SPINAL V1 INHIBITORY INTERNEURON CLADES DIFFER IN BIRTHDATE, PROJECTIONS TO MOTONEURONS AND HETEROGENEITY.

Abbreviated title: Diversity of V1 spinal interneurons

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ABSTRACT

Spinal cord interneurons play a crucial role in shaping motor output, but their precise identity and circuit connectivity remain unclear. Focusing on the cardinal class of inhibitory V1 interneurons, we define the diversity of four major V1 subsets according to timing of neurogenesis, genetic lineage-tracing, synaptic output to motoneurons, and synaptic inputs from muscle afferents. Birthdating delineates two early-born (Renshaw and Pou6f2) and two late-born V1 clades (Foxp2 and Sp8) suggesting sequential neurogenesis gives rise to different V1 clades. Neurogenesis did not correlate with motoneuron targeting. Early-born Renshaw cells and late-born Foxp2-V1 interneurons both tightly coupled to motoneurons, while early-born Pou6f2-V1 and late-born Sp8-V1 interneurons did not. V1-clades also greatly differ in cell numbers and diversity. Lineage labeling of the Foxp2-V1 clade shows it contains over half of all V1 interneurons and provides the largest inhibitory input to motoneuron cell bodies. Foxp2-V1 subgroups differ in neurogenesis and proprioceptive input. Notably, one subgroup defined by Otp expression and located adjacent to the lateral motor column exhibits substantial input from proprioceptors, consistent with some Foxp2-V1 cells at this location forming part of reciprocal inhibitory pathways. This was confirmed with viral tracing methods for ankle flexors and extensors. The results validate the previous V1 clade classification as representing unique interneuron subtypes that differ in circuit placement with Foxp2-V1s forming the more complex subgroup. We discuss how V1 organizational diversity enables understanding of their roles in motor control, with implications for the ontogenetic and phylogenetic origins of their diversity.

SIGNIFICANCE STATEMENT: Spinal interneuron diversity and circuit organization represents a key challenge to understand the neural control of movement in normal adults and also during motor development and in disease. Inhibitory interneurons are a core element of these spinal circuits, acting on motoneurons either directly or via premotor networks. V1 interneurons comprise the largest group of inhibitory interneurons in the ventral horn and their organization remains unclear. Here we present a comprehensive examination of V1 subtypes according to neurogenesis, placement in spinal motor circuits and motoneuron synaptic targeting. V1 diversity increases during evolution from axial-swimming fishes to limb-based mammalian terrestrial locomotion and this is reflected in the size and heterogeneity of the Foxp2-V1 clade which is closely associated to limb motor pools. We show Foxp2-V1 interneurons establish the densest and more direct inhibitory synaptic input to motoneurons, especially on cell bodies. This is of further importance because deficits on motoneuron cell body inhibitory V1 synapses and on Foxp2-V1 interneurons themselves have recently been shown to be affected at early stages of pathology in motor neurodegenerative diseases like amyotrophic lateral sclerosis.

INTRODUCTION

The spinal cord contains a diversity of interneurons that lend to its vast computational power with inhibitory interneurons critically modulating and patterning motoneuron firing to adjust timing and force of muscle contractions. We focus in here on a major group of ventral inhibitory interneurons known as V1 that originate from p1 progenitors, express the transcription factor (TF) Engrailed-1 (En1) and send ipsilateral axons to the ventral horn where they densely innervate motoneuron cell bodies and proximal dendrites (Alvarez et al., 2005). Based on differential expression of calcium buffering proteins and synaptology this early study proposed a diversity of phenotypes and circuit roles for V1 interneurons. Later electrophysiological and modeling studies showed that V1 interneurons play crucial roles in shaping motor output by modulating locomotor speed, governing flexion-extension at the level of central pattern generator (CPG) half-centers and/or last-order reciprocal inhibition of antagonistic motoneurons, and providing recurrent feedback inhibition of motoneuron firing (Sapir et al., 2004; Zhang et al., 2014; Britz et al., 2015; Falgairolle and O'Donovan, 2019, 2021; Shevtsova et al., 2022). Accordingly, there is significant interest in defining the molecular identity and circuit organization of different subtypes of V1 interneurons. This is of further significance because recent findings suggest that early V1 disconnection from motoneurons in motor neurodegenerative diseases such as amyotrophic lateral sclerosis contributes to dysfunction and presages motoneuron death (Wootz et al., 2013; Salamatina et al., 2020; Allodi et al., 2021).

Prior work revealed that V1 diversity can be organized into four major clades according to their positions and molecular identity at postnatal day 0 (P0) (Bikoff et al., 2016). Each clade was defined by expression of unique transcription factors. Co-expression of V-maf musculoaponeurotic fibrosarcoma oncogene homologs A and B (MafA/MafB) defines a clade composed of Renshaw cells, the only homogenous functional V1 subgroup (Benito-Gonzalez and Alvarez, 2012; Stam et al., 2012; Bikoff et al., 2016). The other three clades are respectively defined by expression at P0 of the TFs P.O.U. domain (Pou6f2), Forkhead box P2 (Foxp2) and Specificity protein 8 (Sp8) (Bikoff et al., 2016). Within these three clades, additional diversity was uncovered by further combinations of TF expression and positions (Bikoff et al., 2016; Gabitto et al., 2016; Sweeney et al., 2018). A recent harmonized atlas of several mouse spinal cord transcriptomic studies (Russ et al., 2021) identified seven possible V1 groups, including Renshaw cells, Pou6f2-V1s and three Foxp2-V1 groups. V1 genetic diversity parallels the functional diversity described in physiological and modeling studies, but whether V1 clades occupy specific

functional niches in spinal motor circuits remains unclear in part because of lack of information about their synaptic inputs and outputs and their origins.

Here we clarify the origins, diversity, and synaptic relations with motoneurons of the four major V1 clades. V1 neurogenesis was previously divided into early (E9.5 to E10.5) and late phases (E11 to E12.5), each producing distinct V1 interneurons (Benito-Gonzalez and Alvarez, 2012). Early-generated V1 interneurons include MafB positive Renshaw cells, whereas late-generated V1 interneurons comprise Ia reciprocal inhibitory interneurons (IaINs) expressing Foxp2. It is now well accepted that temporal and spatial properties during neurogenesis intersect to create diversity from each spinal cord progenitor domain (Sagner and Briscoe, 2019; Deska-Gauthier et al., 2020; Deska-Gauthier and Zhang, 2021; Osseward et al., 2021; Sagner et al., 2021). Using further nodal intersections between transcriptomics and neurogenesis, one recent report described seven embryonic V1 groups defined by TFs with temporally restricted expression (Delile et al., 2019). However, because of the dynamic nature of TF expression in spinal interneurons, it is difficult to match embryonic TFs in this study to the V1 clades previously defined at P0. Using clade-defining TFs in combination with 5-ethynyl-2'-deoxyuridine (EdU) birthdating, we identified a relationship between neurogenesis timing and clade identity and uncovered additional diversity within Foxp2-V1 interneurons, the largest V1 clade. To study Foxp2-V1 interneurons we used intersectional genetics to lineage label their cell bodies and axons. This revealed subdivisions according to location and expression of additional TFs. We also found that Foxp2-V1s establish the highest density of V1 synapses on proximal somatodendritic membranes of limb-related lateral motor column (LMC) motoneurons. They also include subgroups receiving dense proprioceptive inputs with some having connectivity of reciprocal IaINs. Together with Renshaw cells they contribute the majority of V1 synapses on motoneuron cell bodies and proximal dendrites, while synapses from other clades (Pou6f2 and Sp8) have minimal representation. We conclude that V1 clades differ in time of neurogenesis, internal heterogeneity and synaptic targeting of motoneurons.

RESULTS

V1 interneurons belonging to different clades have distinct timing of neurogenesis.

To examine the birthdates of the different V1 clades, we lineage traced all V1 interneurons (INs) using *en1*$^{cre/+}$*::Ai9R26-tdTomato* mice (En1^{cre}-Ai9:tdT) (sometimes intersected with *foxp2*$^{flpo/+::}$*RCE:dual-EGFP:* En1-Foxp2-RCE:GFP; see below) and pulse-labeled developing

embryos by injecting pregnant females with EdU at specified 12 hours intervals between E9.5 to E12.5 (Figure 1A). All born pups carrying the genetic V1 lineage markers were analyzed at P5. This age was chosen to preserve as much as possible expression of V1-clade defining TFs (MafB, Pou6f2, Foxp2 and Sp8) for antibody detection. We analyzed spinal cords at E9.5 (n=3; 3 litters), E10 (n=6; 3 litters), E10.5 (n=5; 3 litters), E11 (n=5; 2 litters), E11.5 (n=6; 3 litters), E12 (n=3; 1 litter), and E12.5 (n=4; 2 litters). In all animals we confirmed the expected sequence of cell birthdates in the mammalian spinal cord, from ventro-lateral to dorso-medial locations (Figure 1B) (Altman and Bayer, 2001). One animal pulse-labeled at E11 was removed from the analyses because EdU labeled cells did not correspond to the expected distribution (marked with x in Figure 1D). To ensure only V1 cells that incorporated EdU at the time of injection were included in the analyses, we defined positive neurons as those with more than two-thirds of the nucleus showing homogeneous EdU fluorescence. Neurons with speckles or partial nuclear labeling (Figure 1C) were discarded. Incompletely labeled cells may arise from either partial dilution of EdU after one division cycle or by incorporating EdU during late S-phase or DNA repair events (Packard et al., 1973; Ferreira et al., 1997; Taupin, 2007). Using our labeling criteria, we found varying percentages of V1s are generated at different time points, with a peak at E11 in which 23.4% ±2.9 (mean±SD) of V1s incorporated EdU (Figures 1D,E). After adding together all EdU-labeled V1s at all time points we account for 71.4% of all V1 interneurons. If we include V1 neurons with speckles or partial nuclear labeling, we overrepresent V1s by more than double (255.9%), and obscure differences in EdU labeling at different time points. We nevertheless found overlap among animals' pulse-labeled in contiguous 12-hour time-points. This is expected since our timed-pregnancies have a ±12-hour resolution. Furthermore, within single litters it is common to find animals with developmental differences of 6 to 12 hours. Finally, although EdU has a very short half-life in mice (1.4 ±0.7 mins; (Cheraghali et al., 1994)) and, therefore, its bioavailability should overlap little with injections separated by 12 hr, estimates of S-phase duration during neurogenesis vary between 4 and 17 hrs (Ponti et al., 2013) suggesting other opportunities to create overlap from EdU injections spaced by 12 hr. These sources of variability are inherent to the technique and explain the relatively larger standard deviations on the percentages of V1s labeled at time points with the largest change in birthdates, around peak V1 neurogenesis. In summary, using rigorous criteria for defining EdU labeling and averaging animals from different litters allows to derive the strongest conclusions possible when estimating differences in birthdates.

To analyze birthdates of V1 interneurons identified by clade specific markers (Figure 1F-I), we first compared the number of V1s expressing clade-defining TFs at P5 with previous

estimates at P0 (Bikoff et al., 2016). Overall, MafB-V1s were 9.4% ±1.6 (±S.D.) of the whole V1 population (n = 21 animals) which differs to 25% at P0 form Bikoff et al (2016). Small differences were found for Pou6f2-V1s 8.1% ±4.6 (n = 19) compared to 13% at P0; Foxp2-V1s 32.5% ±8.4 (n = 26) compared to 34% and Sp8-V1s 8.8% ±2.6 (n = 26) compared to 13%. Differences in Pou6f2-V1, Foxp2-V1 and Sp8-V1 cells might be due to small differences in expression due to age and/or immunocytochemical (ICC) sensitivity. In contrast, the large differences found with multi-clade MafB-V1s likely result from rapid downregulation of MafB in some V1 clades after birth. MafB is expressed at P0-P5 in three V1 clades. Ventral MafA-calbindin Renshaw cells, a subpopulation of dorsal Pou6f2-V1s, and subgroups of Foxp2-V1s distributed throughout the ventral horn. MafB is quickly downregulated after birth in Foxp2-V1s, but expression is maintained in Pou6f2-V1s and ventral Renshaw cells (see below). When considering only ventral MafB-V1s located in the Renshaw area (Renshaw V1 clade) we obtained at P5 a percentage not different to the percentage at P0 for the MafA-Renshaw cell V1 clade (5.5% ±1.7, n = 21 vs 5% (Bikoff et al., 2016)). We conclude that TF ICC detection at P5 provides an accurate representation of V1 clades previously defined at P0.

Birthdating divides V1 clades into two groups: early-born and late-born. Most early-born V1 cells are EdU-labeled before E11 and include Renshaw cells and Pou6f2-V1s. Most cells from the two other clades (Foxp2-V1s and Sp8-V1s) are later-born (after E11) (Figures 1F-I). These data derive from 68,562 V1s sampled from 29 animals in 4 ventral horns per animal/TF/Edu time point (average 197.0 V1s per ventral horn). V1 clades differ in their peak times and the spread of birthdates, with Foxp2-V1s and Sp8-V1s showing the larger spreads and later peaks (Figure 1G-H). Pou6f2-V1s, dorsal MafB-V1s, and ventral MafB-V1s display narrower spreads of birthdates and earlier peaks. Normalizing to peaks reveals that ventral MafB-V1 Renshaw cells are generated the earliest (E10.5 peak), followed by Pou6f2-V1s including dorsal MafB-V1s (E11.0 peak), Foxp2-V1s (E11.0-E11.5 peak) and Sp8-V1s (E12 peak). Around 50% of all V1s are born by E11, including most cells in early-born clades and a proportion of Foxp2-V1s and Sp8-V1s (Figure 1I). Less than 10% of Foxp2-V1s and Sp8-V1s are generated before E10.5 and almost no ventral MafB-V1s or Pou6f2-V1s (including the dorsal MafB-V1s) are generated after E11 (Figure 1I). After E12, only Sp8-V1s are generated. Cumulative graphs through all ages show that overall, EdU labeling accounted for 67.3% of all ventral (Renshaw) MafB-V1s, 62.4% of Pou6f2-V1s, 68.2% of dorsal MafB-V1s, 49.8% of Foxp2-V1s and 55.8% of Sp8-V1s. This suggests significant representation from all clades in our EdU analyses (Figure 1I).

Next, we examined whether clade-specific V1 interneurons with different birthdates settle in different positions. We plotted the positions of V1 interneurons from each clade identified by the TFs MafB, Pou6f2, Foxp2 and Sp8 and that are born at different embryonic times (Figure 2). We compared time windows between E10 and E12 because few V1s were found with strong EdU at E9.5 and E12.5 which reduced the accuracy of cellular density plots. V1s within these clades showed subpopulations located at different positions according to birthdate. The earliest-born MafB-V1s accumulate ventrally in the “Renshaw-cell area”. Later-born MafB-V1s occupy more dorsal positions because they are a subgroup of Pou6f2-V1s. Pou6f2-V1s born at different time points always settle in dorsal positions. In contrast, Foxp2-V1s are a large group whose generation lasts the whole V1 neurogenesis period. They show large differences in localization according to birthdate. Foxp2-V1s born at E10 occupy dorsal locations while those born between E10.5 and E11.5 settle laterally: close to the lateral motor column (LMC). There is a dorsal to ventral progression of Foxp2-V1 cell born from E10.5 to E11. Foxp2-V1s born at E12 are located ventro-medially. In summary, Foxp2-V1s follow a clockwise rotation in positioning according to their time of neurogenesis. Finally, early born Sp8-V1 cells are located ventrally while later born Sp8-V1 cells are located more dorsally and medially.

In summary, different V1 clades are generated through overlapping windows of neurogenesis but with distinct peaks which allow classification into early-born (Renshaw cells and Pou6f2-V1s) and late-born clades (most of Foxp2-V1s and Sp8-V1s). Within clades, subgroups with different birthdates settle at specific positions. This is most evident in the largest Foxp2-V1 clade suggesting significant cellular heterogeneity.

MafB-V1s genetic labeling reveals two main types in the mature spinal cord.

We used MafB antibodies to identify the Renshaw cell clade because we were unable to label enough Renshaw cells with MafA antibodies at P5. MafB, however, is also expressed in subpopulations of Pou6f2-V1 and Foxp2-V1 cells at P0 (Bikoff et al., 2016). To better identify these cells, we used a genetic detection strategy by introducing a *mafb*^{GFP} reporter allele (Moriguchi et al., 2006) in En1^{cre}-Ai9:tdT mice (Figure 3). We also used two different antibodies against MafB of differing sensitivity and specificity (Figure 3 Supplemental). The MafB antibody with the highest specificity (Novus NB600-266)(Supplemental Figure 3.1) displayed weaker labeling of Renshaw cells at P5 in spinal cord sections. In contrast, the antibody with highest sensitivity at P5 (Sigma HPA005653) also showed cross-reactivity with other targets in tissue sections and Western blots. Each antibody was directed against different regions of the mouse

mafB gene: aa18-167 for the Sigma antibody and aa100-150 for the Novus antibody. These target sequences are shared with MafA and c-Maf, but the overlap is larger with the Sigma antibody immunogen. Renshaw cells co-express MafA, but Western blots ruled out any cross-reactions with MafA for either of the MafB antibodies (Supplemental Figure 3). However, both antibodies produced a double band between the 37 and 50 kDa markers in Western blots, with only the lower band diminishing with a lower *mafB* gene dose in heterozygotes or absence in knockouts (Supplemental Figure 3). The upper band was not affected by gene dose. In a parallel western blot, the upper band corresponded to c-Maf. This suggests that in Western blots both MafB antibodies cross reacted with c-Maf. In tissue sections only the Sigma antibody cross reacted with other targets, likely c-Maf. This would explain the larger number of dorsal horn interneurons positive for the Sigma antibody compared to the Novus antibody. Many interneurons at this location are known to express c-Maf (Hu et al., 2012; Frezel et al., 2023). Despite these limitations, MafB detection was similar for both antibodies in Renshaw cells and Pou6f2-V1 cells. Expression of the *mafB* gene was further confirmed in these cells with the $mafB^{GFP}$ allele. We preferentially used the more sensitive Sigma antibody because it optimally recognized MafB in Renshaw cells at P5. Intriguingly, immunostaining with either antibody could not label any V1 cells in mature spinal cords despite continuous expression of MafB-Green Fluorescent Protein (GFP was detectable after P15 only after amplification of fluorescence with antibodies). This suggests that in addition to progressive downregulation of gene expression, the amount of detectable protein is further reduced at older ages by control of *mafB* mRNA translation and/or higher TF epitope masking in mature chromatin.

MafB-V1s are best identified in $mafB^{GFP/+}$::En1^{cre}-Ai9:tdT mice, particularly after amplification of GFP fluorescence with antibodies. In these animals, GFP is present in a variety of spinal neurons in the dorsal and ventral horns and in small microglia. In mature (P15) animals, two groups of MafB-V1 interneurons (GFP + tdtomato, tdT) can be identified: one occupies the dorsal region of the V1 cell distribution; the other is located ventrally, is calbindin-immunoreactive (-IR) and corresponds to Renshaw cells (Figure 3A). These two V1 cell groups are also identifiable at P5, but at this age weak GFP is also visible in other V1 cells located in the middle of the ventral horn and that likely belong to the Foxp2-V1 clade. Their numbers vary from animal to animal. This suggests *mafB* gene expression is in the process of downregulation in these cells and explains the high interanimal variability at P5 and the absence of expression at P15. We focused our analyses in dorsal and ventral MafB-V1 populations defined by boxes of 100 µm width at the level of the central canal (dorsal) or the ventral edge of the gray matter (ventral). Together, MafB-V1s in these two regions constitute 13.2% ±2.8 (mean±SD) of all V1s at P5 (n=17 mice) with similar

representation in dorsal (7.1% ±2.4) and ventral (6.0% ±1.2) groups (Figure 3B). These percentages did not change at P15. At P5, on average, 54.2% ±19.4 of dorsal and 70.5% ±10.1 of ventral MafB-V1s had detectable MafB-IR (Figure 3C, n = 9 mice tested). No MafB protein was detected at P15. We tested V1 clade-immunomarkers in ventral and dorsal MafB-V1 cells at P5 (calbindin for Renshaw cells, and Pou6f2, Foxp2 and Sp8 for other V1 clades) (Figure 3D). Dorsal MafB-V1s expressed Pou6f2 (41.1% ±7.4, n=5 mice tested), negligible calbindin (1.5% ±1.8, n=4 mice) and no Foxp2 or Sp8 (0%, n=5 mice). Most ventral MafB-V1s expressed calbindin-IR (79.3% ±8.8, n=4 mice) while expression of other clade markers was negligible (Pou6f2, 0%; Foxp2, 0.4% ±0.9; Sp8. 0.7% ±1.5, n=5 mice) (Figure 1E). Using this genetic model, we confirmed birth dates of dorsal (Pou6f2) and ventral (Renshaw) V1s (Figure 3F, n=2 mice per EdU injection date) and found an excellent match in birthdates of dorsal MafB-V1s identified by either MafB-IR or MafB-GFP. However, we found more ventral MafB-V1s born at E11 using genetic labeling compared to antibody staining, although this difference was not statistically significant. In conclusion, we confirmed both MafB-V1 populations and their early birth dates using a genetic model. These two classes constitute discreet V1 populations. The functional significance of adult dorsal Pou6f2-MafB-V1s is yet unexplored.

