SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse Genetics

All experiments and procedures were performed according to NIH guidelines and approved by the Institutional Animal Care and Use Committee of Columbia University. The following previously published mouse strains were used in this study: *En1::Cre* (Kimmel et al., 2000; Sapir et al., 2004), *MafB::GFP* (Moriguchi et al., 2006), *Nestin::Cre* (Tronche et al., 1999), *Ptf1a::Cre* (Kawaguchi et al., 2002), *Rosa.lsl.eYFP* (Srinivas et al., 2001), *Rosa.lsl.tdT (Ai9* and *Ai14)* (Madisen et al., 2010), *RCE.dual.GFP* and *RCE.lsl.GFP* (Miyoshi et al., 2010), *Tau.lsl.mGFP.IRES.nLacZ* (Hippenmeyer et al., 2005), *Sox14::eGFP* (Crone et al., 2008) and $Sp8^{fl/fl}$ (Bell et al., 2003). The $Pou6f2^{tm1Nat}$ mutant line (Jackson Lab, Stock #009042), in which the last exon encoding the DNA binding domain was deleted, was generated by J. Nathans, replacing an EcoRI-EcoRI fragment of ~3.5 Kb (encompassing the exon and flanking intronic regions) with a PGK-Neo selectable cassette.

To generate $Sp8::FlpoER^{T2}$ mice, FLPo, a codon-optimized version of FLP recombinase (Raymond and Soriano, 2007) was fused to ER^{T2} and inserted into the ATG of the endogenous *Sp8* locus, generating a null allele that simultaneously allows lineage-tracing. To induce recombination, tamoxifen (Sigma) was administered by a single intraperitoneal injection to p0-p1 mice at a dose of 125 μg per gram body weight. *FoxP2::Flpo* mice were generated by inserting FLPo into the ATG in the 4th exon of the *FoxP2* genomic locus, disrupting the endogenous *FoxP2* allele. In both $Sp8::FlpoER^{T2}$ and *FoxP2::Flpo* mice, the neomycin selectable cassette was removed using *Protamine::Cre* mice (Jackson Lab, Stock #003328), which recombines in the male germline. *Otp::Flpo* animals used to validate antibody specificity (Figure S1D,E) were generated in a similar fashion and will be described elsewhere. Mouse strains were maintained on a C57BL/6J background, and were backcrossed for > 6 generations.

Microarray Screen

To isolate V1 and dI4/dIL^{A} interneurons, *En1::Cre; Rosa.lsl.eYFP* or *Ptf1a::Cre; Rosa.lsl.eYFP* mice were obtained at either e12.5 (date of plug defined as day 0.5), p0, or p5. Isolated spinal cords from 3-4 mice were minced in ice-cold aCSF (125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM D-glucose, 2 mM sodium pyruvate, 0.4 mM ascorbic acid, equilibrated with 95% O_2/5% CO_2, with final osmolarity at 315-325 mOsm), and dissociated with the Papain Dissociation Kit (Worthington). eYFP^{+} interneurons were purified by fluorescence-activated cell sorting (FACS) using a Beckman Coulter Altra Hypersort cell sorter (laser excitation and power: 488 nm at 300 mW; shealth pressure: 10 PSI; eYFP detection bandpass: 520 nm/20), and total RNA was isolated with the Absolutely RNA Nanoprep Kit (Stratagene) and quantified with the RiboGreen RNA quantitation kit (Invitrogen) on a 20/20n Luminometer (Turner Biosystems). Purified RNA (between 1-5 ng) was amplified with the Ovation Pico WTA System (NuGEN), followed by fragmentation and biotin labeling of 5 μg of single-stranded cDNA with the Encore Biotin Module (NuGEN).

Microarray chip processing was conducted at the Herbert Irving Cancer Center Genomics Technologies core facility (Columbia University). The Affymetrix GeneChip Hybridization control kit and GeneChip Hybridization,

Wash, and Stain kit were used to hybridize samples to Affymetrix 430 2.0 GeneChip arrays. Fluidics were performed on the Affymetrix GeneChip Fluidics Station 450, and scanned using the Affymetrix GeneChip Scanner 7G.

