not significant, *p<0.05).
- B. Representative HPLC trace showing elution of anti-sense:PNA complex overlayed with ion-exchange gradient (dashed blue line). Trace shown from a cortex sample of L2-siRNA^{NTC}.
- C. Raw peaks for L2-siRNA^{NTC} standard curve (in fmol units).
- D. Quantification of peaks represented with a bilinear fit for L2-siRNA^{NTC} standard curve.



Supplementary Figure 8: Comparing Htt-EG18 silencing of *htt* with preclinical ASO.

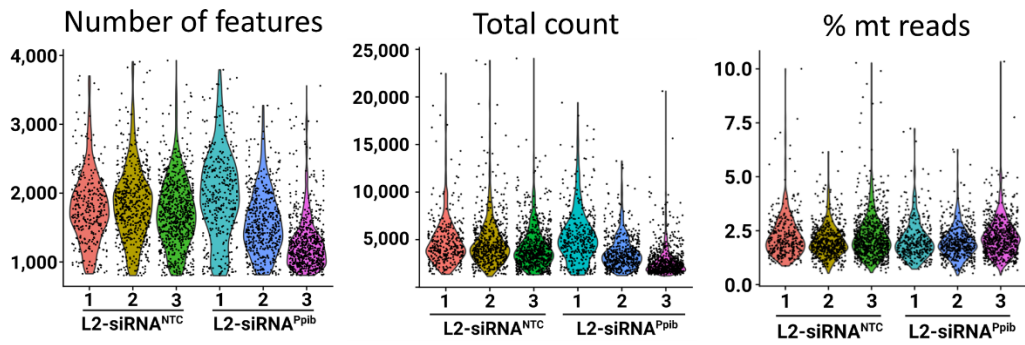
Mice were administered 15 nmol of ASO^{Htt} or L2-siRNA^{Htt} ICV and knockdown was normalized to a vehicle (0.9% NaCl) control. The ASO^{Htt} sequence targeting was designed elsewhere¹ and ordered from IDT. *Htt* levels were determined by RT-qPCR one month after injection. Since the ASO^{Htt} and L2-siRNA^{Htt} knockdown studies were performed on different days, each sample is normalized to their respective cohort of vehicle controls, and only the vehicle from the ASO^{Htt} study is plotted here. Each point represents a mouse (N=6) and bars represent mean \pm SD. One-way ANOVA was performed for each region comparing ASO^{Htt} to vehicle, L2-siRNA^{Htt} to ASO^{Htt} (Bonferroni's correction for multiple comparison).



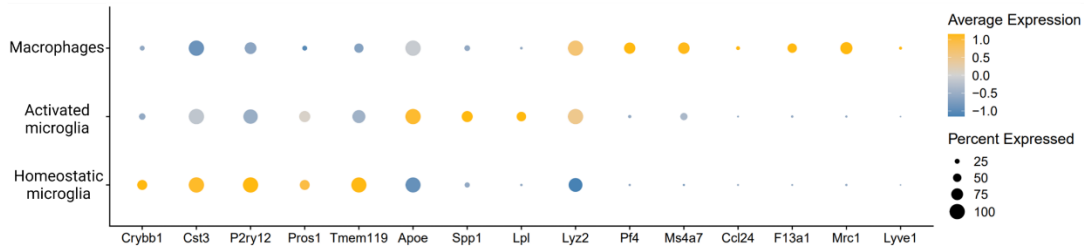
Supplementary Figure 9: Flow cytometry assessment of cell-specific uptake

