Supplementary Information

EXTENDED EXPERIMENTAL PROCEDURES

SUPPLEMENTAL FIGURES S1-S6

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EXTENDED EXPERIMENTAL PROCEDURES

Mouse

Chodl^{PLAP} mutant mice: The Chondrolectin genomic clone was subcloned using PCR from genomic DNA of embryonic stem (ES) cells followed by sequencing. We constructed the targeting vector by inserting the hPLAP-ACN cassette (Zylka et al., 2005) into the translation start ATG of the Chondrolectin gene. Targeted ES cells were generated and confirmed by Southern blotting. To detect the Chodl^{PLAP} mutant allele by PCR, PCR primers were designed as follows: *ks131*, 5'-GATGCTTACAGGCATCCTAG-3'; *ks135*, 5'-AACCTAGTGCAGAGTACTCC-3'; *hPLAP/06*, 5'-AGAACCCGGACTTCTGGAACC-3'; *hPLAP/07*, 5'-TTCATCACGGAGATGACCTC-3'. The wild-type allele produces a ~300 bp fragment with *ks131* and *ks135* primers, whereas the mutant allele is detected by a ~700 bp fragment with *hPLAP/06* and *hPLAP/07* primers.

Pv::CreERt2 mice: The Pv::CreERt2 mice were generated using a strategy analogous to the one described previously (Hippenmeyer et al., 2005), but integrating CreERt2 instead of Cre into the Pv locus. Method for genotyping is also the same as described (Hippenmeyer et al., 2005). Pv::Cre mice (stock#008069) was purchased from the Jackson Laboratories.

RΦAP, RΦGFP and RΦtomato mice were all generated in our Laboratory using the same strategies (Arenkiel et al., 2011; da Silva et al., 2011; Que et al., 2008). The numbers of reporter gene expressing TG neurons were similar between the reporter mice (35±5 reporter labeled cells / single 10x field of view on 20μm sections of trigeminal ganglion). Therefore, the recombination efficiency and expression pattern of the reporter genes are essentially the same.

All experiments were conducted according to protocols approved by The Duke University Institutional Animal Care and Use Committee.

Tamoxifen Injections

Tamoxifen (Sigma) was dissolved in corn oil (10 mg/ml). The tamoxifen solution (0.05-0.1mg/g (Body Weight)) was injected subcutaneously into the back to Pv::CreERt2; RΦtomato mice at P1 (for P8 sample collection) or at P5 (for P28 sample collection). At P8 or P28, brains and mystacial pads were dissected and processed for immunostaining as described below.

Deficient rabies virus retrograde tracing experiment

Pv::Cre; RΦtomato; Chodl$^{PLAP/+}$ mice were anesthetized with ketamine/xylazine (50 mg/kg, 5 mg/kg, respectively, i.p.) at P4 and placed on a stereotaxic frame (David Kopf Instruments) equipped with a mouse and neonatal rat adaptor (Stoelting). Deficient rabies virus ΔG-GFP-RV (200 nl; ~1 × 10^6 infectious units per ml) was injected into right side of the VPM and PoM regions of thalamus with a microinjector (Nanoject II, Drummond Scientific) equipped with a pulled fine glass capillary. The following stereotaxic coordinates relative to Bregma were used: AP -0.7 mm, ML 1.2 mm, DV -2.75 mm. Brains were collected 3 days post infection.

Immunofluorescence

For brain and mystacial pad sections, coronal 50 µm (for P28 brains), 60µm (for P7 and P8 brains) and 150µm (for whisker pad) sections were cut on the cryostat. Sections were collected sequentially into the 24-well plate containing PBS (pH 7.4). The sections were washed with PBS and blocked with 10% Blocking One (nacalai tesque) in PBS containing 0.3% Triton X-100 (0.3% PBST) at 4°C overnight. Tissue sections were incubated with primary antibodies diluted in blocking solution at 4°C for 3 days. Then, sections were washed with PBS, and incubated with appropriate secondary antibodies diluted in blocking solution at 4°C for 2 days, washed again with PBS and mounted slide glasses. For PLAP immunostaing, tissue sections were incubated in 10mM sodium citrate buffer (pH8.5) at 80°C for 30 min before blocking. For 4-color immunostaining (GFP, PLAP, vGluT1 and tomato), tissue sections were incubated with Image-iT Signal Enhancer (Invitrogen) at room temperature for 1h after antigen retrieval.

The primary antibodies used for this study were: rabbit anti-RFP (Rockland, 600401379, 1:1000), chicken anti-GFP (Invitrogen, A10262, 1:1000), guinea-pig anti-vGlut1 (Millipore, AB5905, 1:1000), mouse anti-PLAP (Sigma, A2951, 1:1000), rat anti-CK8 (anti-Troma1) (Univ of Iowa/DSHB, 1:1000), rabbit anti-NF200 (Sigma, 1:1000). The secondary antibodies used were: Alexa 488 conjugated goat anti-mouse and -rabbit antibodies, Alexa 633 conjugated goat anti-guinea-pig antibodies (Invitrogen), Cy3 conjugated goat anti-rat antibodiy, Cy3 conjugated donkey anti-rabbit, Alexa 488 conjugated donkey anti-chicken, Alexa 405 conjugated donkey anti-guinea-pig, Alexa 647 conjugated donkey anti-mouse (Jackson ImmunoResearch).

Alkaline phosphatase staining.

Placental alkaline phosphatase (AP) staining was performed as previously described (Hasegawa et al., 2007). Tissue sections were collected with a cryostat at 20-30 µm thickness. The sections were postfixed with 4% PFA/PBS for 1 hour, followed by incubation at 65°C for 6 hours in PBS. For the detection of AP activity, the sections were rinsed with wash buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl) and then incubated in developing buffer [1:200 NBT/BCIP stock solution (Roche), 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM MgCl2].