Analysis of differential enrichment of gene expression in V1 relative to dI4/dIL^{A} interneurons was performed in Partek Genomics Suite (Partek Inc.), using the GC Robust Multi-array Average (GCRMA) normalization algorithm, followed by one-way ANOVA. Two transcription factor databases, the AnimalTFDB (Zhang et al., 2012; Zhang et al., 2015) and the RIKEN transcription factor database (Kanamori et al., 2004), were referenced to identify the list of transcription factor candidates for enrichment in V1 or dI4/dIL^{A} interneurons. Subsequent analysis focused on those with >3-fold enrichment ($p \leq 0.02$, by one-way ANOVA) in V1 interneuron in at least one developmental age, and with mosaic expression in the ventral spinal cord (Sunkin et al., 2013; Visel et al., 2004). MafA (not present on the microarray) and Prox1 (not enriched) were also included, as they exhibit scattered expression in the ventral spinal cord (Misra et al., 2008; Lecoin et al., 2010). Scatter Plots representing mean expression values were generated in Partek, and heatmaps indicating fold-enrichment were generated in Matlab. All FACS experiments were performed in triplicate, with the exception of p5 *Ptf1a::Cre; Rosa.lsl.eYFP*, which was performed in duplicate. RNA amplification, cDNA fragmentation and labeling, and microarray hybridization for samples of a given age were performed in parallel. Microarray data is available at http://www.ncbi.nlm.nih.gov/geo/ according to established guidelines.

Retrograde Labeling of Motor and Sensory Neurons

Motor neurons and sensory neurons innervating gluteal (GL, hip extensor), tibialis anterior (TA, ankle flexor), and intrinsic foot (IF, plantar flexor, ventral footpad) muscles were retrogradely labeled *in vivo* at p14-p16 via intramuscular injection of ~0.5 µl of 1% unconjugated cholera toxin B subunit (CTB, List Biologicals). Injections were supplemented with 1% rhodamine-dextran (3000 molecular weight (MW), Invitrogen), which enabled us to verify the specificity of muscle injection following the dissection of hindlimb muscles under fluorescence guidance. After 5-7 days to allow time for synaptic accumulation of CTB, animals were processed for analysis. This enabled a determination of the relative dorsoventral position of CTB-backfilled GL, TA, and IF motor pools in ~p21 mice, by measuring the distance from the dorsal boundary of the ventral funiculus to the midpoint of individual motor pools. Similarly, the dorsoventral and mediolateral positions of V1^{R} and V1^{Sp8} cells in relation to GL, TA, and IF motor pools were quantified by measuring the distance from individual V1^{R} or V1^{Sp8} cell soma to the midpoint of the dorsoventral or mediolateral position of each motor pool.

Immunohistochemistry and Analysis of Transcription Factor Co-expression

Immunohistochemistry for p0 and older spinal cords was performed on mice transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer, followed by a 2 hour postfixation. Tissue was then washed, cryoprotected by equilibration in 30% sucrose in 0.1M phosphate buffer, embedded in OCT, frozen on dry ice, and cryostat-sectioned in the transverse plane at 20 µm (p0 animals) or 40 µm (~p21 animals). Alternately, after postfixation tissue was vibratome-sectioned at 100 µm. Embryonic spinal cords were fixed in 4% paraformaldehyde in phosphate buffer for

1 hour at 4°C, and then cryoprotected, embedded, and sectioned as above.

Immunohistochemistry was performed on tissue through sequential exposure to primary antibodies overnight at 4°C, and fluorophore-conjugated (Alexa Fluor 405, DyLight 488 or Alexa Fluor 488, Cy3, and Cy5) secondary antibodies for 1 hour at room temperature. Sections were mounted using Fluoromount-G (SouthernBiotech) and coverslipped for imaging. Confocal images were obtained on a LSM 710 Meta Confocal microscope (Carl Zeiss) at 1024x1024 resolution, using either a Plan-Apochromat 20x/0.8 M27 objective (nuclear staining) or a 63x/1.4 Oil DIC M27 objective (synaptic staining).

