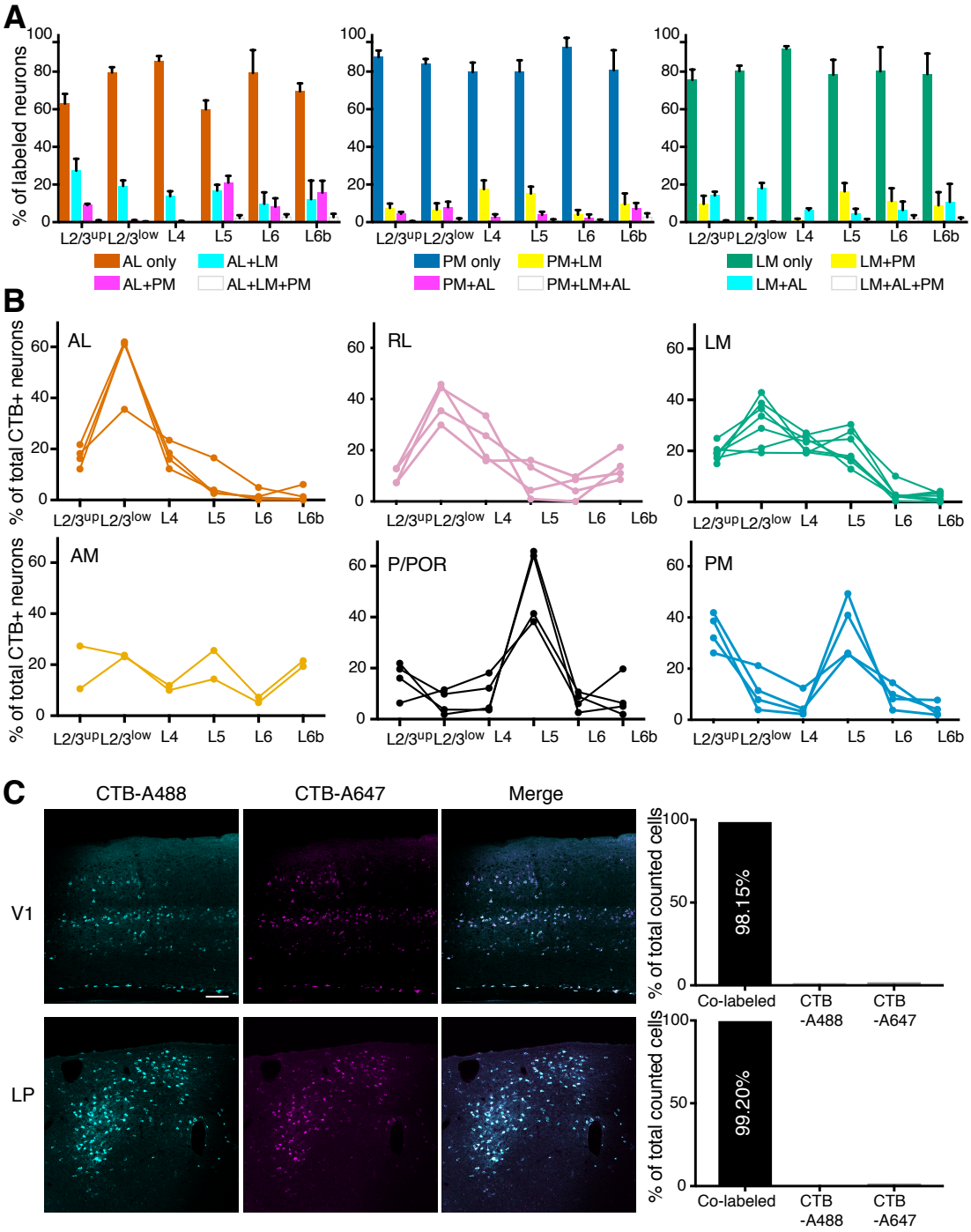

Figure S1. Additional Data to Analyze CTB-labeled V1 Neurons Projecting to HVAs, Related to Figure 1. (A) Distributions of single, double, triple labeled V1 CCPNs in specific layers after CTB injections to AL, PM, and LM. Plots displayed for AL-projecting neurons (left), PM-projecting neurons (middle), and LM-projecting neurons (right). **(B)** Distributions of V1 neuronal locations projecting to AL, RL, LM, AM, P/POR, and PM for individual animals. **(C)** Labeling efficiency of CTB. A mixture of CTB-A488 (final 0.33%) and CTB-A647 (final 0.33%) was injected into a lateral V2 region of mouse brain. Co-labeled neurons were counted in V1 (top) and LP (bottom). Scale bar, 100μm.