Genetic labeling of the Foxp2-V1 lineage reveals twice the number of neurons relative to postnatal Foxp2 expression.

To label the Foxp2-V1 interneuron lineage independent of developmental regulation of Foxp2 expression, we utilized an *en1* and *foxp2* intersection model by generating $en1^{cre/+}::foxp2^{flpo/+}::R26^{RCE\text{-}dualGFP/Ai9\text{-}tdT}$ mice. In these animals we expected that neurons expressing *en1* and not *foxp2* would be labeled with tdT from the R26-Ai9 cre reporter, and that neurons that expressed both *en1* and *foxp2* would be labeled with GFP from the R26-dual (Cre&Flpo) RCE:GFP and not with tdT because the Ai9 cassette is flanked by FRT sites that can be removed by Flpo. We confirmed either tdT or GFP labeling in most V1 interneurons, but a few expressed both fluorescent proteins (Figure 4A). We believe this is the consequence of Flpo recombination inefficiency in the larger FRET-flanked Ai9 cassette compared to the much shorter FRET-stop signal in the RCE:dualGFP. We thus interpret “yellow” V1 neurons as cells that express the *foxp2* gene either for a short period of time, weakly, or both. For mapping purposes, we included “yellow” cells into the Foxp2-V1 clade since we used only the RCE:dualGFP reporter in many follow-up analyses. The distribution of Foxp2-V1s (green) and non-Foxp2 V1s (red, not green) overlapped in the ventral horn (Figure 4B). Their proportions were constant across

postnatal ages (n=6 ventral horns from 1 mouse at P0, P15 and adult and 36 ventral horns from 6 mice at P5; Figure 4C) and across spinal segments from lower thoracic to sacral level at P15 analyzed in 2 mice (n=6 ventral horns per segment and mouse, Figure 4D). Respectively in each mouse, 51 and 52% of V1 cells were EGFP labeled on average, 34% and 39% were tdT labeled and 8% and 14% had both labels. The whole population of Foxp2-V1 interneurons (green+yellow) cells represents between 59% to 66% of all V1s. However, the data presented before suggest that only between 32% to 34% of V1s express Foxp2-IR at P0 and P5. This indicates that Foxp2 expression is downregulated in around half of lineage-labeled Foxp2-V1 cells before P5. Correspondingly, around half (48.9% ±3.8; n=3 mice) of genetically identified Foxp2-V1 cells were Foxp2 immunolabeled at P5 (Figure 4E-G). The locations of Foxp2-V1 cells with and without Foxp2 immunoreactivity at P5 overlapped in the ventral horn (Figure 4F).

In summary, lineage labeling of cells that express Foxp2 at any time during development result in doubling the size of the Foxp2-V1 clade compared to estimates based on Foxp2 expression at P0 (Bikoff et al., 2016). Percentages remain constant throughout postnatal development and into adulthood suggesting no new expression of Foxp2 in V1 cells after birth.

Foxp2-V1 neurons are only half of all Foxp2 expressing spinal neurons but non-V1 Foxp2 cells distribute to very different regions.

Foxp2 protein is found postnatally in many non-V1 cells (Figures 1F and 4E). To study these populations, we used RC::FLTG reporter mice. These mice carry a dual-conditional allele with a FRT-flanked stop and loxP-flanked tdT::STOP preventing transcription of eGFP. Therefore in *en1*^{Cre}*::foxp2*^{Flpo} mice, cells that express only *foxp2* are labeled with tdT, while additional *en1* expression (Foxp2-V1 clade) results in eGFP fluorescence and removal of tdT. We analyzed two mice at P5 and two at P10, and the sections were counterstained with NeuN antibodies for neuronal confirmation (Figure 4H). We found several non-overlapping populations of Foxp2 cells. Most non-V1 Foxp2 cells are located medially in the ventral horn and can either be neurons or astrocytes (by morphology and lack of NeuN). In addition, glial cells and cells in the central canal and spinal cord midline were strongly labeled. This distribution suggests Foxp2 is transiently expressed in some progenitors, other than p1 (no eGFP astrocytes are observed in En1/Foxp2 derivatives). A few other non-V1 cells correspond to deep dorsal horn neurons (NeuN+). The number of Foxp2 neurons per section diminished in the ventral horn from P5 to P20, as expected, because of the reduction in cellular density as the spinal cord matures and grows. This decrease

did not occur among dorsal horn cells (Figure 4I), which suggests some dorsal horn neurons might upregulate Foxp2 postnatally.

In summary, there are at least three broad different types of Foxp2 neurons in the spinal cord: 1) medio-ventral non-V1 neurons that express Foxp2 postnatally and/or at the progenitor stage, sharing labeling with glial cells; 2) dorsal horn non-V1 neurons in which Foxp2 expression increases during postnatal development; 3) V1 neurons that upregulate Foxp2 in embryo and then remain a stable population postnatally (see also (Benito-Gonzalez and Alvarez, 2012). Overall, Foxp2-V1 interneurons comprise 55.7% ±4.7 (mean±SD) of all genetically labeled Foxp2 neurons in the spinal cord (n=4 mice, 2 P5 and 2 P20, Figure 4J).

Foxp2-V1 neurons from lower thoracic to sacral segments follow motoneuron numbers in a 2:1 or 3:1 ratio.

To obtain insights into the possible function of Foxp2-V1 interneurons we analyzed possible shifts in localization and numbers according to spinal cord segment in the lumbosacral region concerning control of lower body and hindlimbs. We examined spinal cord sections from segments Th13 to S1 in P20 mice (n=5) expressing eGFP in Foxp2-V1 interneurons combined with choline acetyltransferase (ChAT) immunoreactivity to identify the motor pools. Motoneurons were defined as any Chat-IR neuron in lamina IX (Figure 5A). Spinal segments were identified by the characteristic distribution and size of the somatic lateral, hypaxial and medial motor columns (LMC, HMC and MMC), and by the presence of autonomic sympathetic (Th13-L2) or parasympathetic (S1) neurons. We did not attempt to distinguish Lumbar 4 from 5 because of their similarity. From these sections we constructed cell plots for each animal (4 ventral horns per animal/segment, Figure 5B) and transformed these into density plots (Figure 5C) by combining all cell plots from all animals analyzed in each segment (n=3-5 mice depending on segment). Foxp2-V1 interneurons are located throughout the ventral horn but accumulate laterally. In segments in which the LMC expands, Foxp2-V1 interneurons border medially the LMC. Contour density plots indicate that the highest density of Foxp2-V1 interneurons lies adjacent to the LMC, suggesting a close relationship between Foxp2-V1 interneurons and the control of limb musculature. This is consistent in segments where the LMC emerges (L2), disappears (L6) or where the LMC reaches its maximal size (L4/5). Correspondingly, Foxp2-V1 neuron numbers significantly increase in segments innervating limb muscles (L3-L6) compared to segments involved with axial (Th13, S1) and hypaxial muscles (L1) ($p<0.0001$, one-way ANOVA followed by post-hoc Bonferroni t-tests summarized in Figure 4D and Supplement Table S3). The limb-

innervating LMC is responsible for most of the change in motoneuron numbers across different spinal cord segments. Consistent with V1 interneurons and motoneurons increasing in number in parallel, the ratio of Foxp2-V1 interneurons per motoneuron remained relatively constant from Th13 to L5 (2:1 to 3:1 ratio) (Figure 5E). Differences between Th13 and L6 are non-significant (post-hoc Bonferroni t-tests, details in Supplemental Table S3). The larger ratio at S1 was significant compared to L3 and L4/5 (see Supplemental Table S3 for details). Estimated ratios at S1 were highly variable in the three animals studied likely because sampling issues: the number of motoneurons quickly diminishes in S1 depending on exact section level. Examination of cell plots in segments lacking LMC limb motoneurons show that most Foxp2-V1 interneurons are located dorsally and distal to the motor pools with relatively lower density close to MMC motoneurons. Foxp2-V1 interneurons located further away from the motor pools might have roles other that direct modulation of motoneuron firing. Finally, a sparse group of Foxp2-V1 interneurons is dispersed in the medial ventral horn in all segments with the best representation from L2 to L6. They correspond to the latest born subgroup (and they have a different genetic make-up, see below), suggesting a unique identity.

Foxp2-V1 interneurons project to LMC, HMC, and MMC motoneurons, but not to autonomic motoneurons.

To gain further insight into the functional roles of Foxp2-V1 interneurons. we examined their synapses on different types of motoneurons and compared them to other V1 groups at P20: a time point after critical windows of synapse proliferation and pruning on V1 neurons (Mentis et al., 2006; Siembab et al., 2010). We examined mice in which Foxp2-V1 axons contain eGFP, and non-Foxp2-V1 axons express tdT (n=3) (Figure 4A). Yellow axons were included in the eGFP/Foxp2+ group. We also analyzed mice in which all V1 axons express tdT through the Ai9 reporter (n=2). In these animals we immunostained axons with calbindin antibodies to identify synapses from Renshaw cells. Sections were further immunostained using antibodies against ChAT (to identify motoneuron cell bodies in lamina IX) and with antibodies against the vesicular GABA/glycine amino acid transporter (VGAT) to reveal synaptic vesicle accumulations in genetically labeled axon varicosities contacting ChAT motoneurons. Motoneurons were sampled in different motor columns from Th13 to S1 segments (Figures 6A-B) and examined at high magnification for synaptic contacts from Foxp2-V1, non-Foxp2-V1, all V1 and Renshaw-V1 axons (Figures 6C1 and 6C2). We estimated synaptic contact densities on 3D reconstructions of cell body surfaces using rigorous methodology (Figure 6C3). We calculated overall V1 synaptic

densities (green and red axons in eGFP/tdT dual color mice and all red axons in tdT single color mice) on motoneurons located in motor columns and segments. This included HMC motoneurons in the Th13 segment (ventral body musculature); LMC motoneurons in segments L1/2 (hip flexors), L4/5 (divided into dorsal and ventral pools, innervating distal and proximal leg muscles, respectively) and the dorsal L6 pool (intrinsic foot muscles); MMC neurons in segments Th13, L1/2, L3/4 and S1 (innervating axial trunk musculature and the tail at sacral levels) and finally preganglionic autonomic cells (PGC, sympathetic at Th13 and L1/2 and parasympathetic at S1). We analyzed 4 to 9 motoneurons per animal. Initially, we kept the data separated by mouse identity to check for possible differences due to mouse and/or genetics (Figure 6D). A mixed-effects nested ANOVA revealed significant differences in V1 synapse density over different types of motoneurons ($p<0.0001$), and no influence of mouse or genetics (statistics details in supplemental table S4 and Figure 6D table). Post-hoc Bonferroni t-tests demonstrated that HMC and lower lumbar LMC motoneurons receive significantly more V1 synapses than MMC motoneurons, while LMC motoneurons in L1/2 and L6 had V1 synaptic densities not significantly different to MMC motoneurons. PGC neurons received very low densities of V1 input, significantly lower than LMC or MMC motoneurons.

Next, we examined possible differences between Foxp2-V1 and non-Foxp2-V1 neurons (Figure 6E, top graph). In this case we pooled all motoneurons from 3 mice (n=6 to 16 motoneurons per motor column/segment). We found significant differences according to motoneuron identity ($p<0.0001$), type of axon ($p=0.0107$), and their interaction ($p<0.0001$) (two-way ANOVA, statistics details in supplemental Table S5). This was followed by pair-wise comparisons of synaptic densities according to the type of V1 axon for each motoneuron type (Figure 6E, top graph). For HMC and LMC motoneurons we found significantly higher synaptic densities of Foxp2-V1 axons compared to non-Foxp2-V1 axons in all segments, except for L1/2. The synaptic densities of both types of axons are not significantly different in MMC motoneurons, except for S1 MMC motoneurons that received a significantly higher density of non-Foxp2-V1 synapses. Synapses on PGC neurons were at low density and were highly variable with no differences found between either type of V1 axon. We conclude that motoneurons controlling the hindlimb receive more synapses from Foxp2-V1 interneurons. Foxp2-V1 and non-Foxp2-V1 interneurons equally contact the cell bodies of motoneurons controlling axial musculature.

Finally, we compared synapse densities from Foxp2-V1 interneurons to those originating from Renshaw cells, another V1 interneuron that preferentially targets the proximal region of motoneurons. Renshaw cell axons were identified by the presence of calbindin-IR in tdT V1 axons

(in this case we pooled 10 to 17 motoneurons from two mice). Like above, we found significant differences according to motoneuron identity ($p<0.0001$), type of axon ($p<0.0001$) and their interaction ($p<0.0001$) (two-way ANOVA, statistics details in supplemental Table S6). Post-hoc pairwise comparisons revealed that Renshaw cell synapses occurred at significantly lower densities compared to Foxp2-V1s in all LMC motor groups, except for L1/2 (Figure 6E, lower graph). MMC motoneurons showed similar densities of Renshaw cell and Foxp2-V1 synapses in Th13 and L1/2, higher density of Foxp2-V1 synapses in L3/4, and much higher density of calbindin+V1 synapses in S1. The identity of calbindin+ V1 axons in S1 is unclear because of the relatively higher proportions of calbindin-IR V1 interneurons in sacral segments. Further confirmation of Renshaw cell identity for calbindin+ V1s in S1 is required. PGC neurons received almost no calbindin+ V1 axons (Figure 6E, bottom graph). After calculating the synaptic densities originating from Foxp2-V1 and Renshaw cell axons, we estimated the remaining V1 synapses as non-Foxp2 and non-Renshaw V1. Plotting these three categories of synapses by their percent contributions to all V1 synapses shows that the majority of synapses on the cell bodies of HMC, LMC and MMC motoneurons originate from either Foxp2-V1s or Renshaw cells (Figure 6E). PGC neurons mostly receive inputs from V1s that are non-Foxp2 and non-Renshaw cells, but these synapses are of very low density and high variability. The data suggest that the Foxp2-V1 clade is a major source of inhibitory inputs to motoneuron cell bodies where they likely strongly modulate motoneuron firing. However, given the very large difference in cell numbers between Foxp2-V1 interneurons and Renshaw cells, the data also suggest that the amount of divergence in the Renshaw cell output is likely very high. Foxp2-V1 interneurons preferentially target limb motoneurons, consistent with conclusions from cell density plot analyses in Figure 5. There is a correlation between the densities of Foxp2-V1 synapses and their cell density in proximity to different motor columns and segments (see Figure 5). This suggests that Foxp2-V1 synapses on motoneurons likely originate from V1 interneurons that are clustered spatially close to the LMC. Motoneurons in spinal segments with Foxp2-V1 interneurons located further dorsally from motor pools (e.g., S1) receive a relatively lower density of synapses from Foxp2-V1 interneurons. Pou6f2, Sp8 and other possible V1 clades either do not target motoneurons directly, or they target them sparsely or on distal dendrites. This suggests functional differences among V1 clades regarding the strength of direct modulation of motoneuron firing through proximal synapses.

Foxp2-V1 interneurons clustered near LMC motoneurons are genetically distinct.

Birthdate, spatial organization, and synapse densities on different motor columns all suggest that Foxp2-V1 interneurons are heterogeneous and that a laterally-located group close to LMC motor pools might modulate the output of limb motoneurons. To identify potential genetic differences among Foxp2-V1 interneurons, we examined the published TF expression profiles of V1 interneurons (Bikoff et al., 2016), and selected two TFs highly enriched in Foxp2-V1 interneurons for further study: Orthopedia homeobox (Otp) and Foxp4. We used antibodies to reveal V1 interneurons expressing Otp and/or Foxp4 in the P5 spinal cord (all analyses were performed in Lumbar 4 and 5 segments). We first used two animals with Foxp2 and non-Foxp2 V1 interneurons labeled respectively with eGFP and tdT. Both Otp and Foxp4 are almost exclusively expressed by Foxp2-V1 interneurons at P5 with negligible expression in non-Foxp2 V1 interneurons (Figure 7A). Otp was expressed in around 50% of lineage-labeled Foxp2-V1 interneurons and Foxp4 was expressed in around 20%. Foxp4-IR / Foxp2-V1 interneurons always co-localized with Otp-IR, indicating they are a subpopulation of the Otp group. To examine the relationship between these groups with V1 cells that retain Foxp2 expression at P5, we generated different combinations of paired immunolabelings for Otp, Foxp4 and Foxp2 (Figure 7B) in 3 mice with eGFP expression in Foxp2-V1 interneurons and liberating the red channel for TF co-localization. We constructed cell density contours and calculated the percentages of lineage-labeled Foxp2-V1 cells (eGFP) expressing different combinations of Otp, Foxp4 and Foxp2 (Figure 7C). To summarize the most salient results, a large group of Foxp2-V1 cells that co-expresses Otp and Foxp2 at P5 (44% of Foxp2-V1 cells) is localized laterally close to the LMC. It includes a smaller subgroup that also expresses Foxp4 (23% of Foxp2-V1 cells) and that is located more ventrally. Many ventromedial Foxp2-V1 cells express Foxp2, but never Otp or Foxp4 at P5, and they occupy the locations of very late-born Foxp2-V1 cells (Figure 2). A second group of cells expressing only Foxp2 is located more dorsally.

Next, we tested whether Otp and Foxp4 expressing populations match the dorsal to ventral progression of birthdates observed in laterally-located Foxp2-IR V1s (Figure 2). We first investigated possible differences in the birthdate time course of Foxp2-V1 cells labeled genetically (Figure 7D) in comparison with V1 cells identified by Foxp2 expression at P5 (Figure 1G-H and 7F). The time course of neurogenesis was similar, but a few differences were also noted. Like Foxp2-IR V1 cells, most cells in the genetically labeled Foxp2-V1 lineage were born between E10.5 and E12.0, with few born at E10.0 or E12.5 (n=1-2 mice per time point). The peak of neurogenesis for the lineage-labeled population occurred at E11.0; that is 12 hr. earlier than the peak of neurogenesis for V1 cells expressing Foxp2 at P5. This can be explained by a proportion of the earliest born cells in the Foxp2-V1 clade downregulating Foxp2 expression by P5. Next, we

used Otp and Foxp4 to subdivide lineage-labeled lateral Foxp2-V1 cells. To increase sample size, we pooled spinal cords from mice in which we genetically labeled both Foxp2-V1s (eGFP) and non-Foxp2-V1s (tdT) (Figure 7E) together with mice having all V1s lineage-labeled with tdT (n = 4 at E10, 5 at E10.5, 4 at E11, 3 at E11.5 and 2 at E12.5). Pooling data from both genetic models is justified by the above results showing that V1 cells expressing Otp or Foxp4 at P5 are all contained within the Foxp2-V1 lineage. The neurogenesis curves of V1 cells expressing Otp, Foxp4 and Foxp2 at P5 largely overlapped. All three groups had a peak at E11.5 with almost no cells generated at E10.0 or E12.0. The locations of Otp and Foxp4 expressing V1s generated between E10.5 and E11.5 were lateral for Otp and ventral for Foxp4. The location of Foxp4 cells generated at E10.5, E11.0 and E11.5 did not change significantly, but the location of Otp cells generated at E11.5 shifted ventrally compared to those born at E10.5 or E11.0 (Figure 7F). We interpret this result as suggesting that generation of Foxp4-Otp Foxp2-V1 interneurons overlaps with the generation of other Otp cells in the same lineage, but also outweighs other groups towards the end of Otp Foxp2-V1 neurogenesis.