To estimate the fraction of the parental V1 population labeled by our TFs, we simultaneously applied antibodies against 14 of the 19 TFs (Bhlhb5, FoxP1, FoxP2, MafB, Nr3b2, Nr4a2, Nr5a2, Oc1, Oc2, Otp, Pou6f2, Prdm8, Prox1, and Sp8) to sections from *En1::Cre; Tau.lsl.nLacZ* mice, in which V1 interneurons are marked by expression of nLacZ. Five antibodies were excluded for this analysis, including Lmo3, due to the high fraction (74.4 ± 2.1%) of labeled V1 interneurons, as well as FoxP4, MafA, Nr3b3, and Zfhx4, due to nonzero levels of background nuclear staining.

To assess transcription factor co-expression, confocal images were imported into Imaris (Bitplane), and analyzed using the "Colocalization" and "Spots" functions, followed by manual validation. Thresholds were set to exclude non-specific background immunoreactivity. Variations in levels of expression were not taken into consideration, resulting in a determination of either “co-expressed” or “not co-expressed”. For each transcription factor combination, we analyzed two or more lumbar sections from at least three p0 animals, totaling >580 spinal sections and >1100 spinal hemisections. See Gabitto et al., 2016 for details of the Bayesian sparse linear regression algorithm used to infer V1 cell type diversity.

Antibodies

Antibodies developed for this study were generated in guinea pig, rabbit, and rat, and include the following, along with corresponding immunogens: guinea pig anti-FoxP2: DAGSRDGRSSGDTSSEVSTVC; rabbit anti-FoxP4: ASSLLPLSQEDLGVPGEP; guinea pig anti-Lmo3: EEGLMKEGYAPQVR; rat anti-MafA: CGFPREPSPAQAGPGAAKGAPD; guinea pig anti-Nr3b2: amino acids 186-237 from mouse Nr3b2; rat anti-Nr4a2: SGEYSSDFLTPEFVKFSMDC; rat/rabbit anti-Nr5a2: LPPTDYDRSPFVTSP and GYQPYGHFPSRAIKSEY; guinea pig/rat anti-Otp: DPGGHPGDLAPNSDPVEGATC; guinea pig anti-Pou6f2: LRGEDKAATSDSELNE; rat anti-Pou6f2: amino acids 35-184 from human Pou6f2; guinea pig anti-Prdm8: amino acids 228-457 from mouse Prdm8; and guinea pig/rat anti-Sp8: CPELLQPPEPGHRNGLE.

Antibodies generously provided as gifts include: rabbit anti-En1, from Alex Joyner (Davis et al., 1991); rabbit anti-Nr3b2, from Jeremy Nathans (Chen and Nathans, 2007); and rabbit/rat anti-Bhlhb5 and rabbit/guinea pig anti-Prdm8, from Sarah Ross (Ross et al., 2010; Ross et al., 2012).

Other antibodies used in this study include: goat anti-Bhlhb5 (beta-3) (1:2000, Santa Cruz, clone E-17); chick anti-β-Galactosidase (1:5000, Abcam, ab9361); rabbit anti-Calbindin D28K (1:2000, Swant, CB38); mouse anti-

cholera toxin B subunit (1:500, Abcam, ab35988); goat anti-cholera toxin B subunit (1:8000, List Biologicals, 703); mouse anti-Nr3b3 (Err3) (1:2000, R&D Systems, PP-H6812-00); rabbit or guinea pig anti-FoxP1 (1:20000, Jessell Lab); goat anti-FoxP2 (1:500, Santa Cruz, clone N-16); chick anti-GFP (1:4000, Abcam, ab13970); rabbit anti-GFP (1:2000, Invitrogen, A-11122); rabbit anti-MafA (1:2000, Novus Biologicals, NB400-137); rabbit anti-MafB (1:2000, Sigma, HPA005653); rabbit anti-Nr4a2 (Nurr1) (1:500, Santa Cruz, clone M-196); goat anti-Nr5a2 (1:100, Santa Cruz, clone C-17); rabbit anti-Onecut1 (HNF-6) (1:2000, Santa Cruz, clone H-100); rabbit anti-Onecut1 (1:200, Sigma, HPA003457); sheep anti-Onecut2 (1:2000, R&D Systems, AF6294); rabbit anti-Pou6f2 (1:2000, Sigma, HPA008699); rabbit anti-Prox1 (1:2000, Millipore, AF2727); goat anti-Sp8 (1:2000, Santa Cruz, clone C-18); goat anti-vAChT (1:500, Promega, G4481); rabbit anti-vGAT (1:4000, Jessell Lab); guinea pig anti-VGlut1 (1:32000, Jessell Lab); and rabbit anti-Zfhx4 (1:400, Sigma, HPA023837).