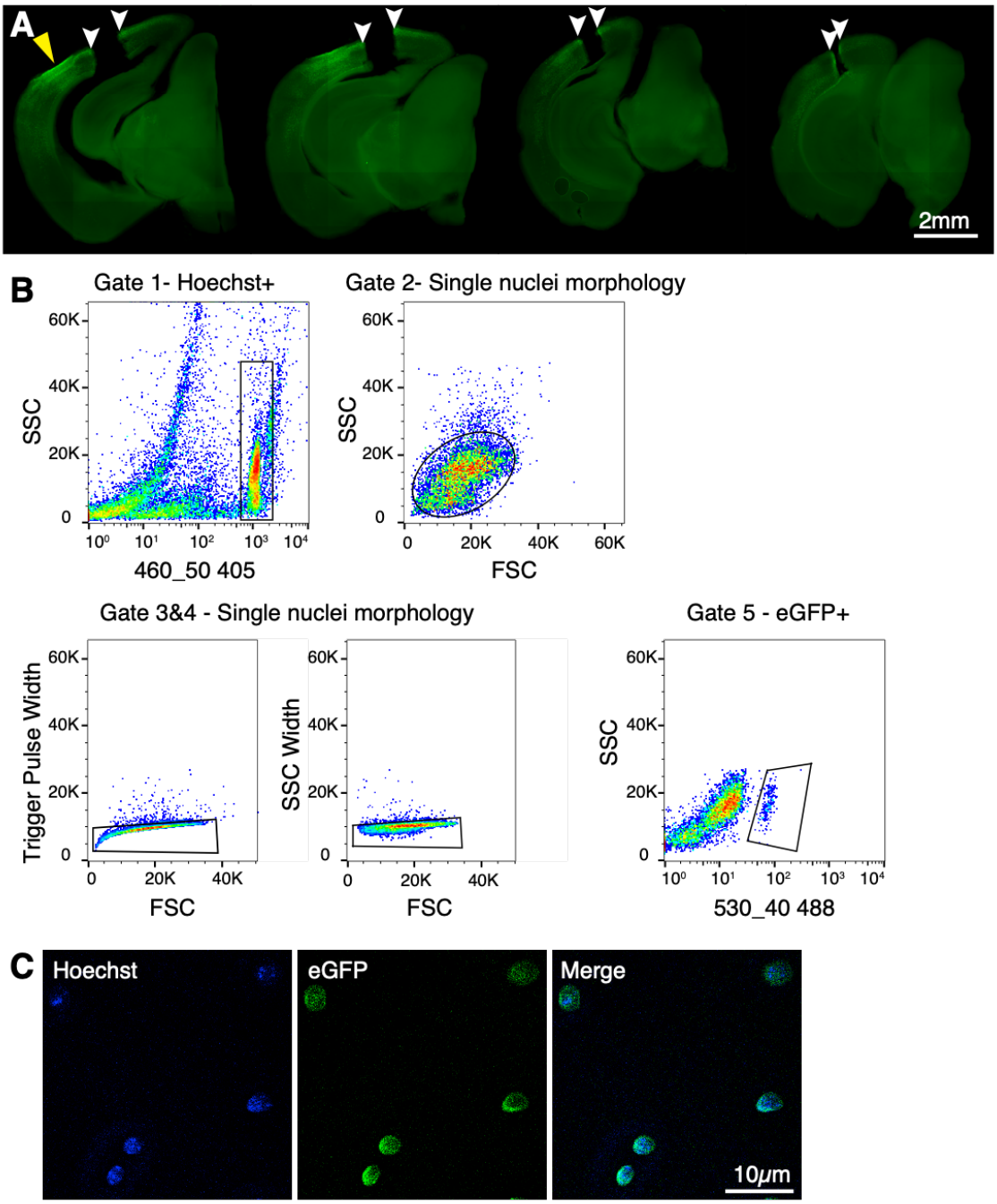

Figure S2. Experimental Details on GFP+ Single Nuclei Dissociation Workflow, Related to Figure 2. (A) Examples of coronal brain slices of *R26R-CAG-loxp-stop-loxp-Sun1-sfGFP-Myc* after V1 dissections (white arrowheads). AAVretro-Cre was injected in AL (yellow arrow). **(B)** Detailed gating information of fluorescence-activated nuclei sorting (FANS). Gate 1 selects Hoechst+ nuclei to exclude debris, while gates 2-4 exclude cell doublets based on single nuclei morphology. Gate 5 selects high eGFP fluorescence. **(C)** Post-sorting examination of single nuclei on the slide for quality control.