For immunostainging combined with AP staining, AP staining was performed first as described above. Sections were then processed for immunostaining as described above.

In situ hybridization

Fluorescent two-color in situ hybridization was performed according to standard methods (Hasegawa and Wang, 2008). The mouse cDNA fragments of the neurotrophic receptors and Chondrolectin were amplified by PCR with the antisense primers containing the T7 promoter sequence. *In vitro* transcription was then performed from the PCR-amplified template using T7 RNA polymerase (Roche, Indianapolis, IN) with Digoxigenin-UTP (Roche) for the synthesis of the antisense probes.

Preparation of confocal stacks for image processing

Z-stack images were taken by Zeiss 710 at optical slice thickness of 1 µm with 0.1 µm overlap between adjacent optical slices to cover the entire thickness of the sections. A z-stack image was imported into ImageJ-based FIji software. The native image type for Zeiss 710 is LSM format which is a multi-channel image from a single z-section. To obtain each single color z-stack image, multi-channel images were split into single color channel (image>color>split channels) and save as *tiff* format. Then, each single color z-stack tiff images were merged and make a multi-color *tiff* format file (image>color>merge channels). This z-stack *tiff* images were used for subsequent image processing.

Generating synapse maps for lanceolate and Merkel-ending neurons

As illustrated in supplemental figure S2B-D, to map the locations of presynaptic termini for longitudinal lanceolate or Merkel-ending neurons within the each barrelette (e.g. D6, C3, B2 etc.) for each z-stack series, we manually crop the barrelette in Fiji. First, we circled the barrelette structure of interest by tracing the outline of vGluT1 immunostained regions. The regions outside the barrelette of interest were selected and filled with black color

(Edit>Selection>Make inverse>Edit>Fill). The cropped z-stack images were manually positioned on the center of image. After alignment, the z-stack images were imported into Imaris software (Bitplane). By using "Coloc plugin" of Imaris software, first, vGluT1 and Chodl^{PLAP} double positive sites, which represented lanceolate-ending presynaptic termini, were extracted from all z-stack images. Similarly, vGluT1 and tomato co-labeled sites, which represented Merkel and a subset of lanceolate-ending synapses, were extracted from all z-stack images. To visualize Merkel-ending-only synapses, Chodl^{PLAP}, vGluT1, and tomato triple-positive spots were removed from the vGluT1 and tomato double-positive sites by using "Mask" function. During the process, the consistency across original, double-positive and extracted signals were re-checked by eyes, to avoid detection of non-specific signals and missing real signals. The extracted synapses were reconstructed in 3D by using the "Surface" function of Imaris.

Generating 2D-projected heatmaps for RA-lanceolate and SA-Merkel synapses

The extracted z-stack images of RA-lanceolate or SA-Merkel synapses were saved as tiff format image sequences (i.e. RA-only or SA-only synapses). All z-stack images from all serial sections for the each barrelette structure was imported, aligned and combined into one large z-stack image using Fiji. Each single optical image of the z-stack is divided into 550x550 (x,y) pixels. The pixel either contains synapse (pixel value=1) or does not contain any synapse (pixel value=0). The total number of pixels in the combined z-stack is thus 550x550xN (N=the total number of single optical images). In order to obtain 2D heatmaps of synaptic densities for each type of afferents, the pixels (and their values) are projected onto three 2D planes: coronal (x, y); lateral/sagittal (y, z), and horizontal (x, z), as illustrated in supplemental figure S4A. The values of the projected pixels are represented as heatmaps by using *colormap* function in MATLAB. The color represents relative synaptic densities in each sample. The highest pixel value in each sample is colored red. The lowest value is colored blue.

Cross-correlation analysis of the RA-lanceolate and SA-Merkel synapse maps

To compare the spatial distribution patterns the RA-lanceolate and SA-Merkel synapses in 4 different D6 barrelettes, a Gaussian filter (20pixel x 20 pixel) was first applied to the z-stack sequences obtained as mentioned above generated for the synapse density analyses for each barrelette. In addition, pixels with an intensity value of zero are removed (threshold was set to be $\geq$1). Subsequently, the similarity of the synapse density distributions between any of the 64 pairs was measured by the Pearson correlation coefficient. Standardization, correlation calculation and heatmap plotting were performed using the MATLAB.

3D reconstruction and image analysis of single-labeled neuron

The cropping, alignment and converting into one z-stack image were performed using the same procedure as described in “generating synapse maps for lanceolate and Merkel-ending neurons”. The outline of barrelette structure was circled by Pencil Tool (FIji) on the middle optical slice image of each z-stack. The volume of barrelette of each section was calculated as follows; “Area” x “Number of z-stack images” x “Voxel depth”. The 3D reconstructed images were made by using 3D Viewer plugin of Fiji or Imaris software. The Imaris software allowed us to calculate the number of voxels (three-dimensional pixel) that the axon or the presynaptic termini occupy on the 3D dataset reconstructed from z-stacks. The normalized collateral volumes were calculated as follows; “Number of voxels of axon collaterals” x “Volume of one voxel” / “Volume of the barrelette”. The synaptic densities were calculated as follows; “Number of voxels of presynaptic termini” / “Number of voxels of axon collaterals”.

Analyzing presynaptic termini onto retrogradely labeled projection neurons (PNs)

A z-stack image that was taken by Zeiss 710 confocal microscope was imported into Imaris software. Chodl^{PLAP}/vGluT1/GFP (GFP labels PNs) triple-positive spots (RA-lanceolate presynaptic loci onto PNs) and Pv-tomato/vGluT1/GFP triple-positive spots (SA-Merkel and a subset of RA-lanceolate presynaptic loci onto PN) were detected by using Spot Detection function of Imaris, and plotted onto the PNs. Chodl^{PLAP}/tomato/vGluT1/GFP quadruple-positive spots were considered as RA-lanceolate synaptic loci. During the process, each identified “spot” was double-checked by eye on the actual 4-color images, to avoid detection of non-specific signals and missing real signals.