Where possible, antibody specificity was validated by showing an absence of staining in knockout animals. Antibodies previously confirmed to be specific against knockout mice include: anti-Bhlhb5 (Ross et al., 2010), anti-FoxP1 (Sürmeli et al., 2011), anti-Nr3b2 (Chen and Nathans, 2007), anti-Onecut1 (Wu et al., 2012), and anti-Prdm8 (Ross et al., 2012). Additionally, all FoxP2, Sp8, Otp, and Pou6f2 antibodies used in this study were validated against knockout mice (Figure S1D-G and data not shown). The Sigma rabbit anti-MafB antibody recognizes MafB, but may also weakly detect other Maf-family members (F.J.Alvarez, unpublished observation), as immunoreactivity is not completely abolished in *MafB::GFP* homozygous null mice. In cases where knockout mice were unavailable, we confirmed that the pattern of immunoreactivity was consistent with previously published expression data obtained from the Allen Brain Institute (Figure S1H-O), Website: ©2012 Allen Institute for Brain Science. Allen Spinal Cord Atlas [Internet]. Available from: http://mousespinal.brain-map.org/.

Spatial Analysis of V1 Interneurons

The position of V1 interneurons in mid/caudal lumbar (L3-L5) spinal segments of p0 mice was analyzed in Imaris (Bitplane), using the "Spots" function. Cartesian coordinates for each interneuron were determined in the transverse spinal cord plane with respect to the midpoint of the central canal, defined as position (0,0). To account for variations in spinal cord size along the rostrocaudal axis, sections were normalized to a standardized spinal cord hemisection (distance from central canal to lateral boundary: 650 μm; distance from central canal to bottom-most boundary: 400 μm). Coordinates were exported from Imaris as .xls files, and plotted using custom Matlab scripts to display the position of each individual cell. Distribution contours were constructed in Matlab using the kde2d function (Matlab File Exchange), which estimates a bivariate kernel density over a set of grid points (Botev et al., 2010).

To characterize the spatial distributions of V1 subpopulations, we developed a number of metrics, including fractional area, mutual segregation, epicenter position, mean pairwise distance, and cell density. Fractional area (F_a) was calculated as the ratio of the kernel density area of a given V1 subpopulation to the kernel density area spanned by the parental V1 population. Kernel density area was defined as the area of a uniform density having the same mean. We note that a uniform density function $f = A$ has a mean density = A, and an area of $1/A$. As the mean of a

kernel density *k(x,y)*, we use

$$\int_{-\infty}^{\infty}\int_{-\infty}^{\infty} k(x,y)^2 dxdy$$

which can be viewed as the expected value of *k(x,y)* itself, or as a weighted average of the density where the density at each point is weighted in the average by its own value. For a discretely defined kernel density k^*generated using the kde2d function and defined over the set *G* of grid points *g* with grid point spacings Δx and Δy in the x and y directions, respectively, we computed the mean density as:

$$\Delta x \Delta y \sum_{g \in G} (k^*(g))^2$$

The kernel density area was then calculated as one divided by the mean density.