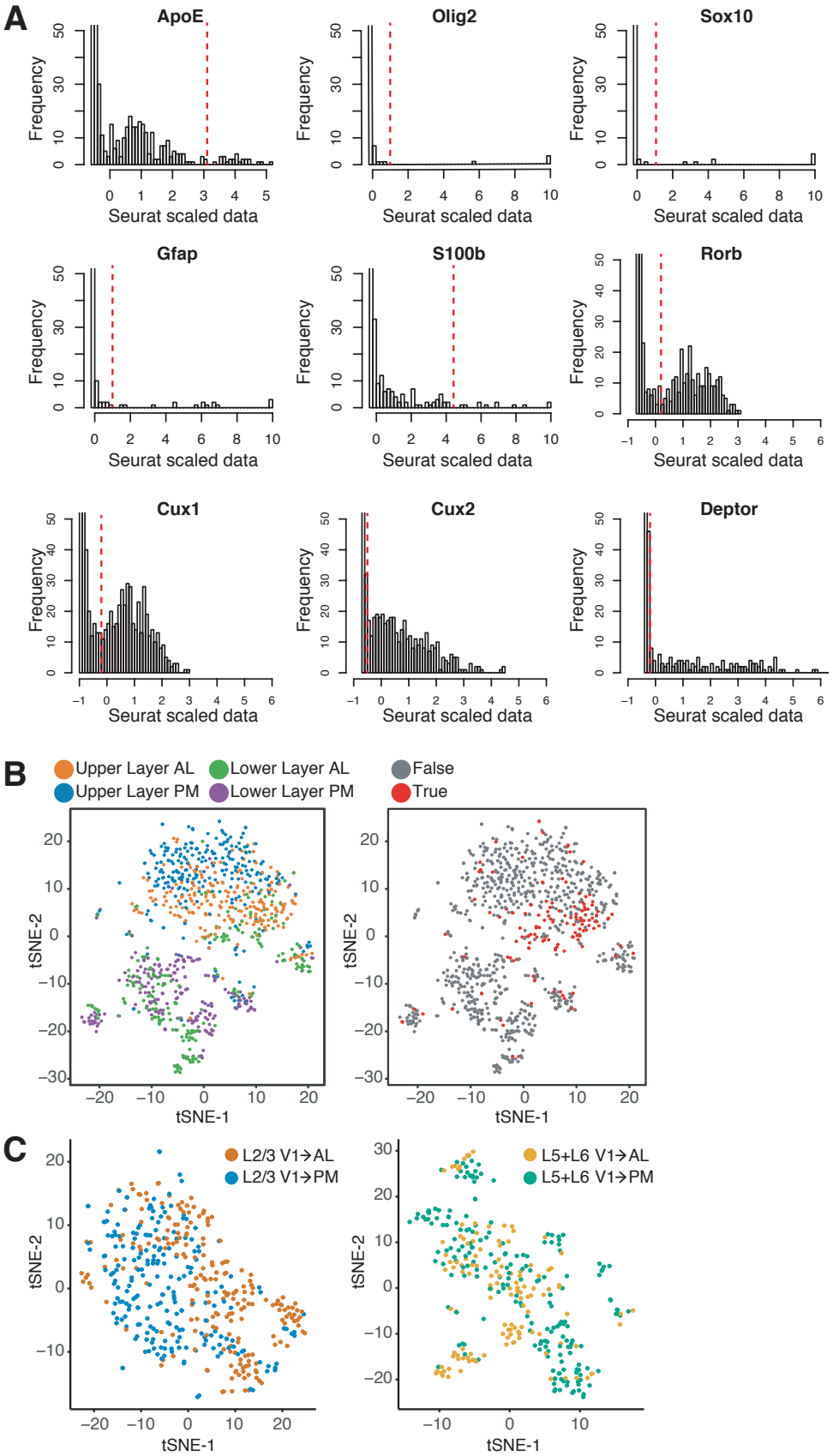

Figure S3. Additional Data for Single-nuclei RNA Sequencing, Related to Figure 2. (A) Data analysis

for filtering out glia cells and L4 neurons. The molecular marker distributions of Seurat scaled logNorm values across all cells to filter glia at specified expression level cutoffs (ApoE: 3.1, Olig2: 1, Sox10: 1, Gfap: 1, S100b: 4.2). The molecular marker distributions of Seurat scaled logNorm values across remaining cells after filtering out glia to define L4 neurons at specified expression level cutoffs (Rorb: 0.2, Cux1: -0.2, Cux2: -0.5, Deptor: -0.2). **(B)** tSNE plot of all neurons labeled by dissected layers and projections (left) and tSNE plot labeled by L4 (true) and non-L4 (false) neurons (right). **(C)** Unsupervised clustering analysis using tSNE plots based on single nuclei level gene expressions of L2/3 (left) or L5 and L6 (right) neurons.