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. Characterization of Chodl^{hPLAP} mice and vGluT1 staining, Related to Figure 1, 2

(**A-C**) Generation of Chodl^{hPLAP} mice. (**A**) Schematic representation of the genetic insertion. hPLAP gene with ACN cassette was inserted at the translational start codon of *Chondrolectin* gene. Exons are represented as white boxes. (**B**) Southern blot analysis of genomic DNA from wild-type and heterozygous mice. The 9.3 and 5.0 kb fragments indicate wild-type and mutant allele, respectively. (**C**) Genomic PCR analysis of the progeny. Wild-type and mutant alleles produced 300 and 700 bp fragments, respectively.

(**D**) Representative TG sections from P7 Chodl$^{PLAP/+}$ mice. 90.5% Chodl+ (AP stained) neurons were co-labeled with NF200 antibody (PLAP and NF200 double positive).
(**E**) Representative TG section from wild type mice. 98.7% NF200+ neurons were co-labeled with vGluT1 antibody (NF200 and vGluT1 double positive).
Scale bars: 100 μm.

Fig. S2. Analyzing Lanceolate- and Merkel-ending afferent inputs into barrelette columns, Relatated to Figure 2

(**A**) Lanceolate- and Merkel-ending neurons project to all brainstem trigeminal nuclei. Immunostaining of vGluT1, PLAP and tomato on the sections of SpC and SpO nucleus from P7 Pv::Cre; RΦtomato; Chodl$^{PLAP/+}$ mice. Grey and Blue, vGluT1+ presynaptic sites; Green, Chodl$^{PLAP/+}$ labeled axon; Red, Pv::Cre; RΦtomato labeled axon. Arrow indicates facial motor neurons. Scale bar: 100 μm.
(**B-D**) Image processing steps for extracting the sites of lanceolate- and Merkel- afferents presynaptic termini**.** Serial sections of SpI and PrV nuclei from Pv::Cre; RΦtomato; Chodl$^{PLAP/+}$ mice were immunostained with vGluT1, PLAP and RFP antibody and Z-stack images (at 1μm step) were taken using confocal microscopy for each serial section. To extract lanceolate and Merkel synapses, all Z-stack images are processed by Fiji and Imaris software. (**B**) PLAP (Chodl$^{PLAP/+}$) and vGluT1 co-localized loci were extracted and pseudo-colored “green” to represent lanceolate afferents presynaptic termini. (**C**) Tomato (Pv::Cre; RΦtomato) and vGluT1 co-localized sites were extracted and pseudo-colored “magenta” to indicate Merkel- plus a subset of lanceolate-ending presynaptic termini. (**D**) From the tomato (Pv::Cre; RΦtomato) and vGluT1 co-localized sites (magenta), those that overlapped with PLAP (Chodl$^{PLAP/+}$) and vGluT1 co-localized sites were removed, the remain loci, representing Merkel-ending afferent presynaptic termini, were pseudo-colored “red”.

Fig. S3. Axonal projections and presynaptic termini distribution maps of RA-longitudinal lanceolate and SA-Merkel ending neurons within representative *large* barrelettes in SpI and PrV nucleus, Related to Figure 2

(**A**) Schematic drawing of projected 2D heatmaps of the synaptic densities onto coronal (C), lateral/sagittal (L), and horizontal (H) planes. The color represents relative synaptic density in each sample for each type of afferent. The highest density is shown as red. The lowest density is shown as blue.

(**B**) Cross-correlation analysis of distributions of the RA-lanceolate and SA-Merkel synapses inside D6 barrelette in SpI and PrV nuclei from four samples. Color scale indicates correlation coefficient values (-0.1-1).

(**C, D**) Presynaptic density maps of the large C3 and B2 barrelettes in SpI (**C**) abd PrV (**D**) from 2 different samples. Left panels shows presynaptic sites extracted from each Z-stacks of the serial sections through each of the C3 or B2 barrelette. Blue: all vGluT1+ loci. Green: presynaptic termini of RA-longitudinal lanceolate afferents (PLAP+ ∩ vGluT1+). Red: presynaptic termini of SA-Merkel afferents ((tomato+ ∩ vGluT1+) – (PLAP+ ∩ vGluT1+)). Right panels shows the synaptic density heatmaps of RA-afferents in each samples on three 2D planes.

Scale bars: 20 μm.

Fig. S4. Additional examples of axon collaterals from single-labeled RA-lanceolate or SA-Merkel neurons inside the barrelette column, Related to Figure 3

(**A**) Representative low magnification images of co-immunostaining of vGluT1 and tomato on the serial sections through the entire SpI in which a single Merkel-ending neuron innervating C2-vibrissa (the ventrally located axons), and another single-neuron innervating a small barrelette (dorsally located axons) were shown. Abbreviations are as follows: A, anterior; P, posterior. Scale bar: 100 μm.

(**B**) Representative coronal and lateral views of the 3D reconstructed collaterals from single-labeled Merkel and single-labeled lanceolate neuron collaterals in the SpI and PrV at P8 and P28, respectively. Red, axon. Yellow, outlines of the barrelette structures. Scale bar: 20 μm.

(**C**) Lateral views of 3D-reconstructed collaterals and identified presynaptic sites (vGluT1+ spots) from a single-labeled Merkel (upper panels) and a single-labeled lanceolate neuron (lower panels) in the SpI and PrV. Red, axon; Green, barrelette structure; Yellow, presynaptic sites.