The mutual segregation between two populations was defined as one minus the overlap between their kernel densities. Overlap was defined as the integral over *G* of the lower of the two densities' values at each *g*. For two discretely defined densities k_1^* and k_2^*, we computed the overlap as:

$$\Delta x \Delta y \sum_{g \in G} \min\{k_1^*(g), k_2^*(g)\}$$

The epicenter of a cell population was its centroid, defined by the mean x- and y-coordinates across cells. The mean pairwise distance of a cell population was the mean of the separation between all possible pairs of cells within the population, calculated using the Matlab function pdist. To estimate a population's three-dimensional cell density in terms of cells per μm^3, we computed the mean number of population members found in a 20 μm section. The resulting value was divided by (20 μm times the population's kernel density area in μm^2) (see above). The divisor here approximates the volume within a section within which cells from the given population were found.

Two-dimensional Kolmogorov-Smirnoff (KS) tests (Peacock, 1983) were performed using the Matlab function kstest_2s_2d (Matlab File Exchange). Differences in the distribution of interneurons along the dorsoventral or mediolateral axes were assessed using the Wilcoxon Rank-Sum test. We tested for differences in the area of V1 subpopulations compared to the parental V1 population using a Monte Carlo approach. In each case, 100,000 bootstrap samples of a size equal to that of the subpopulation were taken from the parental population, and the fractional area relative to the parental population was computed for each bootstrap sample. P values signifying fractional area differences were calculated as (1 + # of bootstrap samples for which the fractional area was lower than that of the subpopulation) / 100,001.

Quantification of Synaptic Inputs

To assess motor neuron axon collateral input onto V1^R or V1^{Sp8} interneurons, GL, TA, or IF muscle groups in *En1::Cre*; *RCE.lsl.GFP*, *En1::Cre*; *Rosa.lsl.tdT*, or *En1::Cre*; *Sp8::FlpoER^{T2}*; *RCE.dual.GFP* mice were injected at p14-p16 with 1% unconjugated CTB (generally supplemented with 1% rhodamine-dextran to visualize site of

muscle injection).

After 4-5 days to allow time for transport of CTB to axon terminals, quantification of CTB^+; $vAChT^+$ synaptic boutons onto $V1^R$ or $V1^{Sp8}$ interneurons was performed using a 63x/1.4 Oil DIC M27 objective and taking 1 μm-thick confocal z-scans of 20-40 μm thick cryostat or 100 μm thick vibratome sections. A motorized stage was used to facilitate the creation of montage images and z-axis image stacks. $V1^R$ cells were defined by the following criteria: (i) V1-derivation (ii) strong calbindin immunoreactivity (Carr et al., 1998), and (iii) a location within 200 μm of the dorsal boundary of the ventral funiculus (Sapir et al., 2004). By these criteria, 90 ± 2% of $calbindin^+$ V1 interneurons in p21 *En1::Cre; RCE.lsl.GFP* mice represent $V1^R$ interneurons (n = 3 animals, 104 cells). Only sections with robust CTB-labeled motor neurons were used for analysis, to exclude the possibility that target cells lacked motor axon collateral inputs because of distance from back-labeled motor neurons. Images were analyzed in Imaris, where dendritic length was measured using the polygon distance function, and the number of colocalized CTB^+; $vAChT^+$ puncta was counted. Quantification of input density was performed only on those cells exhibiting synaptic inputs. Data are represented as mean ± SEM, and were obtained from at least 3 animals. For all experiments, CTB^+ puncta were only considered to be synaptic contacts if they co-labeled with an appropriate marker and were directly adjacent to a cell soma or dendrite in at least 3 adjacent z-stacks.

To measure $V1^R$ input onto CTB-filled GL, TA, or IF motor neurons, synaptic boutons that were both V1-derived and exhibited calbindin immunoreactivity were counted on ~p20 motor neuron somata and ~100 μm of their proximal dendritic arbor, as above. Motor neurons with clear dendritic arbors, $vGluT1^+$ inputs, and cross-sectional areas > 400 μm^2 were imaged, thereby excluding the smaller γ-motor neurons from analysis (Friese et al., 2009). However, because IF motor neurons tended to be smaller, we included IF motor neurons with cross-sectional areas < 400 μm^2 in our analysis if they also received $vGluT1^+$ inputs. Motor neuron soma surface area was quantified in Imaris, from 3-D reconstructions in Surpass mode, using the "Surface" function. $V1^{Sp8}$ interneuron contacts onto motor pools were analyzed similarly, with the exception that these were defined as "contacts" and not "synapses" because we were unable to verify co-expression of vGAT in most contacts due to inability of the vGAT antibody to sufficiently penetrate into sections. After determining the number of $V1^{Sp8}$ contacts onto motor neurons, data was normalized to the number of $V1^{Sp8}$ interneurons in the analyzed cryostat sections, in order to account for variations in tamoxifen labeling efficiency between experiments.