The results can be summarized by proposing at least four groups of Foxp2-V1 cells according to location, TF expression, and birthdate (Figure 7G). Group I is located dorsally, lacks Otp, and has variable expression of Foxp2 at P5. Many are likely generated during early Foxp2-V1 cell neurogenesis (before E10.5). Groups II and III are laterally located and express Otp with or without Foxp2 (group II) or express Otp, Foxp4 and Foxp2 together (group III). These two groups together represent the largest class of Foxp2-V1 cells in L4/5 and are generated during a 24-hr. period from E10.5 to E11.5 with a slight shift in the balance of group II vs III cells at later times of neurogenesis. Finally, medial Foxp2-V1 cells (group IV) are generated very late (after E12: see Figure 2) and lack expression of Otp or Foxp4, but many retain expression of Foxp2 at P5. We failed to identify in this medial Foxp2-V1 group expression of markers for late-born spinal interneurons located in the medial ventral horn, like NeuroD2 and Prox1 (Delile et al., 2019; Osseward et al., 2021), despite presence of many neurons positive for these TFs in the vicinity.

Otp-expressing Foxp2-V1 cells receive proprioceptive synapses.

Previous studies reported that Foxp2-V1 interneurons include a large class of proprioceptive interneurons, some of which could represent reciprocal Ia inhibitory interneurons (IaINs) because they receive convergent synapses from excitatory proprioceptive sensory axons (VGLUT1+) and inhibitory Renshaw cells (calbindin+ V1 axons) (Benito-Gonzalez and Alvarez, 2012). Likely candidates are Otp Foxp2-V1 interneurons in groups II and III because their

localization matches that of electrophysiologically identified IaINs in the cat (Jankowska and Lindstrom, 1972; Alvarez et al., 1997). To examine this question, we first analyzed the types of Foxp2-V1 interneurons receiving proprioceptive inputs. The spinal cords of two mice at P5 (to preserve TF expression) containing Foxp2-V1 lineage-labeled cells (eGFP) were dual or triple immunolabeled for VGLUT1 and Otp and/or Foxp2 (Figure 8A). We analyzed 6 ventral horns at L4/L5 in each animal using high magnification confocal microscopy (60x), tiling the whole ventral region containing all Foxp2-V1 cells. Cells were annotated as receiving no synapses (I in Figure 8A) or low/medium and high density of VGLUT1 synapses (respectively, II and III in Figure 8A). We also noted whether these synapses were located proximally (on cell body and primary dendrites) or more distally (VGLUT1 synapses far from cell bodies on higher order dendrites). In general, cells with proximal VGLUT1+ synapses had higher densities than those with only distal synapses. Overall, we found that 63.0% and 74.3% of Foxp2-V1 interneurons received VGLUT1 synapses in each animal (Figure 8B). Foxp2-V1 cells with no VGLUT1 synapses were found throughout the ventral horn, but those receiving VGLUT1 synapses had a lateral positioning bias (Figure 8C).

Next, we examined Otp and Foxp2 expression in genetically labeled Foxp2-V1 interneurons receiving VGLUT1 synapses (Figure 8D). On average (n=2 mice, 12 ventral horns and 1,116 Foxp2-V1 cells analyzed with similar representation in both animals), 42.5% of Foxp2-V1 interneurons had VGLUT1 contacts and were Otp+ while 22.1% were Otp(-), that means, 65.8% of Foxp2-V1 interneurons targeted by VGLUT1 synapses express Otp. On average only 9.3% of Foxp2-V1 interneurons that were Otp+ lacked VGLUT1 synapses. Thus, we detected VGLUT1 synapses on 82.0% of Otp+ Foxp2-V1 interneurons and 64.9% received these synapses proximally (cell body and primary dendrites) in addition to also having VGLUT1 synapses on more distal dendrites. Sections immunolabeled for Foxp2 revealed that 38.2% of Foxp2-V1 interneurons had VGLUT1 contacts and expressed Foxp2 while 17.4% were Foxp2(-). Moreover, only 14.0% of Foxp2-V1 interneurons retaining Foxp2 expression at P5 lacked VGLUT1 synapses. This suggests that 68.7% of interneurons in the Foxp2-V1 lineage that were targeted by VGLUT1 synapses retain Foxp2 expression at P5, and that 73.3% of Foxp2-V1 interneurons with genetic and antibody labeling received VGLUT1 synapses. Of these cells, 60.2% received VGLUT1 inputs proximally. Moreover, in one of the two animals we also analyzed Otp and Foxp2 co-localization in Foxp2-V1 interneurons receiving VGLUT1+ synapses. We found that 85.6% of Otp+ cells in the Foxp2-V1 lineage and receiving VGLUT1 synapses also express Foxp2 at P5, while 89.8% of dual genetic and antibody labeled Foxp2-V1 cells receiving VGLUT1 synapses are also Otp+. In conclusion, most laterally positioned Foxp2-V1 interneurons that express Otp

and Foxp2 at P5 receive VGLUT1 synapses. VGLUT1 synapses in the ventral horn at P5 originate solely from parvalbumin+ proprioceptors, most likely Ia afferents (Alvarez et al., 2004).

Foxp-V1 interneurons integrated in reciprocal inhibitory circuits between antagonistic muscles.

To examine whether some of these cells have inputs and outputs embedded in reciprocal inhibitory circuits, we combined anterograde labeling of sensory afferents with cholera toxin subunit B (CTB) injected in the tibialis anterior (TA) muscle with retrograde monosynaptic labeling of premotor interneurons using glycoprotein (G) deleted mCherry rabies virus (RV∆G-mCherry) injected in the antagonistic lateral gastrocnemius muscle (LG) of mice with eGFP labeled Foxp2-V1 interneurons (Figure 8E). The sections were immunolabeled for CTB, VGLUT1, mCherry and eGFP. To obtain transcomplementation of RV∆G-mCherry with glycoprotein in LG motoneurons we first injected the LG muscle with an AAV1 expressing B19-G at P4. To optimize muscle targeting and avoid cross-contamination of nearby muscles we performed RV∆G and CTB injections at P15. Analyses were done at P22, a timepoint that is after developmental critical windows through which Ia(VGLUT1+) synaptic numbers increase and mature on V1-IaINs (Siembab et al., 2010).

Unfortunately, motoneuron infection from muscle and transsynaptic retrograde labeling using RV∆G is known to be inefficient after P10 (Stepien et al., 2010). Additionally, at older ages transsynaptic transport is slower and more temporally spread such that fewer interneurons are recovered at single time points after injection. We chose 7 days post-injection for analyses to avoid as much as possible the cell degeneration that occurs at longer survival times after RV infection. We traded the low yield of these experiments for higher specificity when identifying synaptic inputs from TA sensory afferents on Foxp2-V1 interneurons that are premotor to the LG motor pool. We injected 5 animals that were analyzed in serial sections from L2 to L6 segments. All mice showed consistent TA anterograde labeling that in the ventral horn of the L4/L5 segments occupies the dorsal third of LVII and LIX (Figures 8F and 8G). This distribution matches the well-known musculotopic trajectories of central Ia afferents axons in the ventral horn (Ishizuka et al., 1979). In agreement with the known rostro-caudal trajectories of Ia afferent terminal axon collaterals entering the spinal cord, TA-CTB VGLUT1 labeled synapses were found in all lumbar segments, but caudal lumbar segments had the largest density of labeling in the ventral horn. Additionally, there were dense projections to medial LV and to discreet regions in LIII and LIV in

all segments in all animals. In contrast, only 3 mice showed transsynaptic transport of RVΔG-mCherry from the LG motor pool to interneurons, with large variability from animal to animal. In the best animal we recovered 51 transsynaptically labeled interneurons with no evidence of degenerative phenotypes. These cells were found at the same locations, and in similar proportions as was reported by other groups using injections in younger animals where more cells were labeled (Stepien et al., 2010; Tripodi et al., 2011; Ronzano et al., 2022). The interneuron sample included cells in the Renshaw area (n=6 or 11.8%), LVII (15, 29.4%), dorsal horn medial LV (7, 13.7%), dorsal horn LI to LIV (20, 39.2%), and the contralateral spinal cord (3, 5.9%: 1 in LX and 2 in LVIII). Pooling cells with transsynaptic labeling from all three animals, we identified 8 LVII Foxp2-V1 interneurons. Their dendritic arbors were reconstructed in Neurolucida following the mCherry labeling. Five of these cells received more than 1 TA synaptic contact (CTB+ and VGLUT1+) although with large differences in number (5, 9, 12, 17, and 31 synapses). Most synaptic contacts occurred on dendrites, particularly those crossing areas with many CTB-labeled TA afferents. Therefore, the presence and direction of cut dendrites in the section strongly influences the total number of synapses detected in different cells. VGLUT1 contact densities on spinal interneurons are highly dependent on dendritic trajectories with respect to VGLUT1 synaptic fields (Siembab et al., 2016). The Foxp2-V1 cell shown in Figure 8F received the most contacts, which were concentrated on a dendritic segment crossing a field with high density of TA afferent synapses. Within single sections we found LG-coupled Foxp2-V1 and non-Foxp2 LVII interneurons receiving TA synapses on dendrites located in areas with high densities of TA/VGLUT1 synapses (Figure 8G and 8H). Non-Foxp2 IaINs could be derived from V2b or even non-Foxp2-V1 cells. It is known that several genetic subclasses of interneurons contribute to the full repertoire of IaINs controlling different leg joints (Zhang et al., 2014). These results provide proof-of-principle that some Foxp2-V1 interneurons are in synaptic circuits capable of exerting reciprocal Ia inhibition between antagonistic muscles. Clearly, a technique with higher yield and that maintains high specificity is necessary. Additionally, analysis of further extensor-flexor pairs in different joints in both directions will need to be performed to reveal a complete picture of IaIN organization.

Other cell types transsynaptically labeled from the LG included V1-Renshaw cells which did not receive any TA synapses since their dendrites are far away from TA projection areas. Medial LV LG-coupled interneurons (possibly Ia/Ib interneurons not derived from either V2b or V1 classes) are embedded within a region of high density of TA/VGLUT1+ synapses. A few reconstructed neurons at this location (n=3) showed they receive the highest densities of TA synapses with more than 50 contacts on relatively smaller dendrites. Some other LG-coupled

interneurons reside in superficial laminae of the dorsal horn (Figure 8G) where they were contacted by CTB-labeled TA afferent synapses and by RV∆G-mCherry-labeled LG afferents. Muscle afferents ending in superficial laminae are likely non-proprioceptive (i.e., Type III(Aδ) and IV(C) afferents (Ling et al., 2003)). It has also been shown that interneurons at this location can be transsynaptically labeled in the anterograde direction from sensory afferents that incorporate AAV1-G and RV∆G-mCherry from the muscle injection (Zampieri et al., 2014). In our experiments few mCherry-labeled primary afferents were located below LIV suggesting that ventral interneurons, including Foxp2-V1 cells, were most likely transsynaptically labeled in the retrograde direction from LG motor pools.

The timing of these tracing experiments prevented analysis of Otp expression in Foxp2-V1 interneurons because of Otp downregulation at these postnatal times. To best target Otp-V1 interneurons we tried a genetic approach by breeding *en1*$^{cre/+}$*::Ai9-R26tdTomato* mice with new *Otp*$^{flpo/+}$ animals crossed to *RCE:dual-eGFP*. The *Otp*$^{flpo/+}$ mice were generated by inserting Flpo-pA into the ATG in exon 1 of the *Otp* gene (Supplemental Figure 8A). After crossing Otp-flpo with RCE.fsf.GFP reporter mice, we show that genetically labeled Otp cells are distributed throughout the ventral horn of P0 mice and that 98.1% ±2.1% (mean±SD; n=3 mice) of Otp+ cells at P0 are genetically labeled (Supplemental Figure 8B). In dual color *en1*$^{cre/+}$*::Ai9-R26tdTomato Otp*$^{flpo/+}$*::RCE:dual-eGFP* mice, we found at P5 a surprisingly large proportion of V1s labeled (n = 2 mice, 6 L4/L5 ventral horns analyzed in each). The sections were also immunostained for ChAT (for best identification of the LMC) and for Otp to reveal expression at P5 (Figure 8J). Of 1,278 V1 interneurons analyzed in both animals we found 60.8% were eGFP (*en1* and *Otp* intersection), 27.8% were “yellow” (*en1* and “weak“ *otp*) and surprisingly only 11.4% were tdT only (*en1* and no *otp*). As expected, P5 Otp expression detected with antibodies was absent in most tdT labeled V1 cells (93.5% of cells) and “yellow” tdT+eGFP V1 cells (85.4%), but also in a significant proportion of eGFP labeled V1 cells (46.7%) (Figure 8K). This suggests that *otp* is expressed by many V1 cells during embryonic development but is downregulated in around half of them by P5. Cells of the Otp-V1 lineage are located at all V1 medio-lateral and dorso-ventral locations of the V1 distribution (Figure 8J)

V1-cells transiently expressing Otp in embryo included cells of other clades. To examine this we generated a *en1*$^{cre/+}$*::otp*$^{flpo/+}$*::RCE:dual-eGFP* mouse in which only cells at the intersection of *en1* and *Otp* express eGFP. L4/L5 sections from this animal were immunolabeled simultaneously for Pou6f2, calbindin and ChAT and is some serial sections we immunolabeled for Otp. We plotted all Otp-V1 cells (n=519) in 6 sections from this animal according to Pou6f2 or

calbindin immunoreactivity (-IR) and then added the locations of Otp-IR cells from the Otp-V1 lineage in 2 further serial sections (Figure 8L). Pou6f2 was detected in 13.1% of Otp-V1 cells at P5. These cells occupy the dorsal band of the V1 cell distribution typical of the Pou6f2 clade. In addition, 14.7% of Otp-V1 cells were calbindin+ and this included many in the Renshaw cell ventral region and few others located more dorsally. One rare dorsal Otp-V1 cell contained both Pou6f2 and calbindin (included in both percentages above). It is well-known that calbindin in V1s becomes restricted to Renshaw cells throughout postnatal development, mostly in the second postnatal week (Siembab et al., 2010; Lane et al., 2021). By limiting the analysis to ventral Otp-V1 interneurons in the Renshaw area we estimated that 8.3% of them are Renshaw cells. Otp-V1 cells retaining Otp expression at P5 distribute to all dorso-ventral locations but are restricted laterally (Figures 8L and 8M). They represent 36.5% of all lineage-labeled Otp-V1 neurons.

In conclusion, the Otp-V1 lineage includes cells from several V1 clades that downregulate Otp expression before birth. In addition, many medial non-V1 Foxp2 cells also express Otp. Therefore, to specifically target the lateral group of proprioceptive Otp-Foxp2-V1 cells tightly associated to the LMC a triple genetic intersection or alternatively postnatally timed *Otp*-dependent recombination is necessary.

DISCUSSION

A comprehensive inventory of spinal premotor interneurons and circuits requires detailed cataloging of its core components, ideally using multimodal information such as genetic subtypes, timing of neurogenesis, settling positions, incorporation within spinal circuits and their electrophysiological properties and functional roles in the circuit during motor behaviors. In the present work we focus on V1 inhibitory interneurons, a major and heterogenous group of inhibitory interneurons with ipsilateral synaptic projections throughout the ventral horn (Alvarez et al., 2005). Previously, the diversity of V1 interneurons in mice was organized into four clades deduced from Bayesian statistical analyses of combinatorial expression of 19 TFs and their cell locations in the spinal cord (Bikoff et al., 2016; Gabitto et al., 2016). Each group divides into further subgroupings organized in a hierarchical fashion. Here we show that the four V1 clades differ in neurogenesis windows and targeting of motoneurons. This suggests unique early developmental pathways and specific placings in spinal circuits validating the significance of V1 clade divisions as unique functional subtypes.

Two V1 clades, Renshaw cells and Pou6f2-V1 cells have narrow windows of neurogenesis with almost all cells generated before E11, while the Foxp2-V1 and Sp8-V1 clades are generated in a wider temporal window with most born after E11. Within the “early group” many Renshaw cells precede Pou6f2-V1 cells and within the “late group” Sp8-V1 cells lag behind Foxp2-V1 cells. These data confirm a previous report on earlier generation of MafB-V1 Renshaw cells compared to Foxp2-V1 IaINs (Benito-Gonzalez and Alvarez, 2012). Sequential generation of these two V1 cell types seem to be an intrinsic property of p1 progenitors and can be replicated *in vitro* using mouse embryonic stem cells (mESC) to derive p1 and V1 cells (Hoang et al., 2018). The present data now extends the view of sequential determination of cell fate by time of neurogenesis to all V1 clades. However, the results also suggest that while some clades with few neurons and limited diversity have narrow windows of neurogenesis, larger V1 clades like Foxp2-V1 interneurons with wider neurogenesis windows include subtypes that differ in location and synaptic inputs.

Overall, our data agree with a previous report that examined spinal cord subtypes based on the intersection of neurogenesis and transcriptomics by analyzing mouse spinal cords from E9.5 to E13.5 (Delile et al., 2019). This study divided V1 interneurons into seven groups. Groups V1.5 and V1.2 had early birthdates. The V1.5 group’s gene expression profile includes *neurod1,2* and *4*, n*eurod1*, p*rox1*, t*cf4*, *lhx1* and *5*, *pou2f2* and *hes1,* and although it does not clearly match the TF repertoire of the Pou6f2-V1 clade, it occupies a similar birthdate window. In contrast, the V1.2 group shares the gene expression profile of Renshaw cells that includes *calb*, *mafB* and *onecut2*. One difference is that we have consistently found Renshaw cells to be the first born V1 cells and this study place them after the V1.5 group. One possible explanation is that some TFs used to define V1.2 cells, like *mafB*, are upregulated sometime after neurogenesis (Benito-Gonzalez and Alvarez, 2012). V1.1, V1.4 and V1.3 groups follow in neurogenesis timing, and all three express *foxp2*; V1.1 and V1.4 have similar birthdates while V1.3 neurogenesis is slightly delayed. We show similar heterogeneity of birthdates in Foxp2-V1 interneurons. Two subgroups expressing the TF Otp at P5 with or without Foxp4 and/or Foxp2 co-expression have intermediate times of neurogenesis and form a lateral group that is closely related to LMC motoneurons. They both receive VGLUT1/proprioceptive inputs and some form reciprocal inhibitory circuits between antagonistic motor pools. Many Foxp2-V1s located dorsomedially are generated earlier, and Foxp2-V1s located ventromedially are generated last. Ventromedial Foxp2-V1s are not targeted by VGLUT1/proprioceptive inputs which indicates different circuit roles. Finally, groups V1.6 and V1.7 are generated at the end of V1 neurogenesis, and it is tempting to speculate they include the Sp8-V1 clade and the late generated ventromedial group of Foxp2-V1 interneurons. However, it is presently difficult to match gene expression profiles of V1.6 and V1.7 (*neurod1*, *2*, and *6*, *Nfia*,

Nfib, *Nfix*, *prox1*, *tcf4*, *hex6*, *cbln2* and *slit2*) to our V1 clades. One problem is that the high throughput sequencing in Delile's paper is based on samples collected at early embryonic times (E9.5 to E13.5), but gene expression in V1 groups changes throughout embryonic and postnatal development. For example, our efforts to generate a genetic model for Otp-V1s show that the postnatal restriction of this TF to the Foxp2-V1 clade does not occur until after embryogenesis. A similar situation occurs with the Sp8-V1 clade that is best defined by V1 interneurons retaining Sp8 expression at P0 (Bikoff et al., 2016), as Sp8 expression is widespread within ventral spinal progenitors during embryogenesis. On the other hand, we obtained preliminary evidence that the medial Foxp2-V1 group upregulates Foxp2 expression in late embryo (after E14) by difference to the lateral group that upregulates Foxp2 expression as they emerge from progenitors (Benito-Gonzalez and Alvarez, 2012). Thus, Foxp2 expression might not be captured in this group of Foxp2-V1 interneurons when examining V1 interneuron gene expression from E9.5 to E13.5. Despite these differences, there is general agreement in that Renshaw cells are early born and that several Foxp2 groups are generated later and can also be separated by differences in neurogenesis.