Fig. S5. Retrograde labeling of brainstem projection neurons that send axons into thalamus, Related to Figure 4

(**A**) Schematic of the retrograde labeling of projection neurons with deficient rabies virus (ΔG-GFP-RV).

(**B**) Representative images of ΔG-GFP-RV retrogradely labeled projection neurons on the sections of PrV, SpO, SpI-r (rostral SpI) and SpI-c (caudal SpI) nucleus from P7 Pv::Cre;

RΦtomato; Chodl^{PLAP} mice. Blue, vGluT1; Green, projection neurons; Red, tomato; Magenta, PLAP. Note that SpI-c contains barrelettes, whereas SpI-r does not. Scale bar: 100 μm.

Fig. S6. Convergence of SA- and RA- afferent inputs onto projection neurons, Related to Figure 4

Representative high magnification images of sections from SpI nucleus (**A**) or PrV nucleus (**B**) from the 4-color labeling experiments (i.e. ΔG-GFP-RV injected into Pv::Cre; RΦtomato; Chodl^{PLAP} mice at P7). Rightmost panels show synaptic termini of SA-Merkel-ending or RA-lanceolate-ending neurons onto the projection neurons. Red dots: SA synapses; Green dots: RA synapses. White dash lines encircle the barrelette structure. Insets: zoom of the yellow boxed region.

Scale bars: 20 μm.

SUPPLEMENTAL MOVIES

Movie S1, Related to Figure 2

This movie shows representative maps of presynaptic sites extracted from each Z-stacks of the serial sections through the D6 barrelette in SpI nucleus. Blue: vGluT1+ single-positive loci. Green: presynaptic termini of RA-longitudinal lanceolate afferents (PLAP+ ∩ vGluT1+). Red: presynaptic termini of SA-Merkel afferents ((tomato+ ∩ vGluT1+) – (PLAP+ ∩ vGluT1+)). From left to right: anterior-posterior serial sections.

Movie S2, Related to Figure 2

This movie shows representative maps of presynaptic sites extracted from each Z-stacks of the serial sections through the D6 barrelette in PrV nucleus. Blue: all vGluT1+ loci. Green: presynaptic termini of RA-longitudinal lanceolate afferents (PLAP+ ∩ vGluT1+). Red: presynaptic termini of SA-Merkel afferents ((tomato+ ∩ vGluT1+) – (PLAP+ ∩ vGluT1+)). From left to right: anterior-posterior serial sections.

Movie S3, Related to Figure 3

This movie shows a representative three-dimensional reconstruction of collaterals from a single-labeled Merkel neuron in the SpI at P8. Red, axon; Yellow, outline of barrelette.

Movie S4, Related to Figure 3

This movie shows a representative three-dimensional reconstruction of collaterals from a single-labeled lanceolate neuron in the SpI at P8. Red, axon; Yellow, outline of barrelette.

Movie S5, Related to Figure 3

This movie shows a representative three-dimensional reconstruction of collaterals from a single-labeled Merkel neuron in the PrV at P8. Red, axon; Yellow, outline of barrelette.

Movie S6, Related to Figure 3

This movie shows a representative three-dimensional reconstruction of collaterals from a single-labeled lanceolate neuron collaterals in the PrV at P8. Red, axon; Yellow, outline of barrelette.