To measure proprioceptive afferent input onto $V1^R$ interneurons, we imaged $V1^R$ soma and dendrites as above and analyzed the number of CTB^+; $vGluT1^+$ synaptic puncta. The vast majority of GL sensory afferent inputs were located on $V1^R$ dendrites (96.2%, or 76/79 total CTB^+; $vGluT1^+$ inputs). Experiments were conducted similarly for $V1^{Sp8}$ interneurons.

Electrophysiology

Intact spinal cord-hindlimb preparation: The *in vitro* spinal cord-hindlimb preparation (Mentis et al., 2006;

Shneider et al., 2009) was obtained from p2–p5 *En1::Cre; Rosa.lsl.tdT* mice (n=9 animals). Briefly, animals were decapitated and the spinal cord (in continuity with both the hindlimb and peripheral nerves) was dissected and removed under cold (~12°C) aCSF containing the following (in mM): 128.35 NaCl, 4 KCl, 0.58 $NaH_2PO_4 \cdot H_2O$, 21 $NaHCO_3$, 30 D-glucose, 1.5 $CaCl_2 \cdot H_2O$, and 1 $MgSO_4 \cdot 7H_2O$. The spinal cord-hindlimb preparation was then transferred to a customized recording chamber equipped with a confocal microscope (Leica SP5) containing three lasers (488, 543, and 650 nm). The preparation was continuously perfused with oxygenated (95% O_2/5% CO_2) aCSF (~13 ml/min). In all preparations the ventral roots L4–L5 were cut and placed into suction electrodes for stimulation. Bipolar concentric needles (Microprobes, Gaithersburg, MD) were placed into GL, TA and IF muscles for stimulation.

The whole-cell recordings from visually and physiologically identified $V1^R$ interneurons were obtained with patch electrodes advanced through the ventral aspect of the spinal cord. The electrode solution contained the following (in mM): 10 NaCl, 130 K-gluconate, 10 HEPES, 11 EGTA, 1 $MgCl_2$, 0.1 $CaCl_2$, and 1 Na_2ATP, pH adjusted to 7.2–7.3 with KOH. Alexa 488 hydrazide (~30 μM) was added for intracellular labeling (final osmolarity: ~305 mOsm). Cells were visually targeted after removal of the dura and pia mater from the ventral aspect of the cord over the L4–L5 spinal segments. The patch electrode was controlled by a motorized four-axis micromanipulator (Scientifica). $V1^R$ interneurons were identified initially by a graded synaptic response following ventral root stimulation. Laser excitation was subsequently used to confirm co-localization of tdTomato signal with that of the intracellular solution.

$V1^R$ interneurons were accepted for analysis if the following three criteria were met: (i) a resting membrane potential ≤ -45 mV, (ii) overshooting evoked action potentials, and (iii) at least 30 min of recording. The mean resting membrane potential for $V1^R$ interneurons recorded in this study (n = 11) was -55.1 ± 2.1 mV (range, -48 to -64 mV). The average latency of the EPSP following ventral root stimulation was 3 ± 0.4 ms. All measurements of synaptic potentials were made at -60 mV using continuous current injection as necessary. Synaptic potentials (filtered from DC to 3 kHz) were recorded from individual $V1^R$ interneurons or motor neurons (Multiclamp 700A; Molecular Devices) in response to orthodromic stimulation (A365 current stimulus isolator; WPI) of the peripheral muscles GL, TA and IF. Muscles were stimulated at 1.2, 1.5, 2, and 5x threshold, where stimulus threshold was defined as the current at which the minimal evoked response was recorded in three out of five trials. Recordings were fed to an analog-to-digital interface (Digidata 1320A; Molecular Devices) and acquired with Clampex (version 10; Molecular Devices) at a sampling rate of 10 kHz. Data were analyzed offline using Clampfit (Molecular Devices). Measurements were made on averaged traces (3–10 trials).