- A. Cell-specific uptake assessed 48 hours after ICV injection of L2-siRNA or siRNA (10 nmol). Data reported as mean Cy5 intensity of live cells. Statistics computed as unpaired, two-tailed t-tests corrected for multiple comparisons using the false discovery rate (FDR) approach with $Q=5\%$. $N=3$ mice, data presented as mean \pm SD.
- B. Validation of astrocyte gating strategy by performing RT-qPCR on fluorescence activated cell sorting (FACS) purified ACSA2+/O1- and ACSA2-/O1- cells.
- C. Gating strategy for identifying cell types and measuring Cy5 uptake. All samples are pre-gated to separate cells from debris, single cells from doublets, and live cells from DAPI+ dead cells. Neurons are defined as Thy1+/O1-, endothelial cells as CD31+/O1-, Astrocytes as ACSA2+/O1-, Microglia/macrophages as CD11b+/O1-. Within these cell populations, Cy5+ events are gated using fluorescence-minus one (FMO) controls unique to each cell type. Representative plots are shown (median of each triplicate). AF488 = alexafluor 488, PE = phycoerythrin, FSC = forward scatter, SSC = side scatter.

A Quality control and filtering of CD11b^{POS} cells



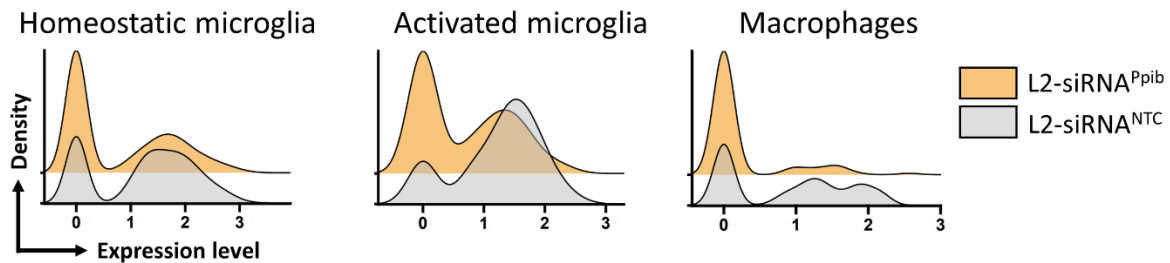
B Cell-type annotation



C Table of cell yield per sample

	Total CD11b+	Homeostatic microglia	Activated microglia	Macrophages	Unknown
L2-siRNA ^{NTC} 1	343	293	28	18	4
L2-siRNA ^{NTC} 2	573	480	53	29	11
L2-siRNA ^{NTC} 3	771	645	72	49	5
L2-siRNA ^{Ppib} 1	368	319	25	23	1
L2-siRNA ^{Ppib} 2	505	437	24	33	11
L2-siRNA ^{Ppib} 3	701	615	25	51	10

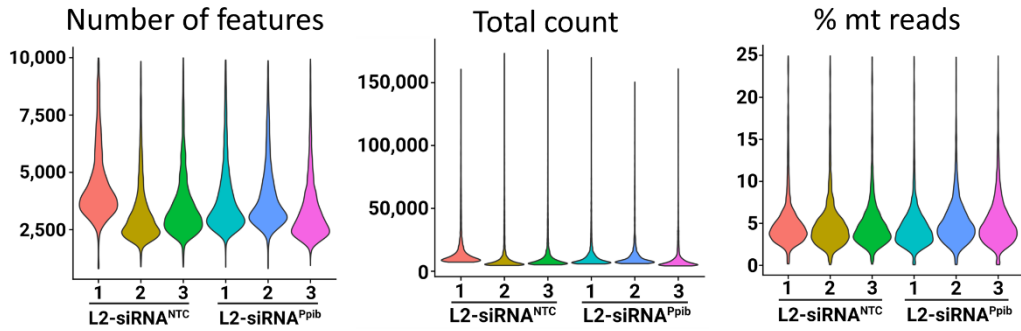
D *Ppib* expression density plots



Supplementary Figure 10: CD11b^{pos} scRNA-seq supporting information

- A. Standard quality control metrics were employed for each sample processed with T2 PIPseq kits. Cells were filtered based on number of features, keeping those containing 800-4,000 uniquely expressed genes. Total count represents the absolute number of transcripts detected and percent mitochondrial (% mt) reads is the fraction of transcripts associated with mitochondrial mRNA.
- B. Dotplot of canonical Cd11b^{pos} population markers.
- C. Number of cells identified in each CD11b^{pos} population for all replicates.
- D. Smoothed ridgeline density plots showing changes in *Ppib* expression between L2-siRNA^{NTC} and L2-siRNA^{*Ppib*}.