Figure S1

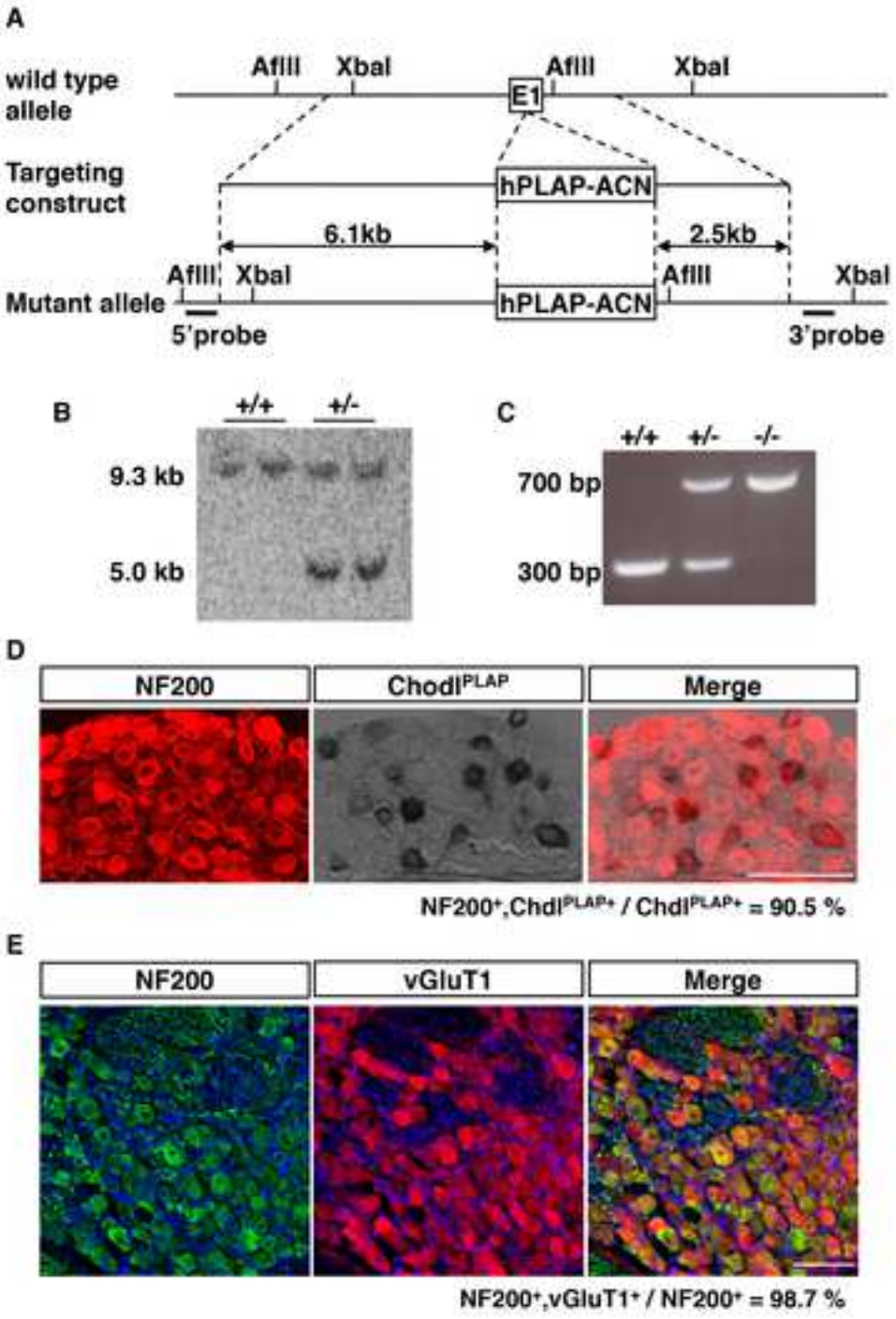
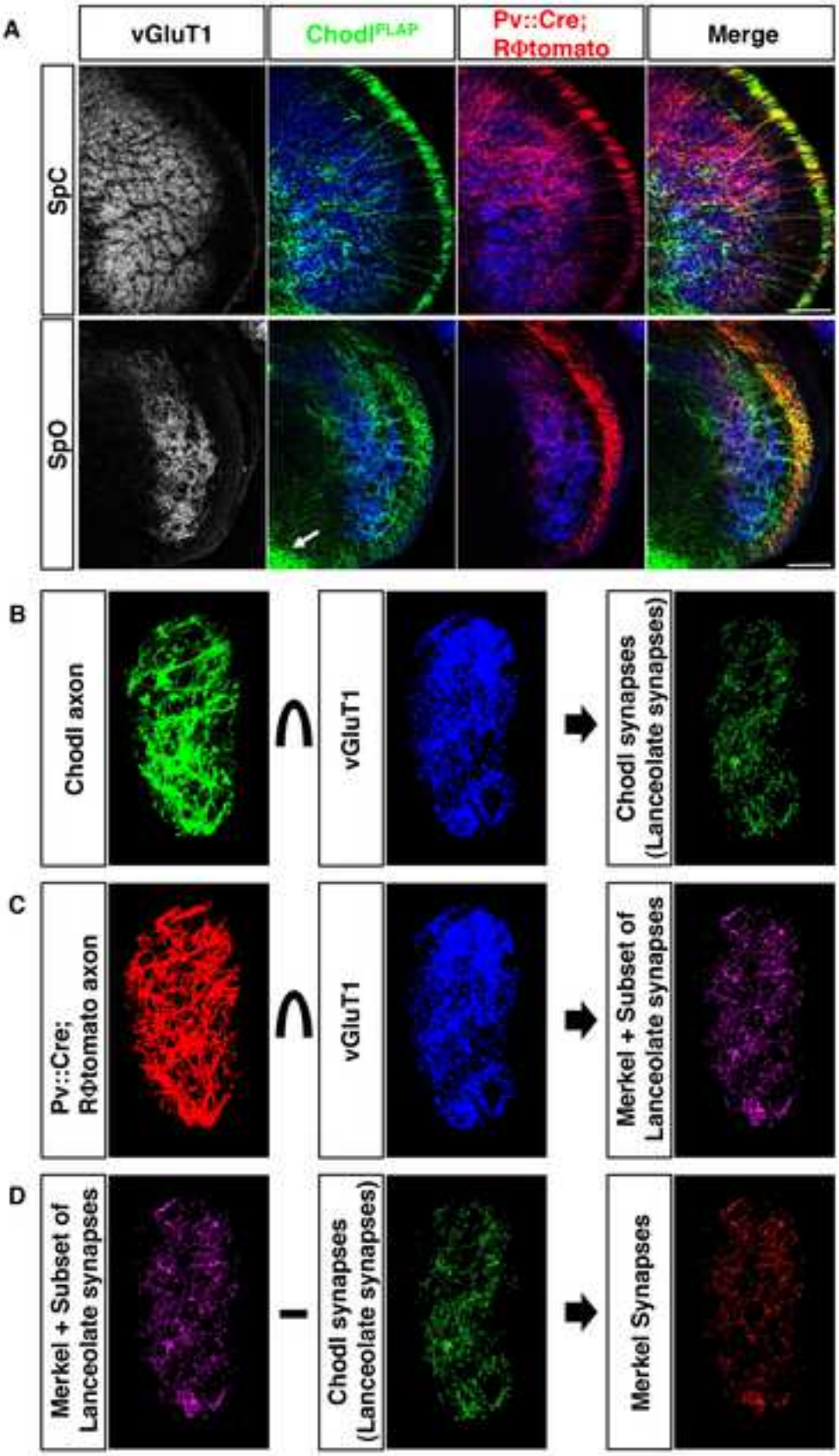