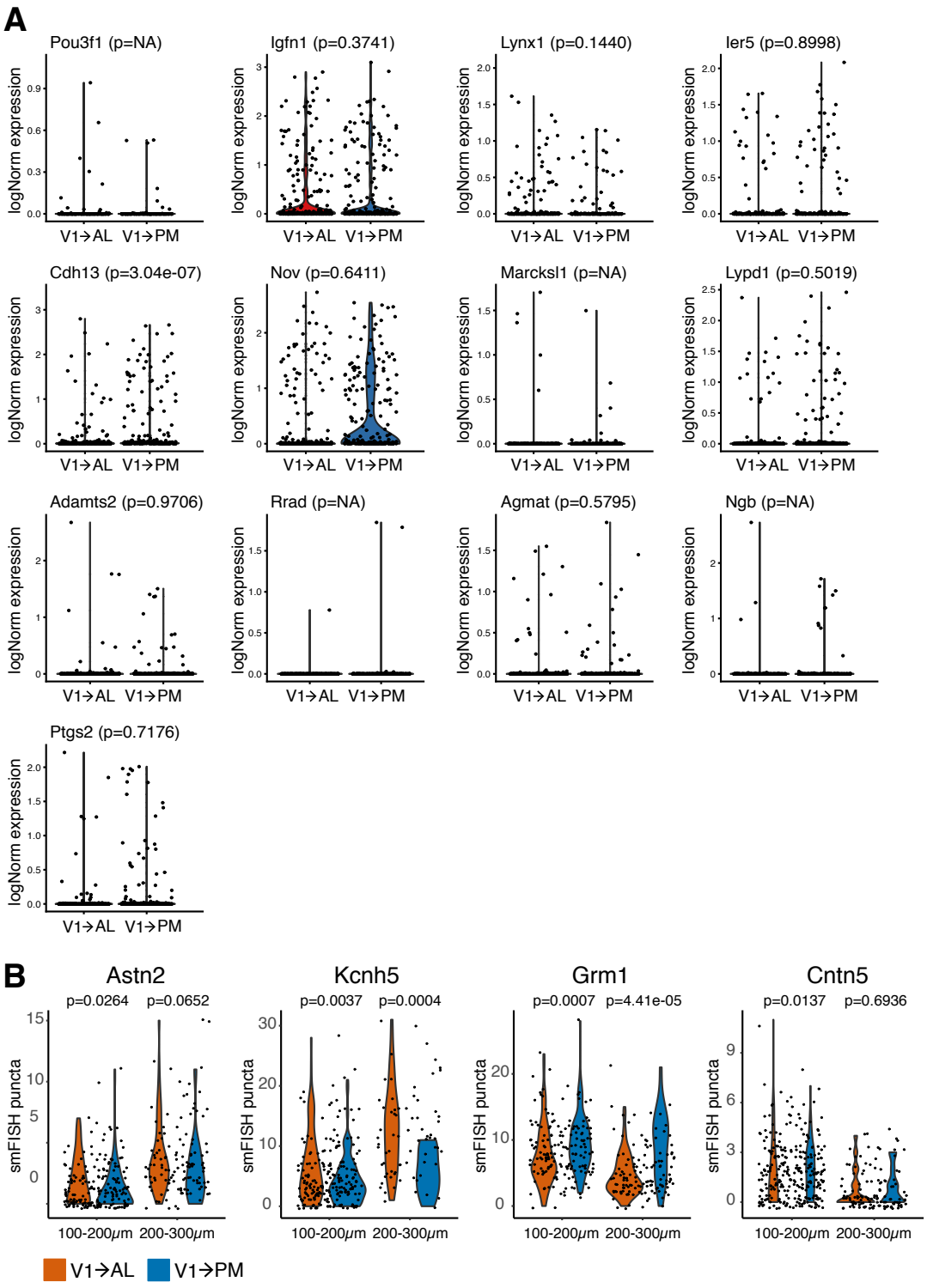

Figure S4. Additional Data for Single-nuclei RNA Sequencing and Single Molecule FISH (smFISH), Related to Figure 2. (A) Gene expression profiles of the reported L2/3 sub-cluster molecular markers on V1→AL and V1→PM neurons. L2/3 sub-cluster makers published in Hrvatin et al. 2018 (Hrvatin et al., 2018) are Pou3f1, Igfn1 Lynx1, Ier5, Cdh13, Nov, Marcksl1, and Lypd1. L2/3 sub-cluster makers published in Tasic et al. 2018 (Tasic et al., 2018) are Adamts2, Rrad, and Agmat. L2/3 sub-cluster makers

published in Tasic et al. 2016 (Tasic et al., 2016) are Ngb and Ptgs2. P values were reported as NA when the gene was expressed in less than 5% of cells. **(B)** smFISH puncta counts within two different cortical depth bins from the pia for V1→AL and V1→PM neurons. Violin plots displaying smFISH puncta count of four differentially expressed gene markers per cell on V1→AL and V1→PM types. Counts were done with two bins, 100-200μm and 200-300μm from the pia. Wilcoxon rank-sum test was used for statistical analyses in three animals (except Kcnh5 for two animals).