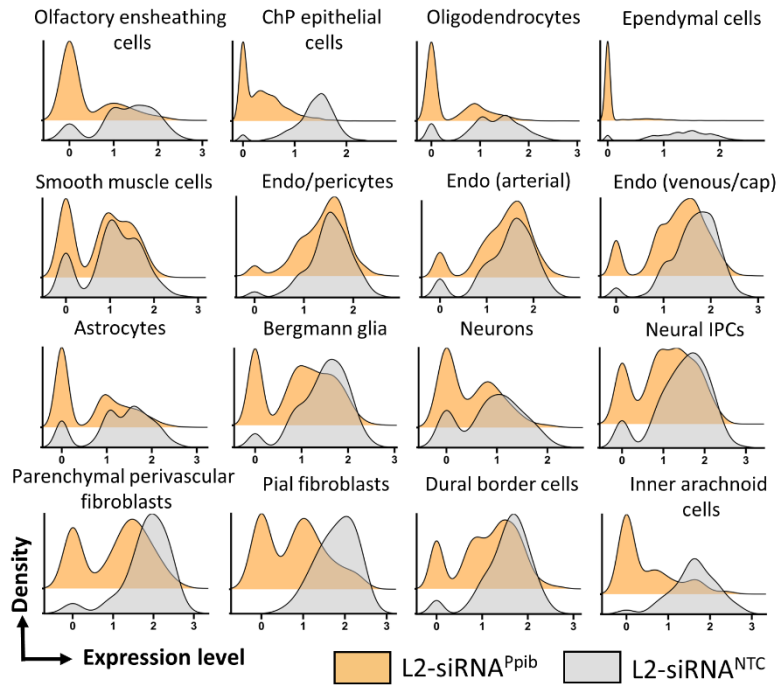
A Quality control and filtering of CD11b^{neg}