A
wild type allele
AflII
XbaI
AflII
XbaI
E1
Targeting construct
hPLAP-ACN
6.1kb
2.5kb
AflII
XbaI
AflII
XbaI
Mutant allele
hPLAP-ACN
5'probe
3'probe
B
+/+
+/-
9.3 kb
5.0 kb
C
+/+
+/-
-/-
700 bp
300 bp
D
NF200
ChodlPLAP
Merge
NF200+,ChdlPLAP+ / ChdlPLAP+ = 90.5 %
E
NF200
vGluT1
Merge
NF200+,vGluT1+ / NF200+ = 98.7 %

Figure S2


A
vGluT1
ChodlFLAP
Pv::Cre;
RΦtomato
Merge
SpC
SpO
B
Chodl axon
vGluT1
Chodl synapses
(Lanceolate synapses)
C
Pv::Cre;
RΦtomato axon
vGluT1
Merkel + Subset of
Lanceolate synapses
D
Merkel + Subset of
Lanceolate synapses
Chodl synapses
(Lanceolate synapses)
Merkel Synapses

Supplemental Figure 3

Figure S3

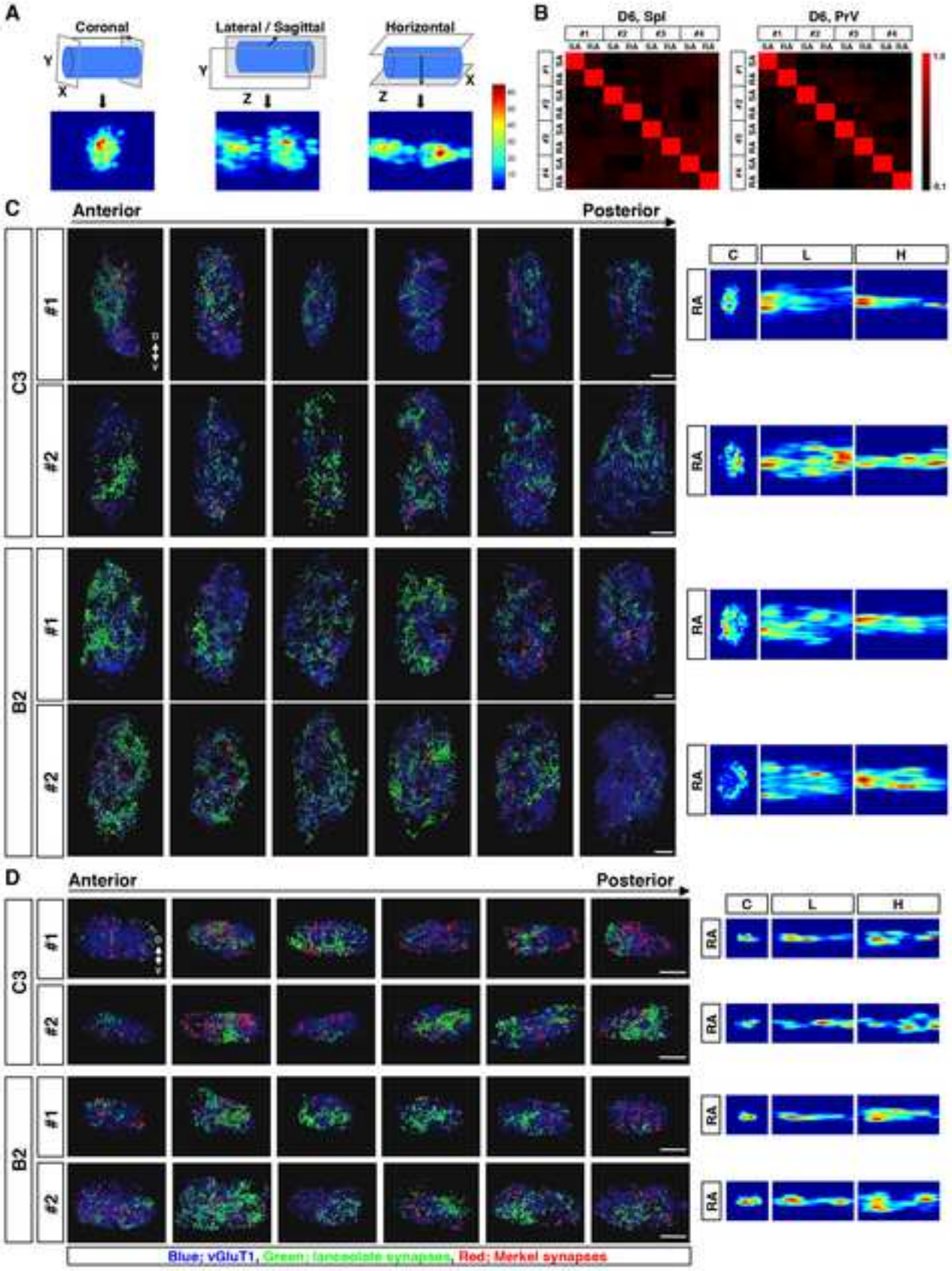


Figure S4

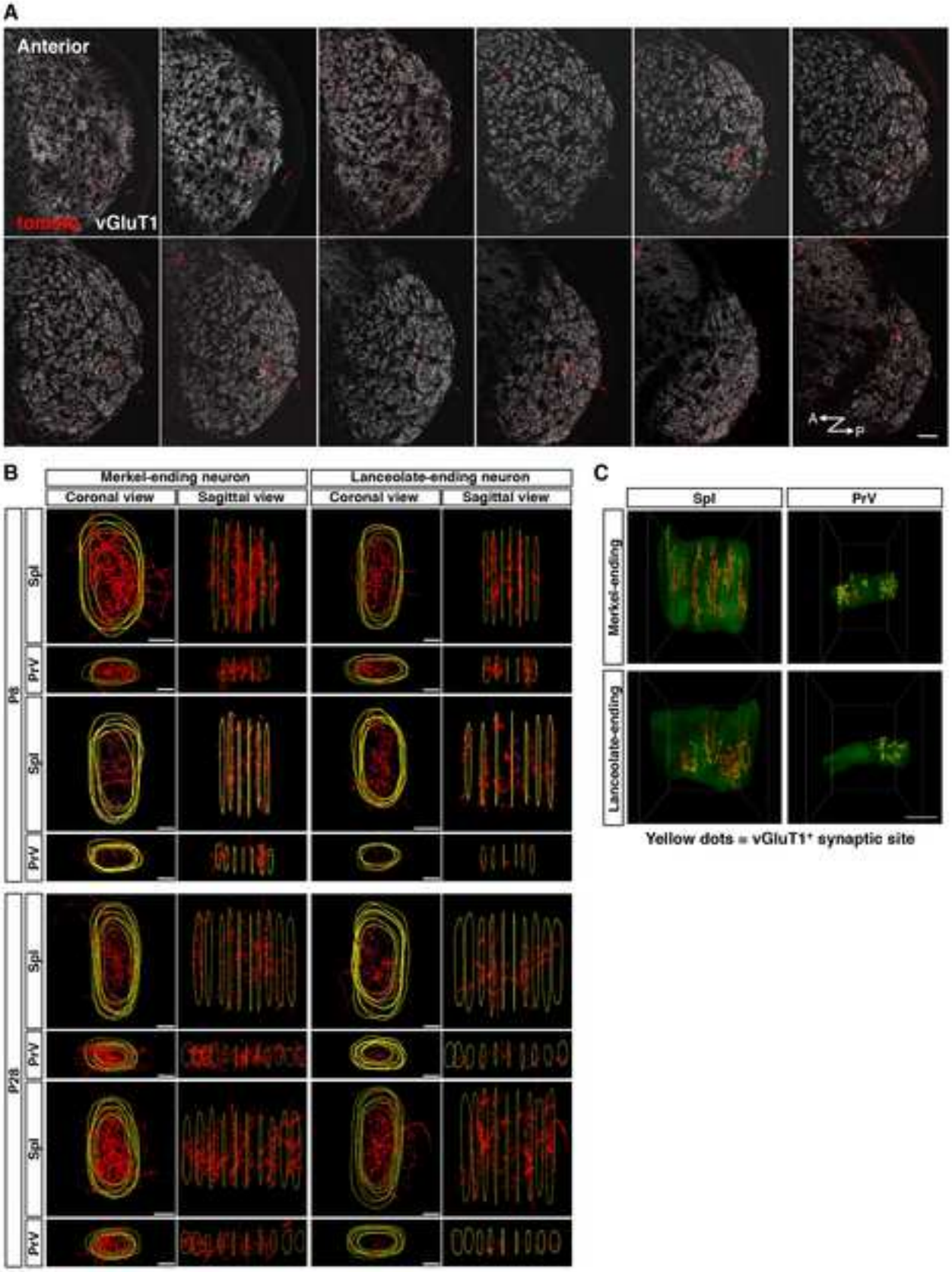

A
Anterior
vGluT1
A
Z
P
B
Merkel-ending neuron
Lanceolate-ending neuron
Coronal view
Sagittal view
Coronal view
Sagittal view
P4
P2it
Spl
PrV
Spl
PrV
Spl
PrV
Spl
PrV
C
Spl
PrV
Merkel-ending
Lanceolate-ending
Yellow dots = vGluT1+ synaptic site