In addition to differences in neurogenesis, we uncovered differences among V1-clades in motoneuron synaptic targeting which supports the view that they constitute unique functional subsets. Renshaw cells and Foxp2-V1 interneurons are the major sources of V1 synapses on motoneuron cell bodies and proximal dendrites, while the Pou6f2-V1 and Sp8-V1 clades either do not provide much input to the motoneuron or these occur quite distally. We previously reported that the density of Sp8-V1 synapses on motoneuron cell bodies and proximal dendrites is between one and two orders of magnitude less dense compared to Renshaw cells synapses at this location (Bikoff et al., 2016). This analysis included a variety of motoneurons innervating flexor and extensor muscles at various hindlimb joints ruling out the possibility that each interneuron preferentially target specific motor pools. Unpublished data from our group of intracellular fills of dorsal MafB-V1 interneurons belonging to the Pou6f2 also show no axon projections to LIX. Given that V1 axons have limited projections outside the ventral horn, it is fair to conclude that Pou6f2-V1 and Sp8-V1 cells likely modulate activity in ventral premotor spinal networks and perhaps modulate synaptic integration in motoneuron distal dendrites traversing LVII, but they do not establish proximal synapses for efficient modulation of motoneuron firing and excitability. That role seems exclusive to Renshaw cells and Foxp2 interneurons within V1 interneurons, although these cells can also exert modulation of other premotor network elements. Within the Foxp2-V1 clade it is not possible at present to define which subgroup provides proximal synapses to motoneurons. However, given the known placement of IaINs (Jankowska and Lindstrom, 1972)

and the known proximal location of reciprocal inhibitory synaptic inputs (Burke et al., 1971), it is reasonable to expect that many Foxp2-V1 synapses on the cell bodies and proximal dendrites of motoneurons arise from Foxp2-V1 lateral groups (II and III) which occupy spinal cord spaces typical of IaINs and receive VGLUT1/proprioceptive projections.

Intriguingly, hierarchical gene clustering of V1 subgroups shows that Renshaw cells (V1.2) and the three groups of Foxp2 V1s (V1.1, V1.2, V1.4) are closely related but distant from the V1.5, V1.6, V1.7 subgroups (Delile et al., 2019) which may include the Pou6f2-V1 and Sp8-V1 clades. This suggests a closer genetic relationship between Renshaw cells and Foxp2-V1 cells than with other V1 groups. Birthdate order is therefore not predictive of synaptic coupling with motoneurons, but hierarchical closeness according to early genetic programs is. This agrees with the general view that early genetic programs tightly define axonal projections. For example, spinal interneurons of different V-subtypes differ in their axons being mainly ipsilateral, contralateral, bilateral, ascending or descending (Goulding, 2009). Relationships between V1 clades may extend to phylogeny. Most genetic subclasses of spinal interneurons increase in genetic and functional diversity from zebrafish to mice (Wilson and Sweeney, 2023). V1 interneurons are present in all vertebrate species from fishes with swimming locomotion to mammals with limbed terrestrial locomotion, but they show low diversity in zebrafish (Kimura and Higashijima, 2019) and large heterogeneity in mice (Bikoff et al., 2016). Similarly, reciprocal inhibition of ipsilateral muscle flexors and extensors at limb joints first appears in limbed amphibia, a species that lacks recurrent inhibitory circuits (Czeh, 1977). As of today, recurrent inhibition of motoneurons by Renshaw cells has only been detected in mammals and in developing hindlimb motor pools of chick embryos (Wenner and O'Donovan, 1999; Alvarez and Fyffe, 2007). It is thus possible that these V1-clades are phylogenetically more recent and were sequentially added to the V1 interneuron repertoire in parallel with the evolution of limb motor control. Their significance is further highlighted by recent studies in ALS mouse models showing that V1 synapses located specifically on the cell bodies and proximal dendrites of motoneurons, along with Renshaw cells and Foxp2-V1 interneurons, are preferentially affected during early progression of the disease (Wootz et al., 2013; Salamatina et al., 2020; Allodi et al., 2021)

The birthdate distributions of Renshaw cells and Foxp2-V1 cells occupy the whole period of V1 neurogenesis which suggests that birthdate order might not be a predictor of phylogenetic position. In addition, the axial MMC (shared with fish) is also densely innervated by Foxp2-V1s and Renshaw cells, and thoracic axial motoneurons in the cat are modulated by Renshaw recurrent inhibitory axons (Saywell et al., 2013). This suggests that axial motoneurons in limbed

mammals acquire inhibitory controls that are non-existent in aquatic vertebrates. Despite the similarities between Renshaw cells and Foxp2-V1 interneurons, they have very different electrophysiological and firing properties in the mature state. Renshaw cells are burst-tonic firing cells, but Foxp2-V1 interneurons are tonic fast-spiking (Bikoff et al., 2016). This suggests differences in the ion channel ensembles they express at maturity. This divergence develops postnatally. Indeed, the firing properties of Renshaw cells in early embryos (E12) (Boeri et al., 2018; Boeri et al., 2021) greatly differ from those expressed postnatally.

In conclusion, the results suggest that previously defined V1 clades show differences in birthdate, heterogeneity, projections to motoneurons and/or premotor networks, and collectively may represent interneurons that differ in their relationship to the evolution of limb function.

Molecular modulation of V1 neurogenesis.

In a remarkable *in vitro* replication of the V1 neurogenesis sequence using mouse embryonic stem cells (mESC), it was found that 24 hr. treatment with a high concentration of retinoic acid and low smoothened agonist induced mESC-derived p1 progenitors. These progenitors sequentially generated V1 interneurons with genetic profiles of Renshaw cells at day 5 and Foxp2-V1s at day 8 *in vitro* (Hoang et al., 2018). Lengthening the retinoic acid treatment favored differentiation into cells with Renshaw cell characteristics at the expense of Foxp2-V1s. This suggests that Renshaw cells and Foxp2-V1s derive from a similar pool of p1 progenitors and their fates might depend on differences in the morphogenetic signals present at the embryonic times when they are born. For example, in early embryos retinoic acid is highly expressed by the mesoderm adjacent to the developing spinal cord (Novitch et al., 2003) and by motoneurons inside the spinal cord (Sockanathan et al., 2003), while later retinoic acid signals are attenuated by opposing actions from fibroblast growth factor family members (Diez del Corral et al., 2003). Another consideration, however, is that although Hoang et al. also differentiated V1 cells with gene expression profiles typical of Pou6f2 and Sp8 cells, these clades were underrepresented. It is thus possible that p1 progenitors giving rise to Pou6f2 and Sp8 V1 cells have different signal requirements during embryogenesis.

Significance of Foxp2-V1 interneuron diversity

Genetic lineage labeling of Foxp2-V1 interneurons reveals approximately double the number of V1 interneurons than when this clade is defined by postnatal Foxp2 protein expression.

Not surprisingly we uncovered high heterogeneity. We defined four groups according to TF combination/position (Figure 7G), but that also reflect birthdate order. Of special interest were groups II and III which were born at mid neurogenesis times and expressed Otp at P5. These cells were closely related to the limb-controlling LMC. Foxp2-V1 interneurons at this location also receives proprioceptive VGLUT1 inputs and some were in reciprocal inhibitory circuits between the TA and LG. There were also non-Foxp2-V1s cells in LVII close to the LMC with similar connections (input from TA proprioceptive afferents, output to the LG motor pool). This agrees with reciprocal inhibition being mediated by more than one genetically defined group of inhibitory interneurons (Zhang et al., 2014). Proprioceptive TA input densities on different dendrites of single neurons varied. This agrees with the idea that afferent inputs are established on interneuron dendrites according to their location relative to the trajectories of specific primary afferents, a concept established for the major inputs of Renshaw cells (proprioceptive and motor) (Mentis et al., 2006; Benito-Gonzalez and Alvarez, 2012; Siembab et al., 2016). Similarly, motoneurons with genetically altered dendritic arbor structure show altered proprioceptive inputs (Vrieseling and Arber, 2006). In this context, the two groups of Otp-Foxp2-V1 interneurons defined by Foxp4 expression and ventral location (group III) and lack of Foxp4 more dorsal (group II), are likely coupled to primary afferents from different muscle groups.

In conclusion, Foxp2-V1 interneurons are a highly diverse group of interneurons. Types I, II/III, and IV may be broadly related to different motor functions while types II and III may be closely related to proprioceptive pathways and differ in muscle, joint, flexor, and/or extensor specificities.

MATERIALS AND METHODS

All animal procedures were carried out in accordance with NIH guidelines and were approved by the Emory University Institutional Animal Care and Use Committee (Atlanta, GA). Animal experimentation also follow ARRIVE guidelines.

Animal models

To genetically lineage-label all or subclasses of V1-interneurons we used eight transgenic mouse models (Table 1). These mice were crossed for intersectional genetic labeling combining a line in which all V1 interneurons express Cre with lines expressing Flpo dependent on Foxp2 or Otp or in which MafB expressing cells express GFP.

<u>V1 and Foxp2-V1 model</u>: *en1*$^{Cre/+}$ heterozygotes (Sapir et al 2004) were crossed with *Rosa26-Frt-lox-STOP-lox-tdTomato-WPRE-frt* homozygotes (Ai9*R26-tdT*, JAX#007909;

B6;129S6-Gt(ROSA)26Sort $^{M14(CAG\text{-}tdtomato)Hze}$/J) to obtain *en1*$^{cre/+}$*::Rosa26*$^{lsl\text{-}tdT/+}$ and after backcrossing, *en1*$^{cre/+}$*::Rosa26*$^{lsl\text{-}tdT/lsl\text{-}tdT}$ animals (in both animals all V1 cells are lineage-labeled with tdT). Similarly, *foxp2*$^{flpo/+}$ animals (Bikoff et al 2015) were crossed with *RCE:dual-EGFP* homozygotes (RCE:FRT JAX#010675; Gt(ROSA)26Sor$^{tm1(CAG\text{-}EGFP)Fsh}$; initially donated by Dr. Gordon Fishell, Harvard University) to produce *foxp2*$^{flpo/+}$*::Rosa*$^{dualEGFPl/+}$ and *foxp2*$^{flpo/+}$*::Rosa*$^{dualEGFP/dualEGFP}$ mice. Crossing *en1*$^{Cre/+}$*::Rosa26*$^{lsl\text{-}tdT/+}$ or *en1*$^{cre/+}$*::Rosa26*$^{lsl\text{-}tdT/lsl\text{-}tdT}$ mice with *foxp2*$^{flpo/+}$ or *foxp2*$^{flpo/+}$*::Rosa*$^{dualEGFP/+}$ or *foxp2*$^{flpo/+}$*::Rosa*$^{dualEGFP/dualEGFP}$ we obtained mice for experiments with the following genotypes: *en1*$^{cre/+}$*::foxp2*$^{flpo/+}$*::Rosa26*$^{lsl\text{-}tdT/+}$ (V1 red), *en1*$^{cre/+}$*::foxp2*$^{flpo/+}$*::Rosa26*$^{+/dualEGFP}$ (Foxp2-V1 green) or *en1*$^{cre/+}$*::foxp2*$^{flp/+}$*::Rosa26*$^{lsl\text{-}tdT/\ dualEGFP}$ (dual color, Foxp2-V1 green and non-Foxp2-V1 red). In some experiments we substituted the reporter lines Ai9-*lsl-tdT* and RCE:dualEGFP for the RC::FLTG line. This line has in the R26 locus a frt-flanked STOP and loxP-flanked tdTomato::STOP preventing transcription of EGFP (JAX#026932,B6.Cg-Gt(ROSA)26Sor$^{tm1.3(CAG\text{-}tdTomato,\text{-}EGFP)Pjen/}$J). In this line, Flpo recombination in cells with Foxp2 expression induces expression of tdT, while cells with additional Cre recombination (V1 expressing Foxp2 cells) will express EGFP and remove the tdT reporter.

<u>V1-Otp model</u>: Similar breeding schemes and reporter lines were used to combine *en1*$^{cre/+}$ and *otp*$^{flpo/+}$ mice to study Otp-V1 interneurons. These mice were generated as described (Bikoff et al., 2016) and summarized in Supplementary Figure 8. Briefly, Flpo, a codon-optimized version of Flp recombinase was inserted into the ATG in the 1st exon of the Otp genomic locus, generating a null allele. Positive ES cell clones were screened by Southern blot and microinjected into blastocysts, and the resulting chimeric mice were crossed to C57BL/6J females. The neomycin selectable cassette was removed using Protamine::Cre mice (Jax#03328).

<u>V1-MafB model</u>: *en1*$^{cre/+}$*::Rosa26*$^{lsl\text{-}tdT/lsl\text{-}tdT}$ animals were crossed with *mafB*$^{GFP/+}$ knock-in mice (Moriguchi et al 2006) to label cells expressing MafB and assess their overlap with V1 interneurons.

The *en1, foxp2, otp and mafB* genes carry the recombinases or GFP inserted into the genomic loci resulting in null alleles. The animals are maintained and bred in heterozygosis, with homozygotes being knockouts for each of these genes. We generated *foxp2*$^{flpo/flpo}$ and *mafB*$^{gfp/gfp}$ homozygotes to test antibody specificities. *foxp2* knockout mice survive postnatally, but *mafB* knockouts die at birth. Thus, *foxp2* knockout mice were harvested at P5 and *mafB* knockout mice as late embryos. All animals were bred in our colonies at Emory University, and the resulting litters were genotyped using a combination of standard tail PCR or Transnetyx and

fluorescent phenotyping of neonates (each gene combination results in specific patterns of labeling along the body).

Table 1. Mouse models

Mouse	MGI #	RRID #	Brief Description	Donating Laboratory	Reference
En1::cre	MGI:3029756		En1-cre KI/KO	Goulding (Salk Inst.)	(Sapir et al., 2004)
Foxp2::Flpo	MGI:6728072		Foxp2-Flpo KI/KO	Bikoff/Jessell (Columbia U.)	(Bikoff et al., 2016)
Otp::Flpo	MGI:6728073		Otp-Flpo KI/KO	Bikoff/Jessell (Columbia U.)	This manuscript
MafB::GFP	MGI:6145008	IMSR_RBRC02220	MafB-GFP KI/KO	Takahashi (Tsukuba U.)	(Moriguchi et al., 2006)
Ai9-tdT	MGI:3813511	IMSR_JAX:007909	Rosa26-lsl-tdTom	The Jackson Laboratory	(Madisen et al., 2010)
RCE:dual-EGFP	MGI:4420759	MMRRC_032036-JAX	Rosa26-lsl-fsf-EGFP	The Jackson Laboratory	(Sousa et al., 2009)
RC::FLTG	MGI:5617960	IMSR_JAX:026932	Rosa26-FLTG	The Jackson Laboratory	(Plummer et al., 2015)
RCE:FRT	MGI:4420764	MMRRC_032038-JAX	RCE-fsf-GFP	Fishell (Harvard)	(Sousa et al., 2009)

Timed pregnancies

Female mice were caged with males at the beginning of the dark period (7:00 PM), and the next morning (7:00 AM) vaginal plugs were checked. A positive plug was considered E0.5; however, since the exact time of mating is unknown, this procedure has an approximate error of ±12 hr. Moreover, we found embryos within single litters that differ by 6-12 hr. in developmental stage.

Tissue preparation

Mouse pups of different postnatal (P) ages (P0, P5, P15, P30, adult) were anesthetized with an overdose of Euthasol (>100 mg/Kg i.p.) and after transcranial vascular rinsing with vascular rinse and heparin they were perfusion-fixed with 4% paraformaldehyde in 0.1M phosphate buffer (PB). The spinal cords were then dissected and removed, postfixed in 4% paraformaldehyde for either 2 hr, 4 hr, or overnight. Postfixation times depend on antigens targeted in immunocytochemistry experiments. In general, calcium buffering proteins, choline acetyltransferase and synaptic vesicle markers require longer postfixation times and transcription factors reauire shorter postfixation times. After postfixation, the tissues were cryoprotected in 0.1M PB with 30% sucrose and prepared for sectioning. Generally, transverse spinal cord

sections were obtained in a sliding freezing microtome at 50 µm thickness and collected free-floating. Embryos, P0, and sometimes P5 spinal cords were cut in a cryostat at 20 µm thickness from tissue blocks snap-frozen in OCT.

Birthdating experiments

5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) was injected i.p. at a dose of 50 mg/kg weight in timed-pregnant females. The data reported were obtained from 18 pregnant females successfully injected at gestation days E9.5, 10, 10.5, 11, 11.5, or 12 after crossing with appropriate males to generate pups with genetic labels for all V1, Foxp2-V1 or MafB-V1 interneurons. The spinal cords were collected after P5 perfusion-fixation with paraformaldehyde as above. P5 was chosen for analyses to maximize transcription factor antigenicity.

EdU Click-iT reaction. Fifty micrometer thick transverse spinal cord sections were obtained in a freezing, sliding microtome from lower lumbar segments (4 and 5) and processed free floating with Click-iT Alexa Fluor 488 (C10337, Invitrogen) or Click-iT Alexa Fluor 647 Imaging Kits (C10340, Invitrogen) depending on other genetic fluorophores present in the animal. Sections were washed twice (5 min) with 3% bovine serum albumin (BSA, Fisher) in 0.01M phosphate buffered saline (PBS) and then then permeabilized with a solution of 0.5% Triton X-100 (Fisher) in 0.01M PBS at room temperature for 20 min. During this time, the Click-iT reaction cocktail was prepared per manufacturer instructions and applied to the sections for 30 min at room temperature protected from light. Sections were washed with 3% BSA in 0.01M PBS at the conclusion of the incubation. Antibody labeling followed the EdU Click-iT reaction. In one pup EdU labeling did not correspond to the target time point and was discarded (#459.2 in Figure 1D). The littermate (#459.1 in Figure 1D) displayed correct EdU labeling for the injection time.

Immunohistochemistry. The characteristics, RRID numbers, and dilutions of all primary antibodies used are summarized in Table 2. Genetic labels, tdT and EGFP, were always amplified with antibodies to aid in visualization. After blocking the sections with normal donkey serum (1:10 in PBS + 0.3% of Triton-X-100; PBS-TX), we incubated the section in different primary antibody cocktails diluted in PBS-TX. Chicken antibodies were used to detect EGFP, and mouse or rabbit antibodies were used to detect tdT, depending on the hosts of primary antibody combinations. In birthdating experiments we used in serial sections either rabbit anti-MafB (Sigma), goat anti-Foxp2 (Sant Cruz), rabbit anti-Pou6f2 (Sigma), goat anti-Sp8 (Santa Cruz), guinea pig anti-Otp (Jessell lab; Bikoff et al., 2016), rabbit anti-Otp (Jessell lab; Bikoff et al., 2016), rabbit anti-Foxp4 (Jessell lab; Bikoff et al., 2016), or rabbit anti-calbindin (Swant). Depending on color combination for triple or quadruple fluorescent labeling with genetic reporters (EGFP or tdTomato) and the

EdU Click-it reaction (Alexa 488 or Alexa 647), these markers were revealed with either FITC, Cy3 or Cy5 conjugated species-specific donkey-raised secondary antibodies, or with biotinylated secondary antibodies followed by streptavidin Alexa-405 (for all secondary antibodies dilution was from 1:100 to 1:200; all secondary reagents were obtained from Jackson ImmunoResearch). After immunoreactions, the sections were mounted on slides and cover-slipped with Vectashield (Vector Laboratories). Similar immunocytochemical protocols were followed in other experiments.