B Table of cell yield per sample

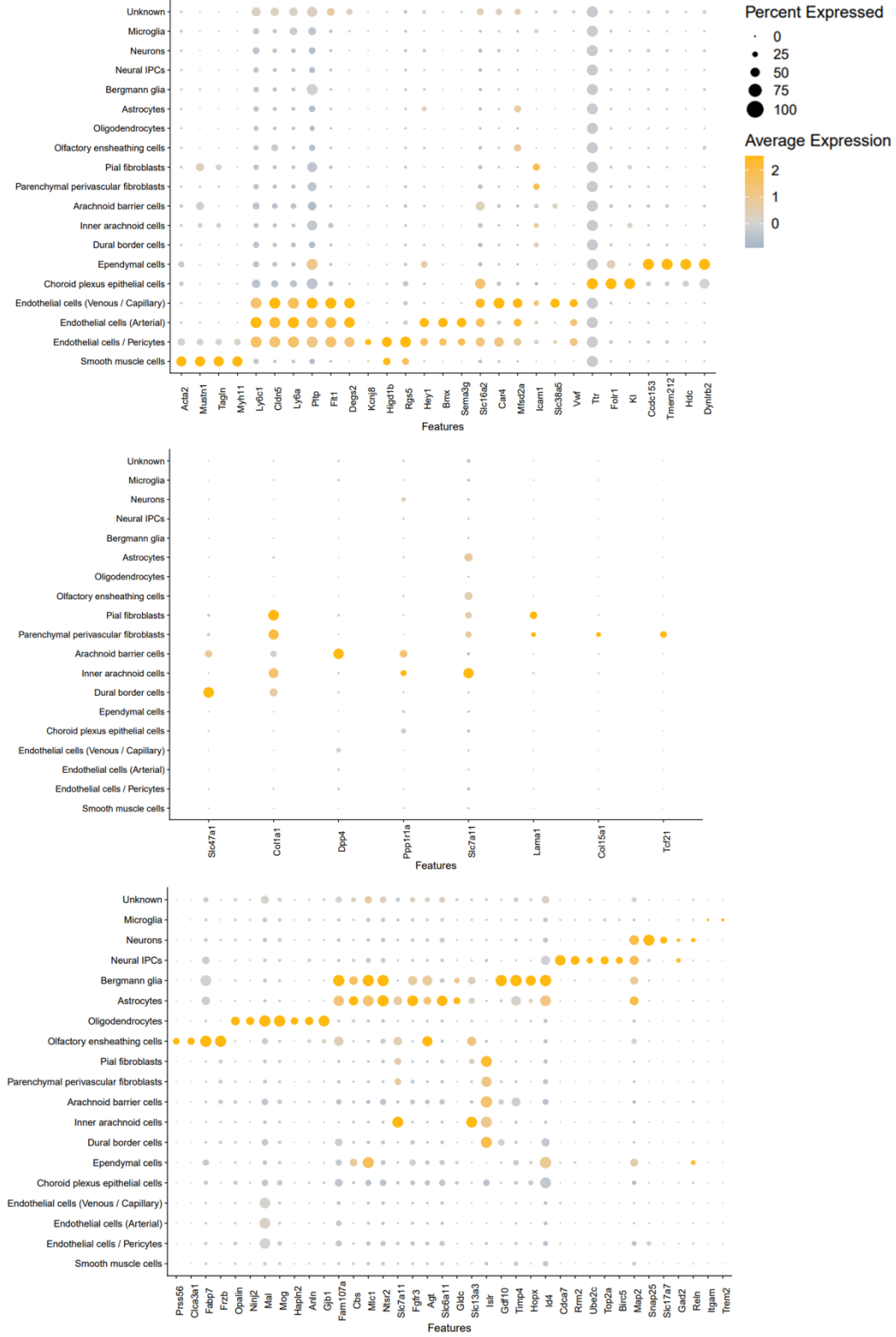
	L2-siRNA ^{NTC}	L2-siRNA ^{Ppib}		L2-siRNA ^{NTC}	L2-siRNA ^{Ppib}
Smooth muscle cells	221	300	Dural border cells	508	368
Endo/Pericytes	284	231	Inner arachnoid cells	107	108
Endo (arterial)	446	517	Arachnoid barrier cells	33	35
Endo (venous/capillary)	1015	1310	Parenchymal perivascular fibroblasts	64	77
ChP epithelial cells	2481	2259	Pial fibroblasts	50	48
Ependymal cells	2725	2195	Olfactory ensheathing cells	101	353
Oligodendrocytes	2548	2368	Neural IPCs	359	308
Astrocytes	770	1965	Neurons	182	104
Bergmann glia	960	416	Microglia	62	50