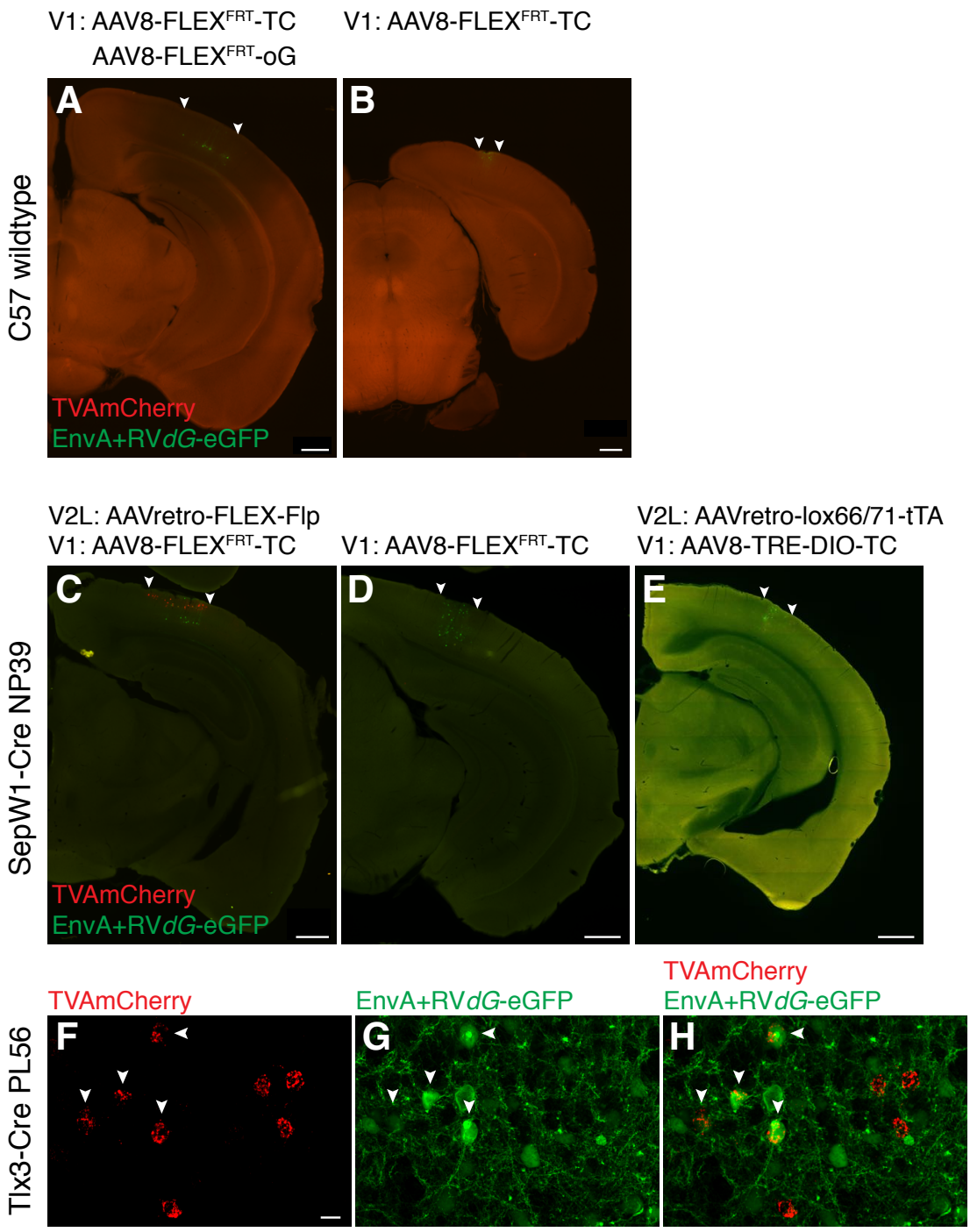

Figure S5. Control Experiments for Rabies Trans-synaptic Tracing, Related to Figure 3. (A-B) Coronal sections of C57 wildtype mouse brains after AAV8-FLEX^{frt}-TVAmCherry(TC) and AAV8-FLEX^{frt}-oG **(A)** or AAV8-FLEX^{frt}-TC **(B)** injections followed by EnvA+RV*dG*-eGFP injection on V1. **(C-D)** Coronal sections of SepW1-Cre NP39 mouse brains after AAV8-FLEX^{frt}-TC injections on V1 and AAVretro-FLEX^{loxp}-Flp on V2L **(C)** or AAV8-FLEX^{frt}-TC **(D)** injection on V1 followed by EnvA+RV*dG*-eGFP injection on V1. **(E)** Coronal section of SepW1-Cre NP39 mouse brain after