Analysis. Confocal images (10X and 20X) were obtained with an Olympus FV1000 microscope. Image confocal stacks were fed into Neurolucida (MicroBrightField) for counting and plotting cells. We analyzed 4 ventral horns per animal in lower lumbar segments (L4-L5). Cells were classified according to genetic labeling, TF immunoreactivity, and EdU labeling. EdU labeled cells were classified as strongly labeled (at least two thirds of the nucleus uniformly labeled) or weakly labeled (speckles or partial nuclear labeling). From Neurolucida plots we estimated: (1) the percentage of V1 INs labeled with EdU (strongly or weakly); (2) the percentage of V1 cells labeled with TF antibodies; (3) the percentage of V1 cells genetically labeled with MafB-GFP; (4) the percentage of V1 INs genetically labeled with Foxp2; (5) the percentage of V1 cells with different genetic or immunocytochemical markers and incorporating weak or strong EdU at different time points; (6) the cumulative numbers of EdU weakly or strongly labeled cells for all V1 cells and for each marker across all embryonic times.

Cell location/density analyses: From Neurolucida plots we constructed cell density profiles for each V1 interneuron type and birthdate, assigning cartesian coordinates to the nucleus location with respect to the dorsal edge of the central canal which was defined as position (0,0). Coordinates were exported as .csv files and plotted using custom Matlab scripts to display the position of each individual cell (Bikoff et al., 2016). 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. We plotted density contours containing from 5% (inner contours) to 95% (most outer contour) of the cell population in 10% increments. Density contours were superimposed to hemicord schematic diagrams in which the distance from central canal to lateral, dorsal, or ventral boundaries was adjusted depending on age and segment from direct measurements obtained in Neurolucida.

Table 2. Antibodies.

Antigen	Immunogen	Host/Type	Manufacturer	Catalog #	RRID #	Dilution
OTP	Recombinant protein [DPGGHPGDLAPNSDPVEGATC]	Guinea Pig, polyclonal	Jessell lab/ HHMI CU	CU1497	AB_2665423	1:3000
OTP	Recombinant protein (aa 1 to 130 from human OTP)	Rabbit, polyclonal	ThermoFisher	PA5-89060	AB_2805328	1:2000
Pou6f2	Recombinant protein (Pou6f2)	Rabbit, polyclonal	Sigma	hpa008699	AB_1079664	1:1000
Pou6f2	Synthetic peptide amino acids 35-184 of human Pou6f2	Guinea Pig, polyclonal	Jessell lab/ HHMI CU		AB_2665423	1:500
Pou6f2	Synthetic peptide amino acids 35-184 of human Pou6f2	Rat, monoclonal	Jessell lab/ HHMI CU		AB_2665427	1:1000
Sp8	Synthetic peptide (C-terminus [C-18] from human Sp8)	Goat, polyclonal	Santa Cruz	sc-104661	AB_2194626	1:2000
Foxp2	Synthetic peptide (N-terminus [N-16] from human FOXP2)	Goat, polyclonal	Santa Cruz	sc-21069	AB_2107124	1:2000 ICC & WB
Foxp4	Recombinant protein [DPGGHPGDLAPNSDPVEGATC]	Rabbit, polyclonal	Jessell lab/ HHMI CU	CU1464	AB_2665415	1:25000
MafA	Recombinant protein (aa 300 to 359 [C-terminus] from mouse v-mafA	Rabbit, polyclonal	Novus	NB400-137	AB_10002142	1:500 WB
MafB	Recombinant protein (Transcription factor MafB)	Rabbit, polyclonal	Sigma	hpa005653	AB_1079293	1:1000 ICC & WB
MafB	Recombinant protein (aa 100 to 150 from mouse MafB)	Rabbit, polyclonal	Novus	NB600-266	AB_2137664	1:250 ICC 1:500 WB
c-Maf	Recombinant protein (aa 150 to 200 from mouse c-Maf)	Rabbit, polyclonal	Novus	NB600-267	AB_2137514	1:500 WB
NeuroD2	Synthetic peptide Mouse NeuroD2 aa 1-100 conjugated to KLH.	Rabbit, polyclonal	AbCam	Ab104430	AB_10975628	1:2000
Prox1	Synthetic peptide from the C-terminus of mouse Prox1.	Rabbit, polyclonal	Millipore Sigma	AB5475	AB_177485	1:500
RFP	Recombinant protein (RFP from *Discosoma sp.*)	Mouse, monoclonal	Rockland	200-301-379	AB_2611063	1:1000
dsRed	Recombinant protein (RFP variant from *Discosoma sp.*)	Rabbit, polyclonal	Clontech	632496	AB_10013483	1:1000
GFP	Recombinant protein (GFP from *Aequorea victoria*)	Chicken, polyclonal	Aves	GFP-1020	AB_10000240	1:1000
ChAT	Human placental enzyme	Goat, polyclonal	EMD Millipore	AB144P	AB_2079751	1:100
NeuN	Synthetic peptide of human RBFOX3/NeuN protein (aa 20-100)	Rabbit, polyclonal	Novus	NBP1-77686	AB_11009597	1:1000
NeuN	Isolated brain cell nuclei	Mouse clone A60	Millipore	MAB377	AB_2298772	1:100
Calbindin	Recombinant protein (calbindin D-28k from rat)	Rabbit, polyclonal	Swant	CB-38a	AB_10000340	1:1000
VGLUT1	Synthetic peptide (aa 456 to 560 from rat VGLUT1)	Guinea Pig/ polyclonal	Synaptic Systems	135-304	AB_887878	1:1000
VGAT	Synthetic peptide (N-terminus from rat VGAT)	Mouse/ monoclonal	Synaptic Systems	131-011	AB_887872	1:100
VGAT	Recombinant protein (N-terminus from rat VGAT)	Guinea Pig/ polyclonal	Synaptic Systems	131-004	AB_887873	1:200
CTB	Cholera toxin B subunit	Goat/ polyclonal	List Labs	#703	AB_10013220	1:200
acetyl-Histone H3	Linear peptide from human Histone H3 acetylated at the N-terminus.	Rabbit/ polyclonal	EMD Millipore	06-599	AB_2115283	1:1000

Analyses of lineage-labeled Foxp2-V1, Otp-V1 and MafB(GFP)-V1 cells across ages and markers.

The spinal cords of animals carrying different combinations of genetic labels were prepared as above for amplification with immunolabeling of their fluorescent reporters and

combination with different markers (for the characteristics of the different samples in terms of number of animals and sections analyzed see the text in results).

Analysis of Foxp2-V1 interneurons in different segments of the adult spinal cord. We used *en1*$^{cre/+}$*::foxp2*$^{flpo/+}$*: :Rosa26*$^{+/dualEGFP}$ mice and amplified the EGFP signal as above. The position of motoneurons was revealed using a goat anti-choline acetyltransferase (ChAT, Millipore) antibody laminae cytoarchitectonics with a rabbit recombinant anti-NeuN antibody (Novus) (not shown in figures). ChAT and NeuN immunoreactivities were respectively detected with Cy3 and Cy5 conjugated donkey anti-goat IgG antibodies (Jackson ImmunoResearch). Confocal microscopy images were obtained (as above), plotted in Neurolucida and cell density contours generated. We analyzed the number of Foxp2-V1 cells and cholinergic motoneurons per section and calculated their ratios (a motoneuron was defined as any cholinergic immunoreactive cell in Lamina IX). Density contours were used to qualitatively compare their distributions in different segments.

Analyses of transcription factor expression in Foxp2-V1 interneurons at P5. To examine Otp and Foxp4 immunoreactivity in lineage-labeled Foxp2 vs. non-Foxp2 V1 cells, we used dual color *en1*$^{cre/+}$*::foxp2*$^{flp/+}$*::Rosa26*$^{lsl\text{-}tdT/\ dualEGFP}$ mice. The sections were immunolabeled with guinea pig anti-Otp (Jessell Lab, CU) and rabbit anti-Foxp4 (Jessell Lab, VU). Otp immunoreactive sites were revealed with biotinylated donkey anti-guinea pig followed by streptavidin Alexa-405 and Foxp4 immunoreactivity was detected with donkey anti-rabbit IgG secondary antibody conjugated to Cy5. These antibodies were combined with immunocytochemical amplification of the EGFP signal (chicken anti-EGFP) and tdTomato (mouse anti-RFP) respectively with FITC and Cy3 conjugated species-specific IgG antibodies raised in donkey. To analyze the populations of lineage-labeled Foxp2-V1 cells expressing Otp, Foxp4 and Foxp2 at P5, we used single color *en1*$^{cre/+}$*::foxp2*$^{flp/+}$*::Rosa26*$^{+/\ dualEGFP}$ mice and amplified EGFP fluorescence with antibodies as above, combined with guinea pig anti-Otp (Jessell, CU)/rabbit anti-Foxp4 (Jessell, CU), rabbit anti-Otp (ThemoFisher)/goat anti-Foxp2 (Santa Cruz) and rabbit anti-Foxp4 (Jessell, CU)/goat anti-Foxp2 (Santa Cruz) antibodies. Triple immunostains were revealed with species-specific secondary antibodies as above using the Cy3 and Cy5 channels for transcription factors. A few sections were immunolabelled with rabbit antibodies against NeuroD2 (Abcam) or Prox1 (Millipore/Sigma). In this case immunoreactivities were revealed with Cy3-conjugated anti-rabbit IgG antibodies. All sections were imaged using confocal microscopy and confocal stacks analyzed in Neurolucida as above. All analyses were done in Lumbar 4 and 5 segments. From cellular plots we estimated co-localizations and cells distributions as explained above.

Analyses of V1 clade markers in MafB-V1 and Otp-V1 interneurons at P5. For these analyses we used *en1*$^{cre/+}$*::otp*$^{flpo/+}$*::Rosa26*$^{+/dualEGFP}$ or *en1*$^{cre/+}$*::Rosa26*$^{lsl\text{-}tdT/+}$*::mafb*$^{GFP/+}$ mice. To analyze Otp-V1 interneurons, EGFP fluorescence was amplified with chicken anti-EGFP antibodies as above, and one of the following additional primary antibodies was added in serial sections: rabbit anti-calbindin (Swant), guinea pig anti-Otp (Jessell CU), guinea pig anti-Pou6f2 (Jessell CU), goat anti-Foxp2 (Santa Cruz), or goat anti-Sp8 (Santa Cruz). All markers were detected in the Cy3 channel. When possible, they were combined with goat anti-ChAT antibodies (EMD Millipore) or mouse anti-NeuN antibodies (Millipore) labeled in the Cy5 channel. ChAT and NeuN immunoreactivities are not shown in results but were used to identify laminae and spinal cord segments according to motor column organization and cytoarchitectonics. In MafB-V1 mice we amplified both EGFP and tdT as above, and combined with the following antibodies: rabbit anti-calbindin (Swant), guinea pig anti-Pou6f2 (Jessell CU), goat anti-Foxp2 (Santa Cruz) and goat anti-Sp8 (Santa Cruz). All marker antibodies were revealed using Cy5 conjugated donkey species-specific anti-IgG secondary antibodies as above. Analyses were done as above: confocal images were imported into Neurolucida for cell plotting and the results expressed as number or proportion of neurons and their position analyzed using cell distribution density profiles. All analyses were done in Lumbar 4 and 5 segments.

Analyses of Foxp2-V1 and Foxp2-non-V1 cells at P5. For these analyses we generated two *en1*$^{cre/+}$*::foxp2*$^{flpo/+}$*::Rosa26*$^{+/FLTG}$ mice. EGFP (Foxp2-V1 cells) and tdTomato (Foxp2-non-V1 cells) were amplified with antibodies as above. The sections were counterstained with mouse anti-NeuN antibodies (Millipore) for segment and laminar localization. Sections from Lumbar 4 and 5 segments were imaged with confocal miscopy and analyzed in Neurolucida. Cell distribution plots, cell numbers, and cell density curves were obtained as above.

Analyses of VGLUT1 inputs on Foxp2-V1 interneurons.

Analyses were done at P5 in *en1*$^{cre/+}$*::foxp2*$^{flp/+}$*::Rosa26*$^{dualEGFP/+}$ mice. The P5 age was selected to preserve TF immunoreactivity. Moreover, at this age VGLUT1 synapses in the ventral horn are specifically contributed by proprioceptive afferents, most likely Ia afferents (Alvarez et al., 2004). For these analyses spinal cord sections were obtained in a cryostat (20 µm thickness). EGFP was amplified with chicken anti-GFP (Aves) and combined with goat anti-Foxp2 (Santa Cruz), rabbit anti-Otp (ThermoFisher) and guinea-pig anti-VGLUT1 (Synaptic Systems). EGFP, Foxp2 and Otp immunoreactivities were revealed respectively with FITC, Cy3 and Cy5 conjugated species-specific secondary antibodies. VGLUT1 was revealed with biotinylated anti-guinea-pig IgG antibodies followed by streptavidin-Alexa 405. The sections were imaged at 10X and 60X

with confocal microscopy. VGLUT1 contacts were analyzed only in high magnification images in which we tiled the whole ventral horn to sample every Foxp2-V1 present. Using Neurolucida, we plotted all Foxp2-V1 cells in each section and classified them according to the presence of VGLUT1 inputs and whether they were at high or low density in a qualitative assessment. Later we pooled all data into presence or abscence of VGLUT1 contacts. From these plots we estimated: (1) the percentage of lineage-labeled Foxp2-V1 interneurons with VGLUT1 contacts; (2) the percentage of these cells with Otp, Foxp2 or both TFs co-localized (for sample attributes with respect to number of animals, sections and cells analyzed, see results).

Identification of Foxp2-V1 interneurons interposed in reciprocal connections between the tibialis anterior muscle and the lateral gastrocnemius motor pool.

For these analyses we combined retrograde monosynaptic tracing of RVΔG-mCherry from the LG muscles with anterograde tracing of muscle sensory afferent synapses using cholera toxin subunit b (CTb) from the TA. RVΔG-mCherry and CTb intramuscular injections were done at P15 to avoid critical windows of synaptic reorganization. To facilitate tracing at this age we applied RVΔG-mCherry at high titer (>10^9 TU/ml) and CTb was injected at high concentration (1%).

Production of RVΔG-mCherry. SADB19ΔG-mCherry rabies virus (RVΔG-mCherry) and the BHK-B7GG (B7GG) cells expressing B19 RV glycoprotein (RV-G) and nuclear GFP for virus propagation and amplification were donated by Dr. Edward Callaway (Salk Institute, La Jolla, CA). Sterile cell culture technique without antibiotics was used throughout all procedures. B7GG cells were placed in cell culture dishes containing DMEM in 10% fetal bovine serum (FBS) (culture medium) and incubated in 5%CO_2 in humid air at 37°C for two hours. Once they adhered to the substrate, the medium was removed, the cultures washed with 10 ml warm PBS and fresh medium applied. Four plates were grown to 90% confluency, and then 4 ml of virus stock was added to each culture and incubated at 37°C/5%CO_2 for 4 hours. After washing three times in warm PBS (to remove as much virus as possible) the cultures were incubated in fresh medium at 35°C/3%CO2 and monitored daily for fluorescence. Four plates of fresh B7GG cells were grown to 90% confluency, washed with 10ml PBS and detached with 6 ml of 0.25% trypsin for five minutes at room temperature (RT) with gentle rocking. A warm culture medium with 10% FBS was added to quench trypsin activity and the cells were dissociated by gentle trituration (approximately 12 passes). The cell suspensions were centrifuged at 800G for 3 mins to pellet cells. After removing the trypsin/culture medium they were re-suspended in warm culture medium. Twelve culture dishes containing 18 ml of culture medium were simultaneously inoculated with 2ml each of the B7GG cell suspension and incubated at 37°C/5%CO_2. The culture medium was

changed after 80-90% confluency and 4ml of viral supernatant added to each plate and incubated for 4 hours at 37°C/5%CO_2., then culture medium was removed, the cells washed three times in PBS and 20ml fresh medium added to each plate. The cultures were incubated at 35°C/3%CO_2 and monitored daily for expression of cytoplasmic mCherry and nuclear GFP. The cell culture medium containing RV∆G-mCherry was collected after 4 days, filtered through 0.45 µm membranes and placed on ice. RV∆G-mCherry was concentrated from 180 ml (10 plates) of cell supernatant via ultra-centrifugation, 2 hrs at 20,000G at 4ºC. The supernatant was aspirated and the six viral pellets re-suspended in 200 µL each of cold Hanks Balanced Salt Solution (HBSS). All supernatants were combined and layered over 1.8 ml of 20% sucrose in HBSS in a 3 ml tube. The virus was pelleted through the sucrose cushion by ultra-centrifugation, at 20,000G for 2 hours at 4ºC. The supernatant and sucrose cushion were gently poured off and any remining fluid aspirated. The pellet was re-suspended in 105 µL of HBSS by gentle agitation and 5 µL aliquots frozen at -80°C for later use.

Virus Titer. Serial dilutions of RVmCH (from one 5 uL aliquot of virus) from the preparation were inoculated onto 1 10^5 293T cells in a 48 well plate. As soon as mCherry was detectable (usually 2 days), the positive cells in the well (dilution) containing 10-100 cells were counted and a titer obtained. For these experiments we used virus at a titer of 1.92 E10 transfection units (TU)/ml.

Production of AAV1-G. Adeno-associated viruses were produced by the Emory Virus Core from AAV2 plasmids expressing the B19 RV glycoprotein under a CMV promoter. This plasmid was donated by Dr. Silvia Arber (Biozentrum, Basel)(Stepien et al., 2010). Virus titer was expressed in genomic copies (GC) and was 2.5XE12 vg/ml by qPCR for the lot used here.

Viral and tracer intramuscular injections. AAV1-G was injected at P4 in the LG (1-2 µL, undiluted), RV∆G-mCherry was injected in the same muscle at P15 (2-3 µL, undiluted) and CTB was injected in the TA at the same time (0.5 µL, 1% diluted is sterile saline). The animals were allowed to survive 7 days after the last injection (P22) at which time they were perfusion-fixed, and 50 µm thick sections were prepared as above.

Immunocytochemistry. Sections were incubated overnight in a cocktail of primary antibodies that included chicken anti-GFP (Aves), rabbit anti-DsRed (Clontech), guinea-pig anti VGLUT1 (Synaptic Systems) and goat anti-CTb (List labs). Primary antibodies were detected with species-specific antibodies coupled to FITC (for EGFP), Cy3 (for tdT), Cy5 (for CTb) or biotin (for VGLUT1). Biotinylated antibodies were exposed with streptavidin-Alexa 405.

Analyses. All sections with mCherry labeled cells were imaged at low (10X), medium (20X) and high (60X) magnification using confocal microscopy. Ia inhibitory interneurons were defined as neurons retrogradely labeled from the LG by RVΔG-mCherry and receiving inputs from TA CTb/VGLUT1 labeled boutons. Because this technique was relatively low yield. all analyses were qualitative. The numbers of animals and yields are reported in results.

Antibody specificities.

The most frequently used antibodies in this study were tested in knockout mice: guinea-pig anti-VGLUT1 and rabbit anti-calbindin (Siembab et al., 2010), rabbit anti-Pou6f2, goat anti-Sp8, and guinea-pig anti-Otp (Bikoff et al., 2016), and goat anti-Foxp2 and two rabbit anti-MafB antibodies (this study, supplemental Figures 1 and 3). In addition, Foxp2 and MafB antibodies used here were further characterized using western blots (see below). Alternative antibodies against Pou6f2 or Otp were first confirmed in dual immunolabeling with validated antibodies. NeuroD2 and Prox1 antibodies were tested and used in recent literature (Osseward et al., 2021). Goat anti-ChAT antibodies gave the well-known patterns of cholinergic immunoreactive neurons in the spinal cord and coincide with genetically labeled neurons in ChAT-IRES-Cre-tdTomato mice. GFP, DsRed and RFP antibodies did not result in any immunolabeling in sections not expressing any of these fluorescent proteins. CTb antibodies resulted in no staining in naïve sections. Rabbit and mouse anti-NeuN antibodies gave identical results. The immunostaining of the mouse anti-NeuN monoclonal in the spinal cord has been amply characterized (Alvarez et al., 2004 and 2005).