C Ridgeline plots of *Ppib* expression



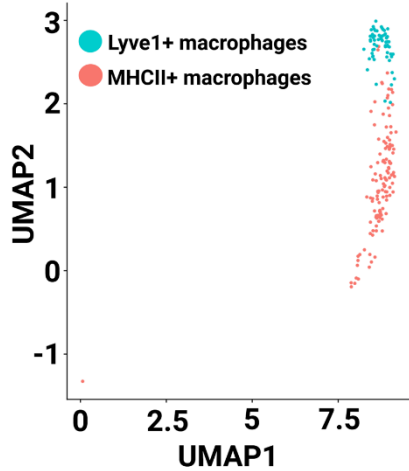
D

Cell type annotation

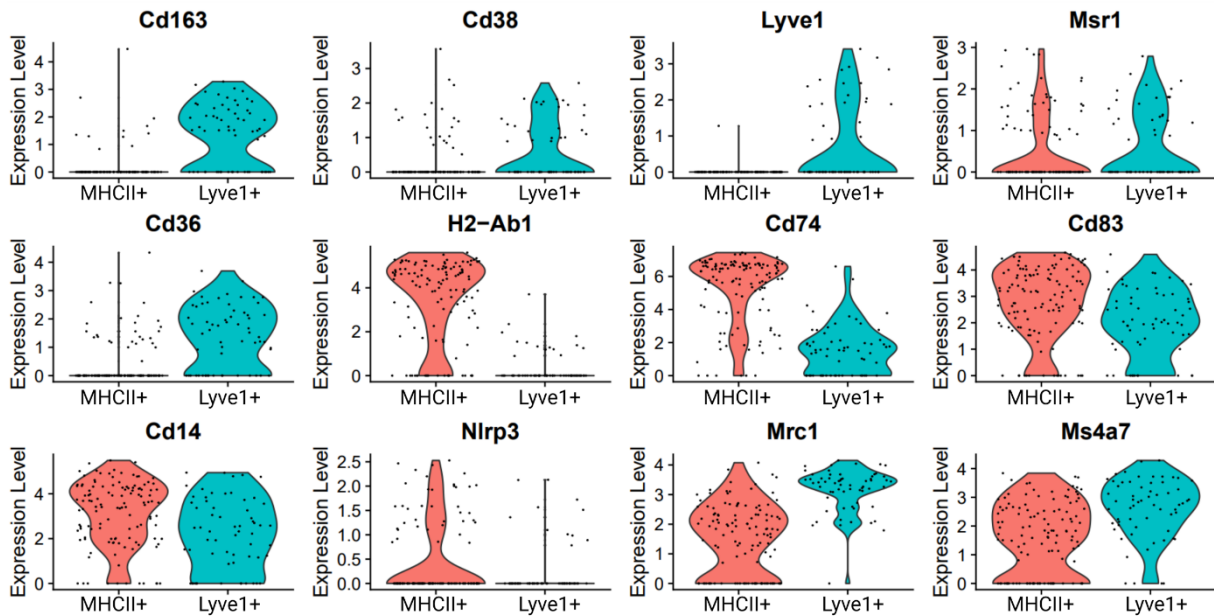


Supplementary Figure 11: CD11b^{neg} scRNA-seq supporting information

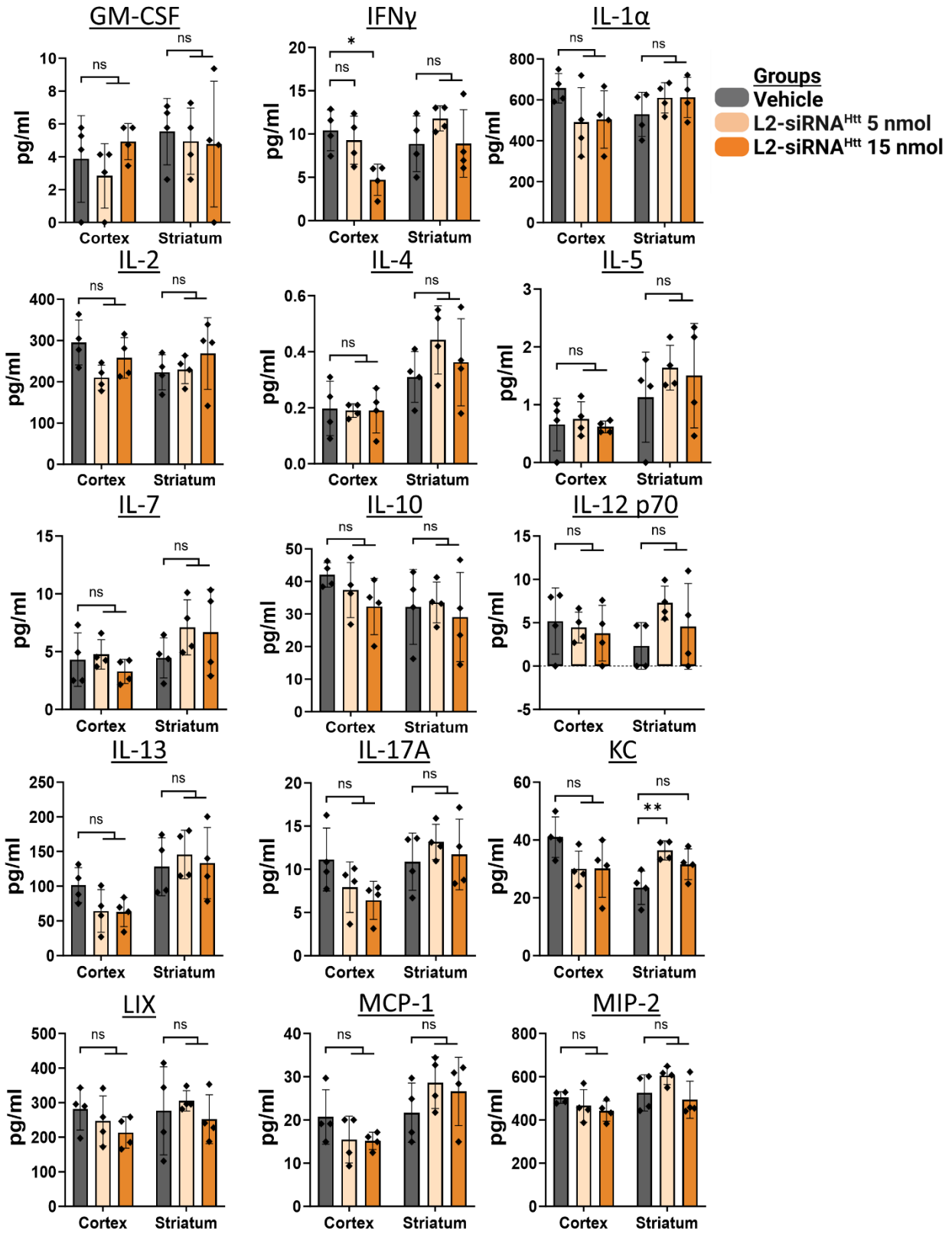
- A. Standard quality control metrics were examined for each sample processed with T20 PIPseq kits. Cells were filtered based on number of features, keeping those containing 800-10,000 uniquely expressed genes.
- B. Table of cell yield for L2-siRNA^{NTC} and L2-siRNA^{Ppib}, reflecting the sum of cells from N=3 samples for each treatment.
- C. Smoothed ridgeline density plots of *Ppib* expression for L2-siRNA^{NTC} and L2-siRNA^{Ppib} in each CD11b^{neg} population identified.
- D. Dotplot of canonical Cd11b^{neg} population markers.

A Macrophage subclusters**B** Cell yield per sample

	MHCII+	Lyve1+
L2-siRNA ^{NTC} 1	9	6
L2-siRNA ^{NTC} 2	16	11
L2-siRNA ^{NTC} 3	36	13
L2-siRNA ^{Ppib} 1	13	8
L2-siRNA ^{Ppib} 2	18	9
L2-siRNA ^{Ppib} 3	31	15