AAVretro-lox66/71-tTA on V2L and AAV8-TRE-DIO-TC injection on V1 followed by EnvA+RV*dG*-eGFP injection on V1. Scale bars, 500μm. **(F-H)** Tangential section of Tlx3-Cre PL56 mouse V1 showing putative TVAmCherry+ eGFP+ starter neurons (arrowheads) after AAV8-FLEX^{frt}-TC and AAV8-FLEX^{frt}-oG followed by EnvA+RV*dG*-eGFP injection on V1. Scale bar, 10μm.

Mouse #	CTB-A488	CTB-A555	CTB-A647
C-01	RL		LM
C-02	RL	LM	
C-03		RL	LM
C-04		P/POR	AM
C-05	P/POR	PM	
C-06	PM	P/POR	LM
C-07		P/POR	AM
C-08	AL	PM	LM
C-09	AL	LM	
C-10	AL	PM	LM
C-11	LM	PM	AL
C-12			RL

P/POR	LM	AL	RL	AM	PM
C-04	C-01	C-08	C-01	C-04	C-06
C-05	C-02	C-09	C-02	C-07	C-08
C-06	C-06	C-10	C-03		C-10
C-07	C-08	C-11	C-12		C-11
	C-09				
	C-10				
	C-11				

Table S1. Summary of Cholera Toxin Subunit B Injected Animals, Related to Figure 1.