Western Blots. Foxp2 and MafB antibodies were further characterized in western blots from spinal cord samples collected from wildtypes, heterozygotes (one null allele) and homozygous knockouts (both null alleles) (supplemental Figures 1 and 3). Lumbar spinal cords were dissected in oxygenated artificial cerebrospinal fluid and immediately homogenized using Cytoplasmic Extraction Reagent Kit from the NE-PER™ nuclear extraction kit (ThermoFisher) with a protease inhibitor cocktail added (5mg/ml. Complete Mini, Roche). The manufacturer's instructions were followed to isolate nuclei and yield aliquots of nuclear proteins. A Bio-Rad DC protein assay was used to determine total nuclear protein content. Protein standards (Bio-Rad) were prepared in NER buffer from the NE-PER kit. Sample absorbance was read on a plate reader at 750 nm. Samples were stored at -80°C until use. For immunoblotting, the samples were prepared in standard SDS-PAGE sample buffer (5X) and 30 µL of nuclear protein from each spinal cord was added to each lane of a Bio-Rad precast 10% polyacrylamide gel. Bio-Rad Kaleidoscope molecular weight markers were added to one lane. Electrophoresis was carried out in Tris buffer

(Bio-Rad) at 180V until the dye front reached the bottom of the gel. The proteins were then then transferred overnight onto PVDF membranes (Bio-Rad) using standard SDS-PAGE transfer buffer (Bio-Rad) and a constant 0.15 A current with gentle stirring at 4°C. For MafB antibodies, three immunostaining procedures were carried out successively on the same membrane. The membrane was washed three times with Tris Buffered Saline and Triton-X-100 (TBST) for 15 minutes and blocked with non-fat dry milk (Carnation, Nestle) for 1hr at room temp on a rocker. The first immunostaining employed a primary antibody against MafB (Novus) at a dilution 1:500 and worked best with 2.5% NFDM in TBST and incubated overnight at 4°C. The primary antibody was detected with donkey anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (HRP, GE Health Sciences) and immunostained bands were revealed using enhanced chemiluminescence (ECL). The blot was then stripped for 20 minutes (Reblot Plus), washed 3 times for 15 minutes in TBS, blocked in 5% NFDM for one hour and re-probed with a primary antibody against MafA (Novus) at 1:500 in TBST and the immunoreactive bands detected via ECL as above. Finally, the blot was stripped a final time using ReBlot Plus (Millipore) for 20 minutes, blocked in 5% NFDM and re-probed with an antibody against MafB (Sigma). Secondary antibody and ECL were identical to the previous two primary antibodies. In a different sequence we substituted the MafB (Sigma) antibody for c-maf (Novus). Similar procedures were used to detect Foxp2 immunoreactivity (Santa Cruz) except that in this case the blot was probed only once, and we used anti-goat secondary antibodies coupled to HRP.

Statistical Analyses.

All statistical analyses were performed in Prism (GraphPad ver 9). In all cases the samples passed the normality test. When comparing multiple groups we used one or two-way ANOVAs depending on the sample structure. To include consideration of repetitive measures in single animals (for example different motoneurons from single animals) we used Nested one-way ANOVAs. Post-hoc pair wise comparisons were always done using Bonferroni-corrected t-tests. When comparisons involved only two groups we used standard t-tests. All statistical details are provided in supplementary tables.

Figure composition.

All images for presentation were obtained with an Olympus FV1000 confocal microscope and processed with Image-Pro Plus (Media Cybernetics) for optimization of image brightness and contrast. Frequently we used a high gaussian filter to increase sharpness. Figures were composed using CorelDraw (version X6) and graphs were obtained in Prism (GraphPad, ver 9).

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Author Contributions

AEW: Analyses of Foxp2-V1 interneuron populations and VGLUT1 inputs. Figure composition. Review and editing original draft.
JTA: Birth dating analyses.
ARL: Helped with birth dating and analyzed V1 inputs onto different motor columns.
LGP: Performed the viral tracing analyses.
AAW: Initial analysis of Foxp4 and Otp populations
RWG: Generating high titer rabies virus for transsynaptic tracing.
AFR: Initial generation and characterization of Foxp2-En1 and the MafB-En1 intersectional mice.
JBB: Generation of Foxp2-flpo and Otp-flpo mice. Review and editing the original draft.
FJA: Funding acquisition, conceptualization, resources, supervision, some data analyses, figures composition. Writing – original draft.

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Data availability

The data generated in this study will be made available at Emory Dataverse. Data files are currently being curated and a doi will be generated in the near future upon final publication.

Any data subsets that support the findings of this study can be made immediately available from the corresponding author upon reasonable request.

Conflict of Interests statement:

The authors declare no conflict of interests.

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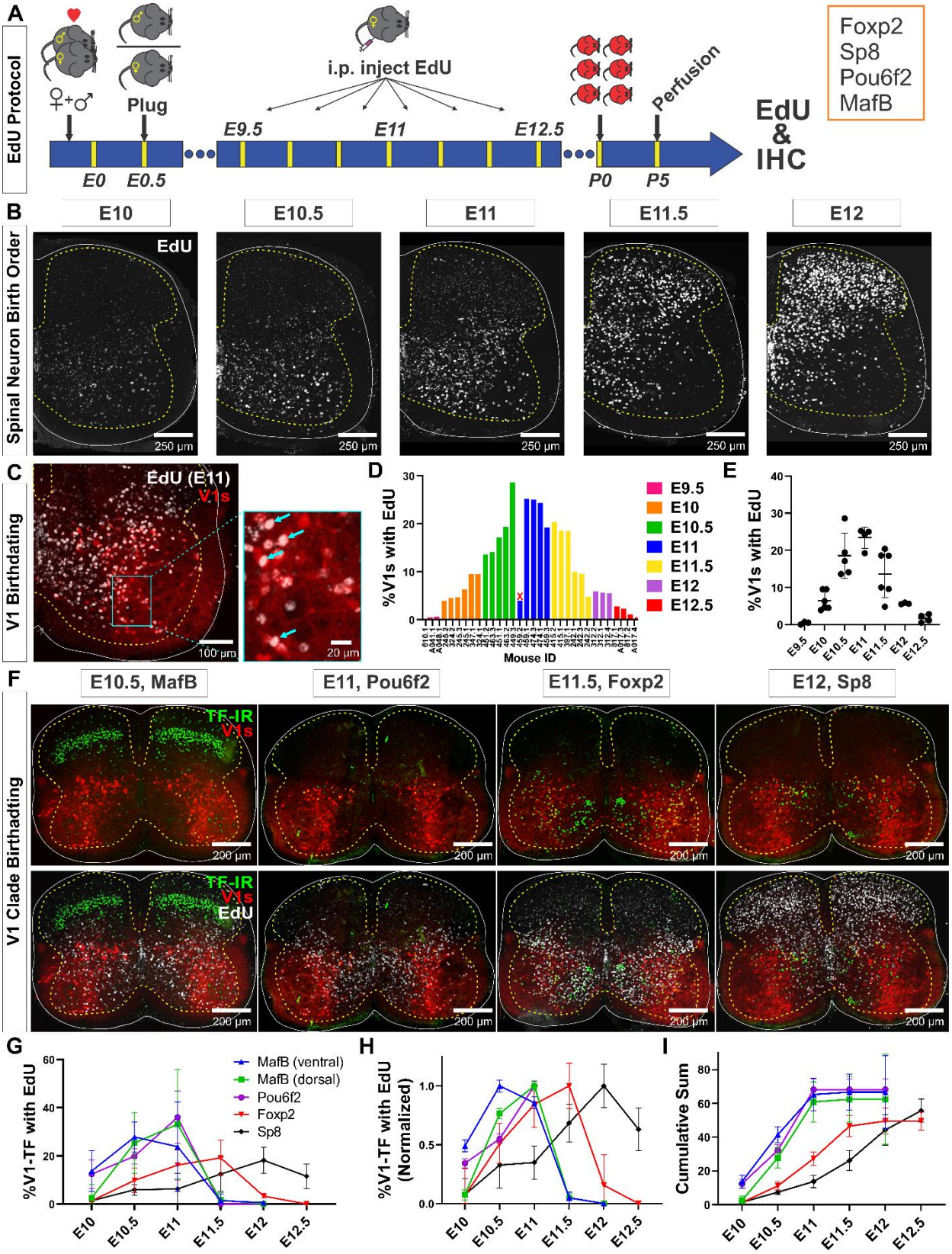
FIGURES


A
EdU Protocol
♀+♂
Plug
i.p. inject EdU
E9.5
E11
E12.5
Perfusion
E0
E0.5
P0
P5
EdU & IHC
Foxp2
Sp8
Pou6f2
MafB
B
E10
E10.5
E11
E11.5
E12
Spinal Neuron Birth Order
EdU
250 µm
C
V1 Birthdating
EdU (E11)
V1s
100 µm
20 µm
D
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Mouse ID
E9.5
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MafB (ventral)
MafB (dorsal)
Pou6f2
Foxp2
Sp8
H
%V1-TF with EdU (Normalized)
I
Cumulative Sum

Figure 1. Neurogenesis order of V1 clades assayed by EdU birthdating. **A,** Experimental design. Timed pregnant En1^{cre}-Ai9:tdT females were EdU injected at one of seven time points between E9.5 and E12.5 and the spinal cords were harvested at P5. Tissue sections were processed for EdU (Click-iT™) and immunostained for transcription factors (TFs) representing major V1 clades. MafB was used to identify the MafA-Renshaw cell clade by location ("ventral MafB-V1 cells"). This is because MafA antibodies in our hands labeled few Renshaw cells. **B,** Patterns of EdU labeling at different embryonic times. Spinal neurons are born in a ventrolateral to dorsomedial sequence. Until E11, mostly ventral neurons are generated. **C,** Example of E11 EdU labeling in an En1-tdT spinal cord. EdU integrated in the DNA at the time of injection is diluted with subsequent cell divisions. To ensure we only counted V1 cells that incorporated EdU during S-phase after their final division, we only counted V1 cells with nuclei largely filled by EdU Click-iT™ reaction (arrows indicate four such strongly labeled V1 EdU+ cells). **D,** Percentages of V1 cells labeled with "strong" EdU in each mouse. The x-axis indicates individual animals ("<litter number>**.**<animal number>"). For the most part there was consistency in the percentage of EdU-labeled V1 interneurons at each time point, although we also noted variability between litters and among animals within a litter. One animal (459.2) showed the wrong EdU pattern for its injection age and was discarded (indicated by an X). **E,** EdU birthdating reveals a peak in V1 neurogenesis around E11 (error bars = SD; each dot represents one animal). At the time points flanking the peak there is a larger amount of variability, suggesting a fast-changing pace of V1 neurogenesis. **F,** Representative images of TF antibody staining combined with EdU labeling to determine birthdates of defined V1 clades. The time points represented were selected according to the maximal or near maximal generation of V1 interneurons in each clade. **G-I,** V1-clade neurogenesis quantification. Graphs represent the average ±SEM calculated from n = 3.9 ±0.3 mice per TF/date (not all TFs were tested in all mice). For each mouse average, we analyzed four ventral horns in Lumbar 4 or 5 segments. ***G,*** Percentage of V1s expressing each clade-specific TF labeled with EdU at each embryonic time point. Ventral MafB-V1s (Renshaw cells) and Pou6f2-V1s are mostly born before E11 (dorsal MafB-V1s are a subgroup of Pou6f2-V1s). Foxp2-V1 and Sp8-V1 interneurons have wider windows of neurogenesis, but most are generated after E11. **H,** Data normalized to the maximum percentage of V1s born in each group to compare peak generation in each clade. Ventral MafB-V1s, Pou6f2-V1s, Foxp2-V1s and Sp8-V1s have progressively later times of peak generation. ***I,*** Cum-sum graphs of V1-clades neurogenesis. Between 50% to 68% of all neurons in each V1 clade are labeled across all ages. By E11 almost all early-clade neurons are generated, while less than half of neurons in late-clades are.

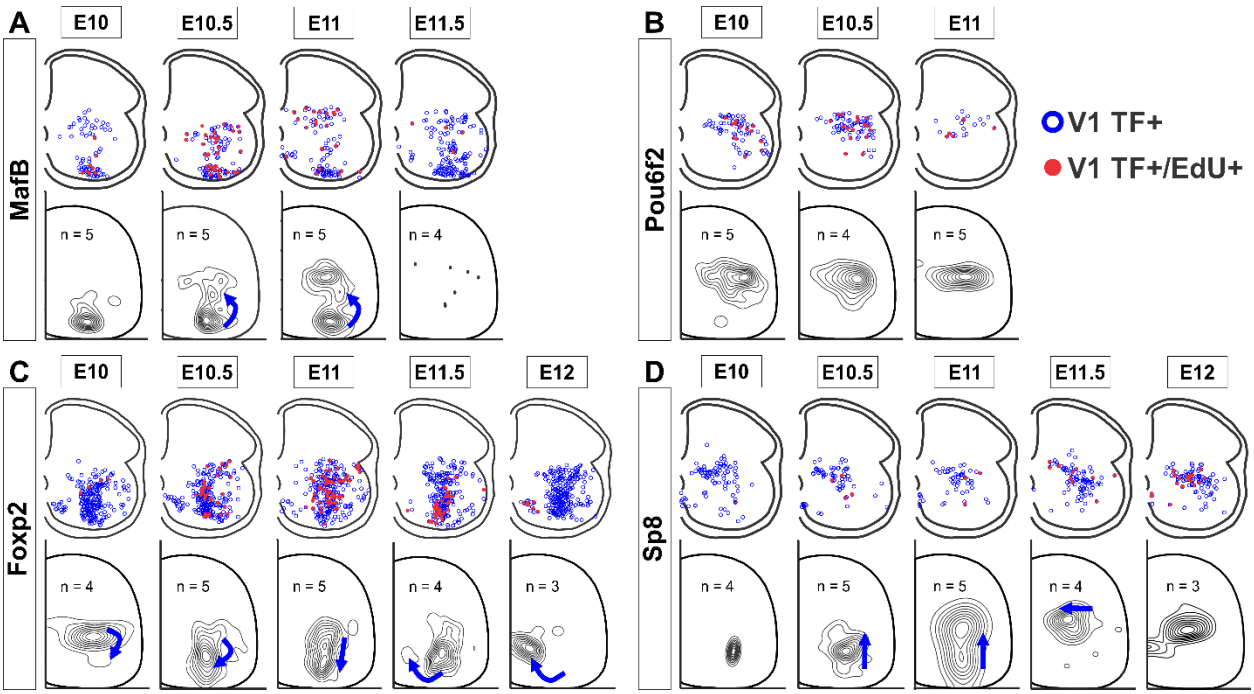

Figure 2. V1 interneurons born at different embryonic times settle in different locations. For each panel in the top row, blue open dots indicate all V1 interneurons positive for each TF at P5, and filled red dots indicate those that also have strong EdU. Each plot is from one representative animal injected at each of the indicated embryonic times (four ventral horns from each animal were analyzed with all cell locations superimposed in the diagram). The bottom rows show cellular density profiles of the V1 interneurons positive for each TF that have strong EdU from all animals EdU-injected at the indicated embryonic times; n indicates the number of animals in each plot. Four ventral horns were analyzed per animal. Contour plots are derived from 2D kernel density estimates of interneuron positions; lines encompass 10% increments. Only timepoints with representative numbers of TF+/EdU+ cells are shown. Blue directional arrows highlight the difference in settling locations for V1s in each clade born at this time point with respect to the previous time point. **A,** MafB-V1s are divided into two groups: ventral MafB-V1s corresponding to Renshaw cells and later-born dorsal MafB-V1s that correspond to a subgroup of Pou6f2-V1s. **B,** Pou6f2-V1s are born between E10 and E11.5 and always settle dorsally. **C,** Foxp2-V1s show large variations in location according to birthdate. Cells of increasingly older birthdates settle dorsally, laterally, ventro-laterally and then ventro-medially. **D,** A few Sp8-V1s are born early and are located ventrally, but the majority of Sp8-V1s are born later and locate dorsally before moving more medially those born the latest. The directional changes in settling positions according to birthdate is different for each V1 clade.

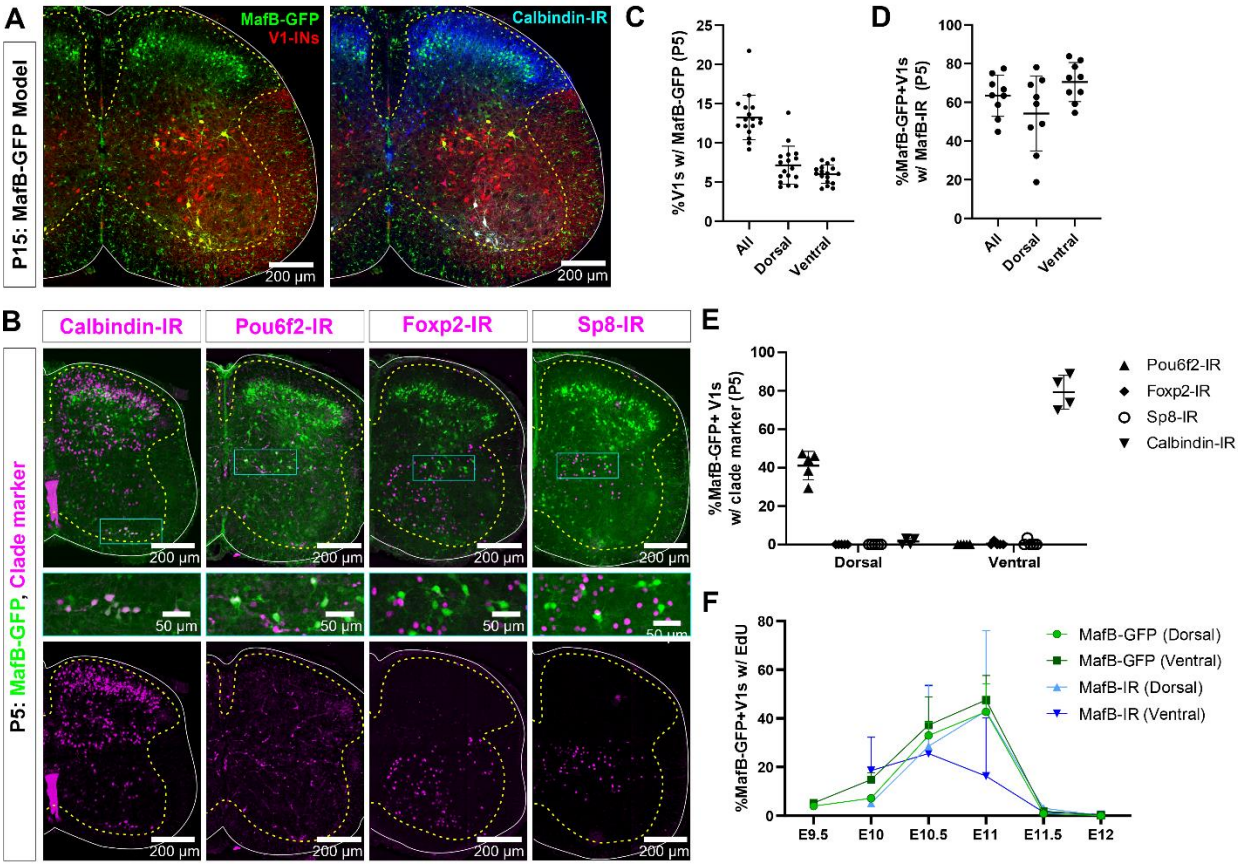

Figure 3. MafB-V1s visualized in a MafB:GFP mouse model. A, In mature (>P15) mice, MafB-GFP+ cells in lamina VII are mostly contained in the V1 lineage (tdTomato) and distribute to the most dorsal and ventral regions of the distribution of V1s. Those that are also calbindin-IR fall in the typical ventral region occupied by Renshaw cells. In addition, there are many dorsal horn non-V1 MafB-GFP+ neurons. The small cells throughout white and gray matter are microglia. **B,** Compared to P15, a few more ventral horn neurons express MafB-GFP at P5, including some motoneurons and microglia whose MafB labeling is weaker. Within MafB-GFP+ V1 neurons the two groups located at the most dorsal and most ventral regions correspond to the V1 neurons that retain MafB-GFP at P15. Like P15, the ventrally located group expresses calbindin. The dorsal group expressed Pou6f2 at this age. Little-to-no Foxp2-IR or Sp8-IR is found in either dorsal or ventral groups of MafB-GFP+ V1s. **C,** Quantitation of MafB-V1 neurons in P5 mice. Around 13% of all V1s express MafB-GFP+, and the percentages located in the Renshaw cell area ("ventral") or dorsal lamina VII ("dorsal") are evenly split (n = 17 mice, 4 ventral horns each. Bars show SD). **D,** At P5, over half of the MafB-GFP+ V1 cells have detectable levels of MafB (Sigma) immunoreactivity in both the ventral and dorsal groups (n = 9 mice, 4 ventral horns each. Bars show SD). **E,** MafB-GFP+ / Pou6f2-IR V1 cells are exclusively in the dorsal MafB-GFP group,

while MafB-GFP+ / calbindin-IR V1 cells are exclusively in the Renshaw cell area, Negligible number of MafB-GFP+ V1 cells express Foxp2 or Sp8. (n=4-5 mice, 4 ventral horns each. Bars show SD). **F,** EdU birthdating reveals similar proportions of EdU+ neurons at P5 for dorsal MafB-GFP vs MafB-IR neurons labeled at each embryonic time (n = 2 mice per timepoint per condition, 4 ventral horns each. Bars show SD). The mismatch in birthdates between MafB genetic and antibody labeling at E11 in the ventral group could arise because some E11-born ventral MafB-GFP V1s quickly downregulate MafB to levels undetectable with antibodies.