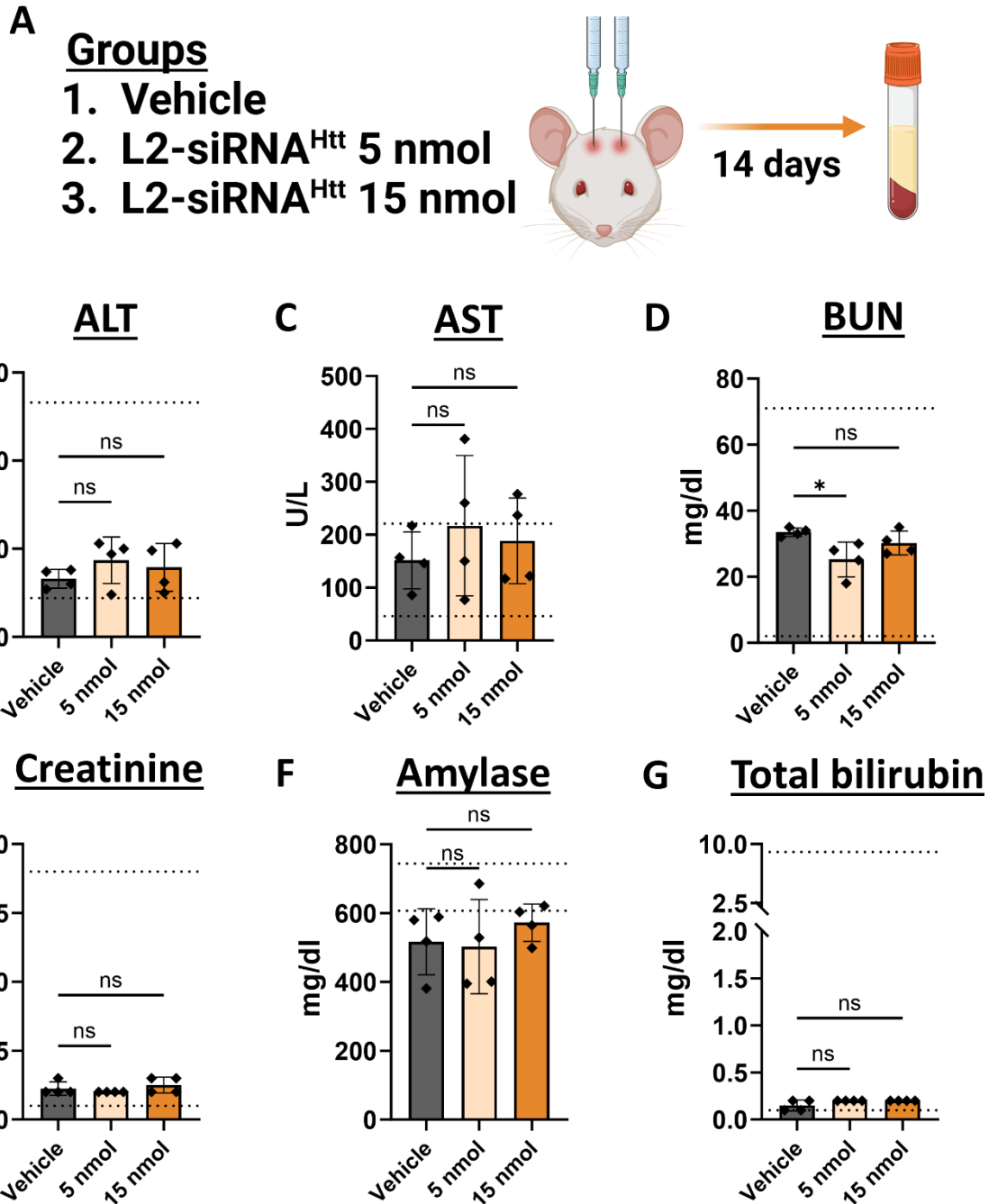
C Annotation of macrophage populations**Supplementary Figure 12: Identifying macrophage subtypes**

- UMAP projection plot showing re-clustering of macrophages into Lyve1+ and MHCII+, as described elsewhere.²
- Number of cells identified in each macrophage population for all replicates combined.
- Annotation of gene expression in Lyve1+ and MHCII+ macrophages.