Acute spinal cord slice preparation: Passive and active properties of V1 subsets were recorded in an acute spinal cord slice preparation from p10 to p14 animals. Briefly, spinal cords were dissected in ice-cold oxygenated (95% O_2/5% CO_2) sucrose-modified aCSF containing the following (in mM): 218 sucrose, 26 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 10 D-glucose and 2 $MgCl_2$. Spinal cords were then embedded in low-melting agarose, and L2 to L5 segments were sectioned in the transverse plane (300 μm thick) in sucrose-modified aCSF using a Leica VT1000P Vibratome. Slices were collected serially and placed in oxygenated chamber trays under continuous circulation and

oxygenation with normal aCSF containing the following (in mM): 133 NaCl, 26 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 10 D-glucose, 2 $MgCl_2$ and 2 $CaCl_2$). After incubating slices at 32° C for 1 hour, they were maintained at room temperature (22-25° C) until use.

Slices were transferred to a submersion style chamber for recording and perfused with oxygenated normal aCSF at room temperature. V1 interneurons were visualized in *En1::Cre; Rosa.lsl.tdT* (Ai9); *MafB::GFP* mice (for $V1^{R}$ or $V1^{Pou6f2}$ subsets) or *En1::Cre; FoxP2::Flpo; RCE.dual.GFP; Rosa.lsl.tdT* mice (for $V1^{FoxP2}$ interneurons) under epifluorescence (Olympus BX50WI microscope, 40x water-immersion), and interneurons primarily within lumbar segment 3 were patched at higher magnification (X2 magnifier) under DIC optics. Patch pipettes were filled with an internal solution containing (in mM): 122.5 potassium D-gluconate, 17.5 KCl, 9 NaCl, 1 $MgCl_2$, 10 HEPES, 3 Mg-ATP, 0.3 Na-GTP, 0.1% Cascade Blue, and sometimes 20 EGTA, pH adjusted to 7.2-7.3. Recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA), using an output gain of 1 (current clamp) and a Bessel filter (10 kHz), and digitized at 16-bit resolution (Digidata 1322A, Molecular Devices).

V1 interneuron passive properties, including input resistance (R_i) and cell capacitance (C_m), were measured in whole cell voltage-clamp (-70 mV holding potential, -5 or -10 mV pulses at 200 Hz, or -5 mV pulses for a duration of 20 msec). Cells were excluded if pipette access resistance (R_a) exceeded 20 MΩ, or if the spike did not overshoot by at least 10 mV. Active properties were measured in current-clamp, in response to 435 msec current pulses. We focused on several firing characteristics, including (i) a bursting or non-bursting phenotype, and (ii) the degree of spike frequency adaptation (SFA, indicated by larger interspike intervals in successive spikes through a pulse), and also assessed the presence or absence of low-threshold depolarizations underlying the initial spiking. Increasing levels of current were injected in 10 pA increments, up to a total of 60 pulses or until the action potential mechanism deteriorated (characterized by a progressive widening and decreased amplitude of successive action potentials during a single pulse). All cells were held between -80 and -87 mV, based on preliminary studies showing that the low threshold depolarizations that account for large differences between $V1^{FoxP2}$ interneurons and $V1^{R}$ or $V1^{MafB/Pou6f2}$ interneurons are revealed only under hyperpolarizing conditions. To assess bursting and the degree of SFA, instantaneous frequency (IF) plots were constructed by measuring peak-to-peak interspike intervals throughout the pulse. Bursting cells were characterized by large instantaneous frequencies at the beginning of the pulse that then quickly drop to steady state firing, represented as either horizontal lines (suggesting no SFA) or lines that slope downward (indicating the presence of SFA). Threshold was measured in the first pulse to elicit an action potential, and defined as the membrane voltage with the maximum velocity depolarization change (i.e., peak acceleration).