L2/3^{Upper}	P/POR	LM	AL	RL	AM	PM
P/POR		ns	ns	ns	ns	**
LM	ns		ns	ns	ns	**
AL	ns	ns		ns	ns	**
RL	ns	ns	ns		ns	****
AM	ns	ns	ns	ns		ns
PM	**	**	**	****	ns	
L2/3^{Lower}	P/POR	LM	AL	RL	AM	PM
P/POR		****	****	****	*	ns
LM	****		****	ns	ns	****
AL	****	****		**	****	****
RL	****	ns	**		ns	****
AM	*	ns	****	ns		ns
PM	ns	****	****	****	ns	
L4	P/POR	LM	AL	RL	AM	PM
P/POR		*	ns	*	ns	ns
LM	*		ns	ns	ns	***
AL	ns	ns		ns	ns	ns
RL	*	ns	ns		ns	**
AM	ns	ns	ns	ns		ns
PM	ns	***	ns	**	ns	
L5	P/POR	LM	AL	RL	AM	PM
P/POR		****	****	****	****	**
LM	****		**	*	ns	**
AL	****	**		ns	ns	****
RL	****	*	ns		ns	****
AM	****	ns	ns	ns		ns
PM	**	**	****	****	ns	
L6	P/POR	LM	AL	RL	AM	PM
P/POR		ns	ns	ns	ns	ns
LM	ns		ns	ns	ns	ns
AL	ns	ns		ns	ns	ns
RL	ns	ns	ns		ns	ns
AM	ns	ns	ns	ns		ns
PM	ns	ns	ns	ns	ns	
L6b	P/POR	LM	AL	RL	AM	PM
P/POR		ns	ns	ns	ns	ns
LM	ns		ns	ns	**	ns
AL	ns	ns		ns	*	ns
RL	ns	ns	ns		ns	ns
AM	ns	**	*	ns		*
PM	ns	ns	ns	ns	*	

Table S2. Two-way ANOVA Analysis with Tukey's Multiple Comparisons Test of CTB+ Neurons between Projection Areas and Layers, Related to Figure 1. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not statistically significant

Animals	T31	T35	T41	T44	T45	T50	T55
# nuclei	195	17	31	193	258	167	82
Projection	AL	PM					
# nuclei	461	482					
Dissection	Upper	Lower					
# nuclei	501	442					
Projection + Dissection	Upper AL	Upper PM	Lower AL	Lower PM			
# nuclei	239	262	222	220			
Plate	T31_1	T31_2	T31_3	T41/T35	T44_1	T44_2	T44_3
# nuclei	74	62	59	48	62	69	62
Plate	T45_1	T45_3	T45_4	T50_1	T50_3	T55_3	
# nuclei	86	84	88	84	83	82	
Sequencing Run	R1	R2	R3	R4	R5	R6	R7
# nuclei	74	121	131	110	167	170	170

Table S3. Summary of Single Nuclei Information for RNAseq, Related to Figure 2.

Supplemental tables below are provided separately as Excel files in a zip file.

Table S4. Single Nuclei Sequencing Sample Metadata, Related to Figure 2.

Table S5. Differentially Expressed Genes List for V1→AL and V1→PM in L2/3 Using Zinbwave-EdgeR, Related to Figure 2.

Table S6. Differentially Expressed Genes List for V1→AL and V1→PM in L5 and L6 Using Zinbwave-EdgeR, Related to Figure 2.

Table S7. Differentially Expressed Genes List for V1→AL and V1→PM in L2/3 Using Zinbwave-DESeq2, Related to Figure 2.

Table S8. Differentially Expressed Genes List for V1→AL and V1→PM in L5 and L6 Using Zinbwave-DESeq2, Related to Figure 2.

Table S9. Virus Injection and Animal Information for Rabies Tracing, Related to Figure 3.

Table S10. Quantification of Rabies Tracing Data, Related to Figure 3.