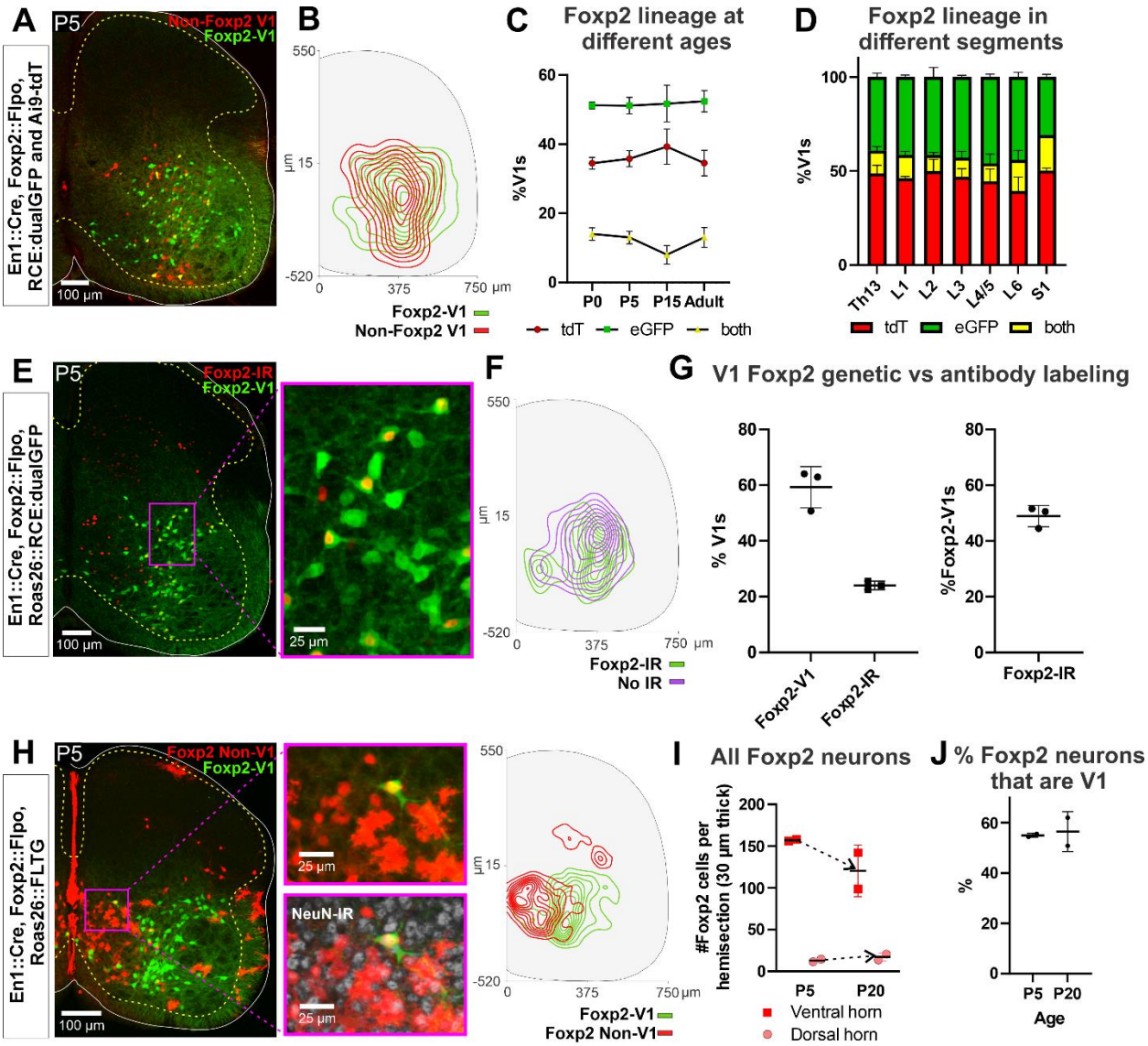

Figure 4. Genetic mouse models used to label the Foxp2-V1 lineage. A, P5 mouse intersection of En1::Cre and Foxp2::Flpo with two reporter alleles (Ai9-tdTomato and RCE:dualEGFP) in the Rosa26 locus. Foxp2-V1s express GFP (green) and non-Foxp2-V1s tdTomato (red). A few "yellow cells" correspond with Foxp2-V1 neurons that failed to remove the tdTomato Ai9 reporter. **B,** Density contours demonstrate high spatial overlap between V1 neurons expressing Foxp2 (EGFP+) or not (tdT+ and EGFP-) (n=6 ventral horns). **C,** Expression of the lineage label is stable throughout postnatal development. This means that there is no additional *foxp2* gene upregulation in new V1s after P0 (n=6 ventral horns per age in one animal, except for P5 in which 6 animals and 36 ventral horns are included; error bars show SEM). **D,** In P15 mice, expression of the lineage labels is also uniform across the lower spinal cord segments from

thoracic 13 to sacral 1 (n = 6 ventral horns in each segment from 2 mice; error bars show SEM). **E,** The En1::Cre, Foxp2::Flpo, RCE:dualEGFP model detects more Foxp2-V1s in the postnatal spinal cord than Foxp2 antibody staining at P5. **F,** Foxp2-V1s with or without postnatal expression of Foxp2 occupy roughly similar areas in the P5 spinal cord (n=3 mice, 3 ventral horns per animal). **G,** Right, Foxp2-V1s make up roughly 60% of all V1s when measured with genetic labeling, but only about 30% of V1s maintain detectable Foxp2 expression at P5. Left, around half of Foxp2-V1s maintain detectable levels of Foxp2 at P5 (n is same as F; error bars show SD). **H,** FLTG reporter mice reveal lineage-labeled non-V1 Foxp2 cells in the spinal cord. eGFP is expressed in Foxp2-V1s and tdTomato in non-V1 Foxp2 cells. The zoomed images of the highlighted region with and without NeuN-IR demonstrate that non-V1 Foxp2 cells include non-neuronal cell types with astrocyte morphologies. Only neurons (NeuN-IR) are included in the contour plots which show non-V1 Foxp2 neurons located in the medial ventral horn and a few in the deep dorsal horn. (n = 2 animals at P5, 6 ventral horns in each). **I,** Most Foxp2-neurons (red, non-V1s or green, V1s) are in the ventral horn and their number in 30 μm thick L4-5 sections decreases with age as the neuropil matures and expands in size. Dorsal horn Foxp2-neurons maintain their numbers despite the growth of the spinal cord. Each point is one animal analyzed through 6 ventral horns (errors bars are SD). **J,** The percentage of Foxp2-lineage-labeled cells that are Foxp2-V1s remains constant throughout postnatal development (n=2 mice, 6 ventral horns each; error bars show SD).

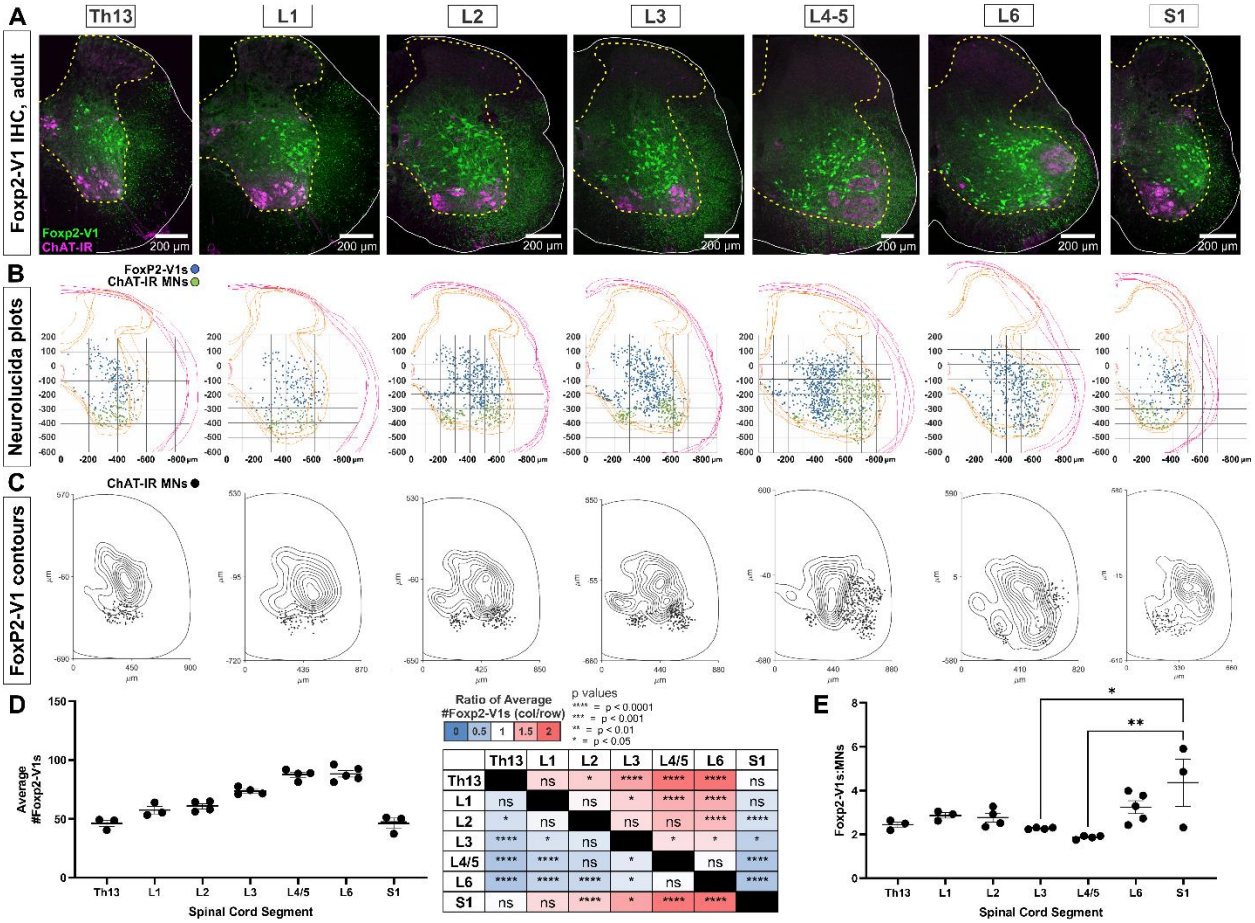

Figure 5. The spatial distribution of Foxp2-V1 interneurons is closely associated with shifting motor columns throughout thoracic, lumbar and sacral levels of the spinal cord. **A,** Foxp2-V1 lineage labeling and ChAT antibody staining for motoneuron identification in adult mouse spinal cords from thoracic to sacral levels. Foxp2-V1 neurons accumulate at the lateral edge of the ventral spinal cord but their locations shift as the LMC expands from L3 to L6. In addition, there is a distinct group of Foxp2-V1 interneurons dispersed at medial locations in all lumbar segments. **B,** Plots of Foxp2-V1 and ChAT-IR motoneuron cell body positions in x,y coordinates with 0,0 at the top of the central canal (n=4 ventral horns, 1 representative animal). **C,** Contour plots of kernel Foxp2-V1 cell density estimations. The highest density of Foxp2-V1 neurons cluster close to LMC motoneurons from L2 to L5 (contours enclose 10% increments, closer lines indicate higher density). Motoneuron numbers progressively increase from Th13 to L5 before dropping in number. **D,** The number of Foxp2-V1s per 50 µm thick section (ventral horn) significantly increases in lower lumbar segments from L3 to L6 compared to S1 (dots represent individual mice; n = 3-5 mice in different segments, each mouse estimate is from 6 ventral horns; bars show SEM; one-way ANOVA, post-hoc Bonferroni corrected t-tests: summary statistical

results are in the right-hand table. See Table S3 for more details). **E,** The ratios of Foxp2-V1 neurons to MNs remain relatively constant at roughly 2.5:1 with no significant changes throughout the lumbar cord. Significance was only found for L3-L5 compared to S1 (*$p<0.05$; **$p<0.01$; post-hoc Bonferroni tests). High variability in S1 is likely due to the sharp rostro-caudal decrease in motoneuron numbers in S1 sections and possible sampling differences among animals.

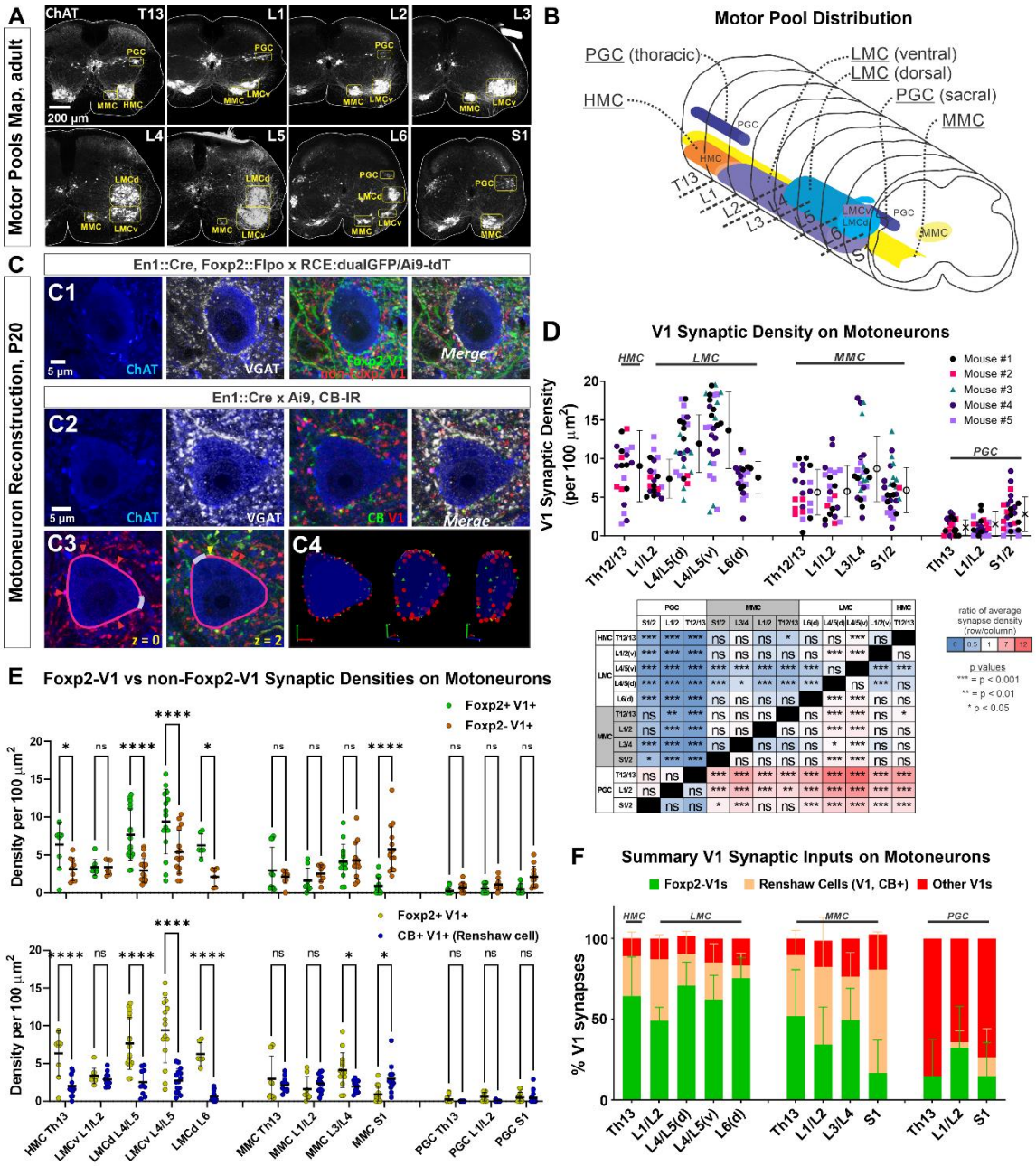

Figure 6. Limb and axial motoneurons are densely innervated by Foxp2-V1s and Renshaw cells. A, Motor column identifications in P20 mice following labeling with ChAT antibodies from lower thoracic to upper sacral spinal cord: PGC = preganglionic cell column; MMC = medial motor column; HMC = hypaxial motor column; LMCd/v = lateral motor column (dorsal/ventral). **B,** Schematic depiction of the rostro-caudal span of each motor column in the spinal cord segments studied. **C,** Synapse quantification. Axons of Foxp2 and non-Foxp2 V1 interneurons were respectively labeled with eGFP and tdT in *en1^{Cre}::foxp2^{flpo}::R26 RCE:dualGFP/Ai9tdT* mice. In *en1^{Cre}::R26 Ai9tdT* mice we identified V1-Renshaw cell axons using calbindin antibodies.