Supplementary Figure 13: Inflammatory protein ELISA panel

Two weeks after ICV injection of vehicle (0.9% NaCl) or L2-siRNA^{Htt} (5 nmol or 15 nmol), cortex and striatum homogenates were analyzed for presence of inflammatory proteins (cytokines, chemokines, growth factors). All samples below the limit of detection on the standard curve were treated as 0 in terms of graphical presentation and statistical analysis. All samples were below the limit of detection for TNF α , and therefore data are not shown. All data are presented as mean \pm SD. A one-way ANOVA with Bonferroni's correction was performed for each region and protein (ns – not significant, * $p < 0.05$, ** $p < 0.01$). GM-CSF, granulocyte-macrophage colony stimulating factor; IFN γ , interferon gamma; KC, keratinocyte chemoattractant; LIX, Lipopolysaccharide-induced CXC chemokine; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2.



Supplementary Figure 14: Serum chemistry assessment of organ toxicity

Mice were injected ICV with a vehicle (0.9% NaCl) or L2-siRNA^{Htt} (5 nmol or 15 nmol), and blood serum was isolated after two weeks. Six standard markers were measured in this panel: alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine, amylase, and total bilirubin. Data are presented as mean \pm SD. Statistics are computed as a one-way ANOVA with Bonferroni's correction compared to vehicle (ns – not significant, * $p < 0.05$). Standard chemistry reference range was obtained from UCLA Division of Laboratory Animal Medicine and is represented by dotted lines.

Name	Sequence (5'->3')	Location	Length	Ref
Htt S	(MeU)*(fA)*(MeU)(fA)(MeU)(fC)(MeA)(fG)(MeU)(fA)(MeA)(fA)(MeG)(fA)(MeG)(fA)(MeU)(fU)*(MeA)*(fA)	10150	20	[24]
Htt AS	VP(meU)*(fU)*(MeA)(fA)(MeU)(fC)(MeU)(fC)(MeU)(fU)(MeU)(fA)(MeC)(fU)(MeG)(fA)(MeU)(fA)*(MeU)*(fA)	10150	20	[24]
PPIB S	(MeA)*(fA)*(MeC)(fA)(MeG)(fC)(MeA)(fA)(MeA)(fU)(MeU)(fC)(MeC)(fA)(MeU)(fC)(MeG)(fU)*(MeG)*(fA)	437	21	[34]
PPIB AS	VP(meU)*(fC)*(meA)(fC)(meG)(fA)(meU)(fG)(meG)(fA)(meA)(fU)(meU)(fU)(meG)(fC)(meU)(fG)*(meU)*(fU)	437	21	[34]
NTC S	(fC)*(MeA)*(fA) (MeU) (fU) (MeG) (fC) (MeA) (fC) (MeU) (fG) (MeA) (fU) (MeA) (fA) (MeU) (fG)*(MeA)*(fA)	NA	19	[29]
NTC AS	VP(MeU)*(fU)*(MeC) (fA) (MeU) (fU) (MeA) (fU) (MeC) (fA) (MeG) (fU) (MeG) (fC) (MeA) (fA) (MeU)*(fU)*(MeG)	NA	19	[29]
ASO ^{Htt}	CTCGActaaagcaggATTTC	4042	20	[8]
Htt PNA probe	5'/N Cy3-OO-TATATCAGTAAAGAGATTAA 3'/C	NA	20	[24]
NTC PNA probe	5'/N Cy3-OO-GGGACTGGCTAGTTAAACA 3'/C	NA	19	NA

Key: S=sense strand, AS=anti-sense strand, *=phosphorothioate, Me=2'-O-Methyl, f=2'fluoro, VP=vinyl phosphonate, O = ethylene glycol linker, lowercase = DNA, for ASO^{Htt} uppercase = 2'-O-(2-methoxy)ethyl

Supplementary Table 1: Nucleic acid sequences used