Figure S5

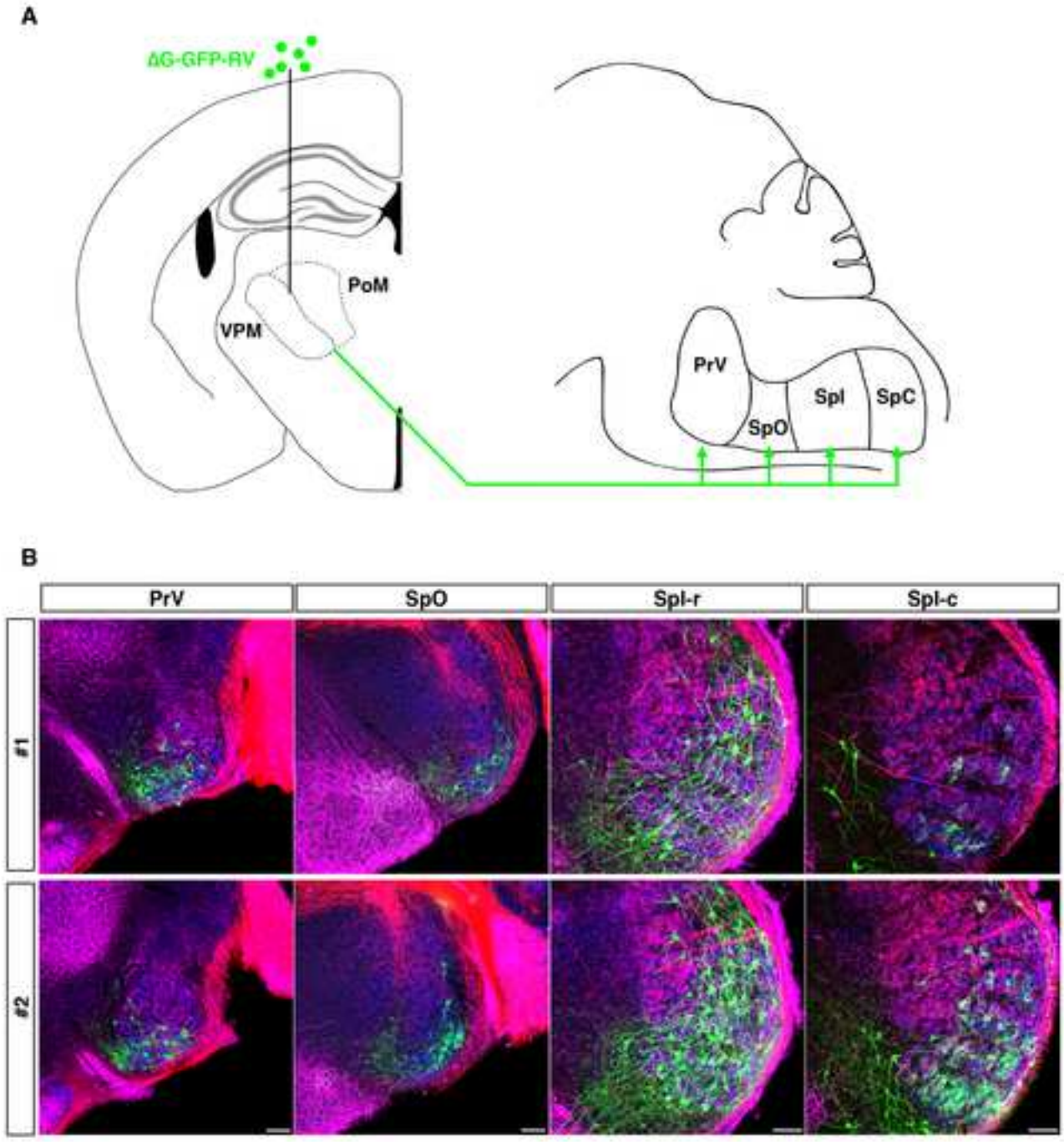
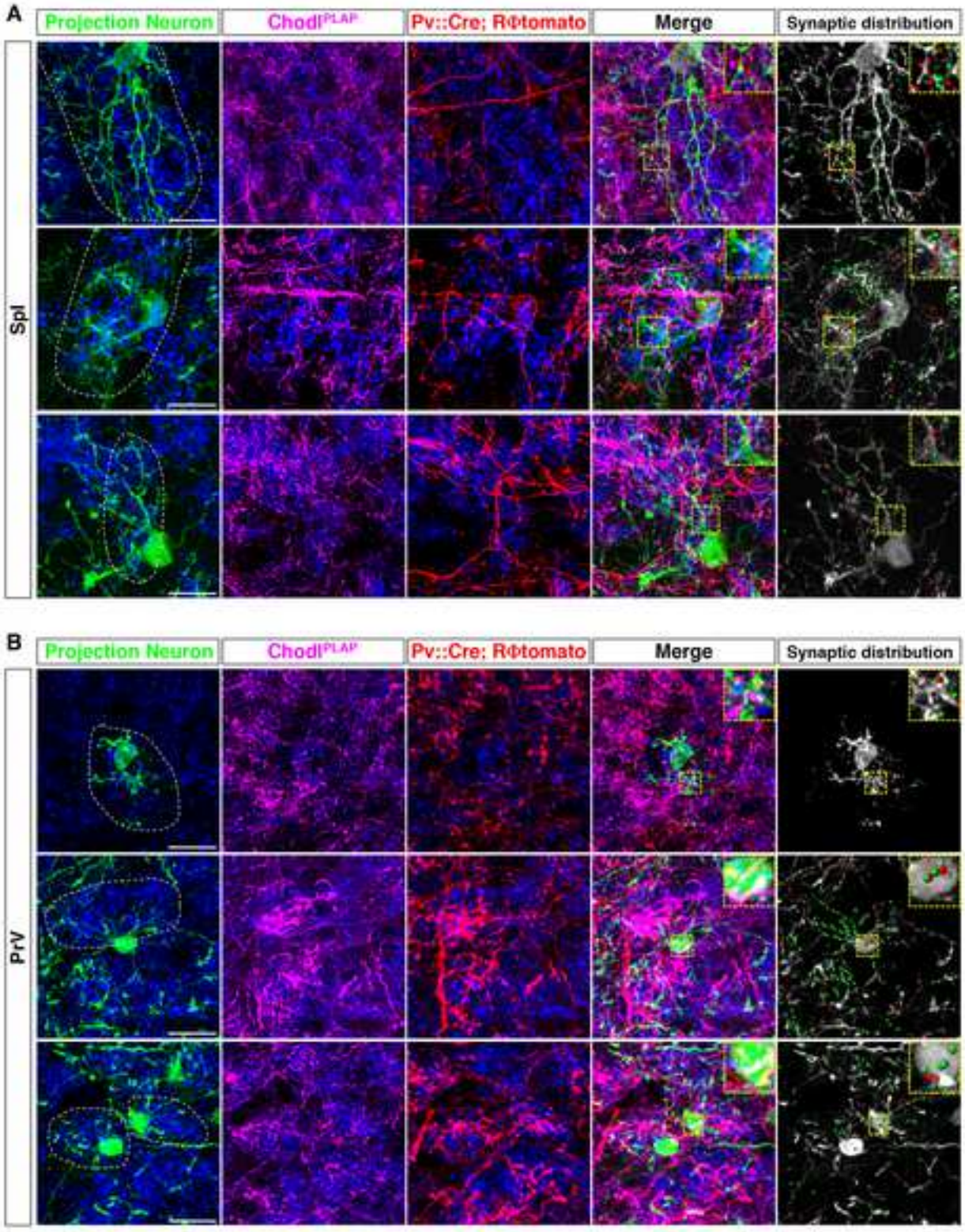


Figure S6


A
Projection Neuron
Chodl^PLAP
Pv::Cre; RΦtomato
Merge
Synaptic distribution
Spl
B
Projection Neuron
Chodl^PLAP
Pv::Cre; RΦtomato
Merge
Synaptic distribution
PrV