Synaptic locations were labeled with VGAT antibodies and the postsynaptic motoneurons with ChAT antibodies. Synapse densities were analyzed in a ribbon of membrane at mid-cell body level (7 optical planes, 1 µm z-step). **C1,** Single optical plane of a L4/5 LMCv motoneuron surrounded by genetically labeled Foxp2-V1 and non-Foxp2-V1 axons. Inhibitory synapses on ChAT-IR motoneurons are VGAT+. **C2,** Single optical image of a L4/5 LMCv ChAT-IR motoneuron receiving synapses from putative V1 Renshaw cells (genetically labeled V1 axons with calbindin-IR and VGAT). **C3-4,** Method for estimating synapse densities on motoneuron cell bodies using C2 as example. **C3,** V1-VGAT (red arrowheads) and V1-CB-VGAT synapses (yellow arrowhead) are marked (VGAT-IR is not shown for clarity), and the cell body contour annotated with regions corresponding to dendrite exits. This process was repeated in 7 consecutive mid-cell body optical planes (cross-sections with well-defined nucleus and nucleolus). **C4,** A membrane surface slab is reconstructed in 3D (two different rotations shown). The surface area corresponding to dendrite exits is subtracted from the total surface area of the slab to calculate the available surface area on the motoneuron cell body. V1-VGAT synapses (red), V1-CB-VGAT synapses (yellow), and CB-VGAT synapses (green) are marked. A similar process was followed for calculating Foxp2-V1 synapse density in dual labeled animals. **D,** Quantification of total V1-VGAT synapse densities on motoneuron cell bodies in different motor columns (n = 21-30 motoneurons per motor column, taken from 5 animals with 4-9 motoneurons per animal per motor column). Each data point is one motoneuron color coded by mouse origin. Average synaptic densities ±SD indicated to the right of scatter plots. A nested ANOVA indicated significant differences among motor column/segments ($p < 0.0001$) with no inter-animal variability ($p = 0.4768$). The table summarizes all post-hoc pairwise comparisons for average V1 synaptic densities of each motor column and segment (Bonferroni corrected t-tests) (Statistical details are in Supplementary Table S4). Colors indicate increased (>1, red) or decreased ratios (<1, blue) of column motoneurons vs row motoneurons. PGC neurons receive significantly fewer V1 synapses than MMC or LMC motoneurons. The LMC (ventral and dorsal) in lower lumbar (L4/L5) had significantly more V1 contacts than MMC motoneurons or L6 dorsal LMC. **E,** Comparison of synaptic densities from Foxp2-V1 and non-Foxp2-V1 neurons (top) or Renshaw cells (bottom). All motoneurons sampled in 2 to 3 animals for each comparison were pooled together. Densities of V1-VGAT synapses from Foxp2-V1s, non-Foxp2 V1s, or calbindin (CB)+ V1s (Renshaw cells) (n = 6-17 motoneurons sampled per motor column/segments, average = 12.1 ±2.9 SD) were compared using a two-way ANOVA for axon type vs motor column and segment. <u>Foxp2-V1 vs non-Foxp2-V1 synapses</u>: significant differences in density were found for type of synapse ($p=0.001$), motor column location ($p<0.0001$), and their interaction ($p<0.0001$). Significant

differences after post-hoc Bonferroni tests are indicated (*p<0.05; ****p<0.0001). In general, synapses from Foxp2-V1 axons have higher density than non-Foxp2-V1 axons on HMC and LMC columns at all spinal segments except for L1/L2 LMC. MMC motoneurons receive similar synaptic densities from both types of V1 axons, except at the sacral level in which non-Foxp2 V1 synapses predominate. PGC neurons receive very low densities of V1 axons and there are no significant differences between either type in any region. Foxp2-V1 vs CB+V1 synapses: significant density differences were found for type of synapse (p<0.0001), motor column location (p<0.0001), and their interaction (p<0.0001). Significant differences between Foxp2-V1 and CB+V1 synapses after post-hoc Bonferroni tests are indicated (*p<0.05; ****p<0.0001). Synapses from Foxp2-V1 axons have higher density than CB+V1 axons in HMC and LMC columns at all spinal segments except for L1/L2 LMC. MMC motoneurons receive similar synaptic densities from both types of V1 axons in upper lumbar regions, but Foxp2-V1 synapse predominate in lower lumbar. In S1 the density of CB+/V1 synapses is significantly higher. The low synaptic densities estimated in PGC neurons for Foxp2-V1s and CB+ V1s are not significantly different. Details of all statistical comparisons are Supplementary tables S5 and S6. **F,** Comparing the numbers of Foxp2 and CB+ (Renshaw) V1 synapses to the total number of V1 synapses, we estimated their respective percentages. From these estimates we calculated that the remainder belongs to non-Foxp2 and non-CB+ Renshaw cells. The large majority of V1 synapses on the cell bodies of LMC, HMC and MMC motoneurons are either from Renshaw cells or Foxp2-V1s. Asterisks denote significant differences as found in E.

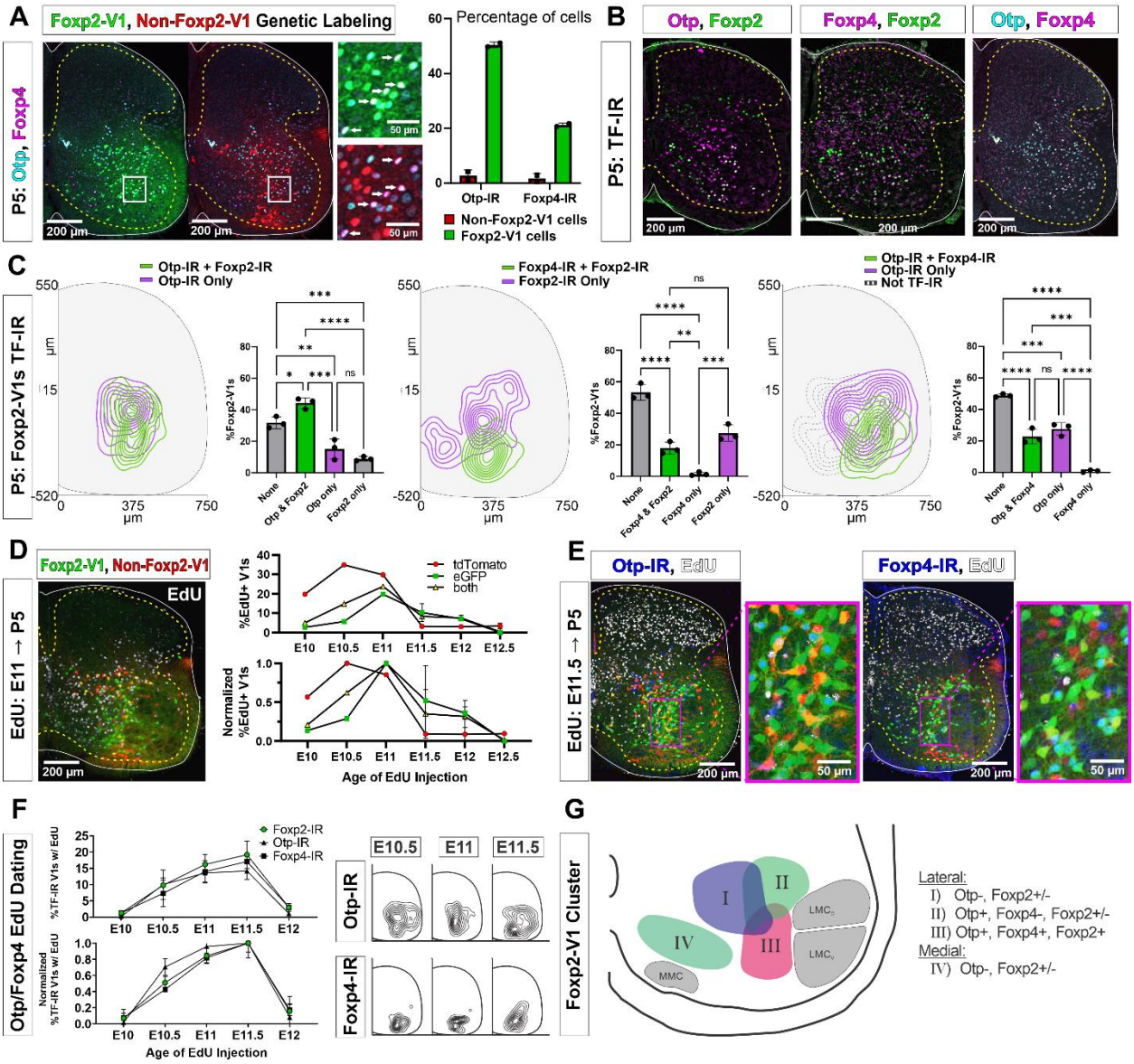

Figure 7. Subgroups of Foxp2-V1 interneurons defined by transcription factor expression at P5 and birthdate. A, Otp (blue) and Foxp4 (white) expression in lineage-labeled Foxp2-V1s (eGFP, green) and non-Foxp2-V1s (tdT, red). The boxed area is shown at higher magnification with different color combinations for clarity. It shows that Foxp4-IR cells in the Foxp2-V1 population always also expressed Otp (arrows). Quantification: 49.8-51.2% of Foxp2-V1s express Otp and 20.7-21.7% Foxp4 (n=2 mice each examined in 3 ventral horns in L4/5). Very few non-Foxp2-V1 cells express either TF (Otp: 1.3-4.2% and Foxp2: 0.5-2.9%). **B,** Images of P5 spinal cords containing Foxp2-V1 lineage labeling (eGFP, omitted for clarity) and double immunolabeled for Otp/Foxp2, Foxp4/Foxp2 and Otp/Foxp4. **C,** Quantification of Foxp2-V1 interneurons with different combinations of TF expression at P5. For each combination, the left panels show cell

distributions and the right graphs show the percentage of Foxp2-V1s with each combination (n = 3 mice each analyzed in 6 ventral horns). To discern groups with significantly different numbers of cells, the data was analyzed with one-way ANOVAS followed by Bonferroni corrected pair-wise comparisons (Statistical details in Supplemental Tables S7, S8 and S9. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05). There are five main groups defined by TF expression patterns: Otp-Foxp2 (44%), Otp-Foxp2-Foxp4 (23%), Otp only (15%), Foxp2 only (9%) and no TF labeling (32% by subtracting all other groups). Some groups associate with specific locations: Otp-Foxp2 cells and the smaller proportion of Otp only cells are located laterally; Otp-Foxp4-Foxp2 cells are located latero-ventrally; medial cells either contain only Foxp2 or nothing; some dorsal cells are either Foxp2 only or do not express any of these TFs. **D,** Example image of EdU birthdating in *en1^{Cre}::foxp2^{flpo}::R26 RCE:dualGFP/Ai9tdT* dual-color mice pulse labeled at E11. Foxp2-V1s (green and yellow cells) are born between E10 and E12, with peak birthdate around E11 and after non-Foxp2 V1s (tdTomato only). The lower graph's data is normalized to highlight the time of peak neurogenesis for each population (n=15.0 ± 4.1 ventral horns from one mouse per time point from E10 to E11 and 2 mice per time from E11.5 to E12.5; total 9 mice, bars show SEM). There is no difference between green (eGFP only) and the smaller population of yellow cells (eGFP and tdTomato). **E,** EdU labeling in the Foxp2-V1 dual-color genetic model combined with Otp and Foxp4 antibody staining. **F,** V1s expressing Foxp2, Otp, and Foxp4 at P5 are mostly born between E10.5 and E11.5 with neurogenesis time courses largely overlapping. The normalized plot indicates that peak neurogenesis for all three populations occurs at E11.5, although a marginally higher number of Otp V1 cells are born earlier (n=4 ventral horns from 3.27 ±1.34 mice per timepoint; error bars show SEM). Contour plots to the right show settling locations of Otp and Foxp4-IR populations born at each time point. **G,** Schematic of the L4-5 ventral horn summarizing Foxp2-V1 subgroups according to location and combinatorial expression of Otp, Foxp4, and Foxp2 at P5. LMC_D: dorsal lateral motor column; LMC_V: ventral lateral motor column; MMC: medial motor column.

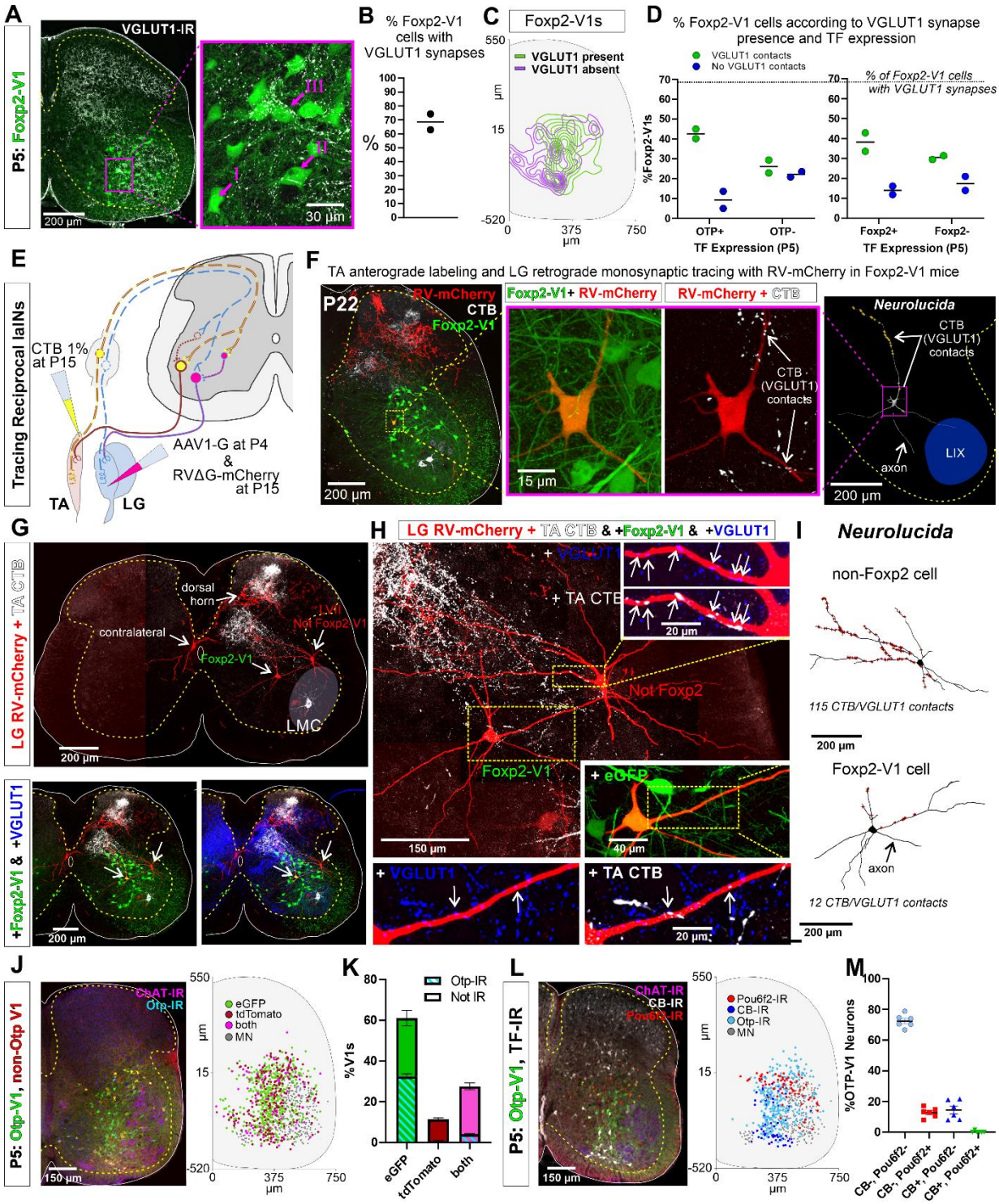

Figure 8. Many Foxp2-V1 interneurons receive proprioceptive inputs, and some are reciprocal Ia inhibitory interneurons (IaINs). A, Left, low magnification confocal image of Foxp2-V1 lineage-labeling (eGFP) combined with VGLUT1 antibody staining to identify primary proprioceptive afferent synapses. The boxed area is shown at high magnification (right) to

demonstrate variability of VGLUT1 synapse density on Foxp2-V1 interneurons (I = absent, II = medium or low, III = high). For simplicity and rigor, we classified Foxp2-V1 interneurons as receiving or not receiving VGLUT1 synapses. **B,** Percentage of Foxp2-V1 interneurons with VGLUT1 synapses (both proximal and distal). Each dot is one animal estimate from 6 or 7 ventral horns with respectively 591 and 525 Foxp2-V1 interneurons sampled. Line represents the average of both animals, 69.6%. **C,** Distribution of Foxp2-V1 interneurons with and without VGLUT1 synapses (green and blue, respectively). The positioning of Foxp2-V1 interneurons receiving VGLUT1 synapses is biased laterally. **D,** Foxp2-V1 interneurons with (green) and without (blue) VGLUT1 synapses according to expression of Otp (left graph) or Foxp2 (right graph). Each dot represents one mouse, and the lines represent averages. The numbers of sections and genetically labeled Foxp2-V1 interneurons sampled in each mouse are as in **B**. In each mouse, this includes 237 and 236 Otp-IR cells and 256 and 225 Foxp2-IR cells. **E,** Experimental design to label spinal neurons that receive inputs from tibialis anterior (TA) muscle primary afferents and also connect to lateral gastrocnemius (LG) motoneurons, forming Ia reciprocal inhibitory connections from TA to LG. TA sensory afferents are labeled anterogradely with CTB followed by antibody detection of CTB and the presynaptic marker VGLUT1. Interneurons premotor to LG motoneurons are labeled by monosynaptic retrograde labeling with RV∆G-mCherry. **F,** Foxp2-V1 IaIN with the most TA/VGLUT1 contacts (31) in our sample (n = 5). Left, low magnification image of Foxp2-V1 interneurons (eGFP, green), RV-mCherry labeling (red) of LG muscle afferents in the dorsal horn and interneurons presynaptic to the LG and of TA afferents anterogradely labeled with CTB (white). The Foxp2-V1 interneuron contains mCherry (yellow cell, inside box). This cell is magnified in two panels to the right, one showing Foxp2-V1 and RV-mCherry and the other RV-mCherry and CTB labeling. Arrows in the zoomed image show examples of CTB synapses (confirmed with VGLUT1) on the dendrites of this neuron. Far right image is the 3D reconstructed cell (Neurolucida) with CTB/VGLUT1 synapses indicated on its dendrites by yellow stars. The axon initial trajectory is indicated (the axon is lost at the section cut surface). The blue area highlights lamina IX. **G,** Low magnification showing TA-CTB afferents (white) and LG-RV-mCherry labeled interneurons (red). Transsynaptically labeled interneurons are categorized according to position and Foxp2-V1 lineage labeling: images below show superimposed Foxp2-V1 eGFP (green) and additional VGLUT1 immunolabeling (blue). The location of the LMC is indicated. Contralateral interneurons were found in LX (as the one in this section) and in LVIII (the other two in this animal, not shown). **H,** High magnification images of two LVII LG-coupled interneurons (RV-mCherry, red) receiving synapses from TA afferents (dendrites in boxed regions are shown at high magnification demonstrating CTB-TA labeling and

VGLUT1 content). The most medial interneuron belongs to the Foxp2-V1 lineage (see inset with +eGFP). **I,** Neurolucida neuronal reconstructions showed that the Foxp2-V1 interneuron contained a medium number of TA/VGLUT1 synapses (13 contacts) in our sample of putative IaINs derived from the Foxp2-V1 lineage (n=5), while the non-Foxp2-V1 interneuron contained the largest (115) of any LVII reconstructed interneuron with mCherry, including many proximal synapses. However, one reason for this difference is that the dendrites projecting toward regions with high densities of TA CTB/VGLUT1 synapses were out of this tissue section. **J,** Intersection of *otp* and *en1* using the dual color strategy with simultaneous expression of the Ai9 tdT and RCE-DC eGFP reporters. V1 cells that expressed *otp* are labeled with eGFP (green). In these cells the Ai9-tdT reporter is effectively deleted by flpo recombination dependent on *otp* expression level: strong (eGFP only) and weak (eGFP and tdT). V1 cells that express tdT only (red) never express *otp*. In addition, the sections were labeled with antibodies for Otp (light blue) to reveal cells that retained expression of Otp at P5 and ChAT (deep blue) to localize the motor pools. Most V1s express eGFP, with tdT-only cells being a minority. Right, cell plot positions of some of the cell types identified in these sections (one mouse 6 ventral horns in L4/L5). Most cells are Otp-V1 and include green (eGFP only) and pink cells (eGFP and tdT). **K,** Quantification of cells with eGFP only (green), tdT only (red), or both (pink) (n=12 ventral horns from 2 mice, error bars show SD). 88.6% of V1s expressed eGFP, most without tdT (61.1% of V1s) and of these around half (53.3%) retained Otp expression by P5. No Otp expression (antibody staining) was detected in tdT cells, and very few cells co-expressing tdT and eGFP ("weak" Otp expression) were Otp-IR. ***L,*** Detection of Pou6f2, calbindin and Otp in genetically labeled Otp-V1 cells. Left confocal image and right cell plot (n=6 ventral horns from 1 mouse). Pou6f2-Otp-V1 cells concentrate in a dorsal band within the ventral horn. Calbindin-IR Otp1-V1 cells concentrate in the Renshaw region (ventral most region) and others are in the dorsal region of the ventral horn. Otp-V1 cells retaining Otp expression at P5 occupy all dorsoventral positions in the lateral spinal cord. **M,** Quantification of 6 ventral horns at L4/L5 from one animal; 397 Otp-V1 cells sampled. Most (72.5%) did not express Pou6f2 or calbindin, 12.8% expressed Pou6f2 and 14.4% calbindin. Only one Otp-V1 neuron in the whole sample co-expressed Pou6f2 and calbindin (0.25%).