V1 subpopulation	Segmental Level	# of animals	# of hemisections	# of neurons	Position-based p-value, x-axis (Wilcoxon Rank Sum test)*	Position-based p-value, y-axis (Wilcoxon Rank Sum test)*	Position-based p-value (2D KS test)*	Fractional area**	3D cell density (cells per 10^6 μm^3)	Mean pairwise distance (μm)***
Bhlhb5	L3-L5	6	20	648	1.99E-01	1.96E-12	9.70E-16	0.615	16.4	185.18
FoxP1	L3-L5	7	17	370	6.47E-01	6.03E-05	2.29E-06	0.692	11.4	176.14
FoxP2	L3-L5	7	16	682	2.93E-01	8.24E-05	3.33E-04	0.855	14.6	195.97
FoxP4	L3-L5	7	16	304	1.88E-15	3.83E-30	2.05E-44	0.444	13.9	133.09
Lmo3	L3-L5	3	13	1028	2.50E-03	9.21E-01	2.36E-05	0.855	32.0	184.07
MafA	L3-L5	7	21	179	6.29E-01	6.94E-50	1.82E-41	0.217	10.3	126.13
MafB	L3-L5	5	14	349	3.75E-02	2.92E-21	1.27E-15	0.602	13.2	206.04
Nr3b2	L3-L5	6	19	296	1.02E-01	6.98E-33	6.32E-32	0.447	10.6	153.40
Nr3b3	L3-L5	5	11	287	1.63E-07	1.19E-09	6.32E-10	0.713	11.3	188.27
Nr4a2	L3-L5	8	18	184	1.45E-01	9.16E-02	5.27E-06	0.528	5.4	152.46
Nr5a2	L3-L5	6	25	256	5.64E-24	8.25E-01	5.17E-27	0.542	5.7	181.10
Onecut1	L3-L5	7	15	218	1.43E-07	1.35E-34	3.54E-26	0.298	13.6	169.51
Onecut2	L3-L5	5	13	248	3.80E-04	2.52E-28	3.36E-20	0.415	12.2	185.87
Otp	L3-L5	6	21	866	1.80E-02	9.31E-01	3.85E-12	0.659	19.6	168.23
Pou6f2	L3-L5	6	14	282	1.50E-21	7.79E-33	1.93E-28	0.583	9.9	164.58
Prdm8	L3-L5	6	22	506	2.48E-14	5.88E-01	4.29E-15	0.648	9.5	209.85
Prox1	L3-L5	12	27	237	2.66E-23	2.05E-15	1.66E-32	0.426	6.3	196.71
Sp8	L3-L5	7	24	384	3.63E-68	4.77E-71	7.29E-88	0.386	12.5	149.33
Zfhx4	L3-L5	4	12	335	4.26E-06	7.50E-02	4.05E-10	0.686	11.6	232.65
Nr5a2 + Nr3b2	L3-L5	8	29	51	1.69E-14	2.76E-06	2.45E-25	0.154	3.1	87.95
Pou6f2 + Lmo3	L3-L4	4	16	180	2.73E-49	2.18E-13	8.56E-45	0.365	9.2	123.71
Pou6f2 + Nr5a2	L3-L4	8	15	61	9.15E-11	8.56E-21	2.31E-25	0.236	5.2	85.16
Prdm8 + FoxP4	L3-L5	7	20	187	1.25E-02	3.33E-38	1.27E-42	0.260	10.8	102.14
Prdm8 + Sp8	L3-L5	8	23	190	1.49E-47	1.85E-60	2.26E-77	0.286	8.9	104.40
Prox1 + Nr3b3	L3-L5	6	22	50	1.68E-26	1.14E-08	1.38E-31	0.085	8.0	62.17

Table S1. Statistical Analysis of Spatial Distributions of V1 Subpopulations; Related to Figure 3

Sumary of spatial metrics and statistical analysis for each of the V1 subpopulations. *p-values correspond to comparisons of the distributions of a given V1 subpopulation and the parental V1 population. **All V1 subpopulations covered a significantly smaller area than the parental V1 population ($p < 0.001$ by one-tailed Monte Carlo test). ***The mean pairwise distance for parental V1 interneurons is 212.09 μm.

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