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Turnee N. Malik April 14, 2014

Characterization of Foxp2 Expressing Spinal V1 Interneurons

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An abstract of
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Abstract

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The large diversity of spinal interneurons contributes to the wide range of complex motor behaviors exhibited by mammals. These interneurons are critical in modulating motor activity, but little is known about the processes by which these cells differentiate. Of particular interest in this study is the population of V1-derived interneurons (V1-INs), which gives rise to various subtypes of inhibitory interneurons modulating motor output. Although each group of V1-INs has unique cellular characteristics and participates in functionally distinct circuits, they have in common their derivation from the same progenitor pool and expression of the transcription factor engrailed-1. The largest subset of V1-INs also express the transcription factor Foxp2, and this allowed development of a dual conditional transgenic model to selectively label Foxp2(+) and Foxp2(-) V1 lineages using combinations of tdtomato and EGFP. The results indicate that the pattern of reporter protein expression in our model is stable from birth to adulthood. Furthermore, we conclude that the adult pattern of EGFP and tdtomato expression is established between embryonic day (E) 11.5 and E15 and appears to be dependent on the dynamics of Foxp2 expression.

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Introduction

Movement requires the successful integration of signals from the periphery and several central structures including the brainstem, cerebellum, motor cortices, basal ganglia, and spinal cord. The necessity for bidirectional communication between these systems highlights the importance of the spinal cord as a bridge between the brain and the body. The spinal cord relays sensory information to higher brain centers and works together with these supraspinal regions to execute a variety of motor behaviors. Ultimately, however, it is the activity of local spinal circuits that produces coordinated muscle contractions.

The function of the spinal cord is well-reflected in its anatomical organization. Spinal cord gray matter can be divided into functionally specialized dorsal and ventral portions. The dorsal horn contains groups of neurons that process sensory information from afferent nerve fibers which enter the spinal cord through a number of roots whose cell bodies reside in the dorsal root ganglia. In contrast, the ventral horn houses neuronal populations that are responsible for executing movement. These cells perform this task by modulating and selecting the temporal firing sequences of different motor neurons, and in this manner, coordinate muscle contractions during movement. Motor output is shaped by the activity of local synaptic circuits established by the interneurons of the spinal cord.

The immense diversity of spinal interneurons (INs) contributes to the wide range of complex motor behaviors exhibited by mammals. Many different types of spinal INs regulate the output of motor neurons, thereby endowing motor circuits with a large degree of flexibility and sophistication. Developmental studies conducted within the last

15 years have shown that the multitude of adult spinal INs arises from only a few progenitor classes, each generating one of just 10 classes of embryonic spinal interneurons (dorsal dI1–6 and ventral V0–3 INs: Ericson et al., 1997; Goulding et al., 2002). The origin of these progenitor domains stems from the dorsoventral concentration gradient established by two morphogens—dorsal bone morphogenetic protein and ventral sonic hedgehog (Jessell, 2000). Though position along the dorsoventral axis confers neuronal identity, the question of how the diversity of adult spinal interneurons is generated from just a few progenitors and embryonic classes remains unresolved. Recent work has outlined some of the basic properties of these canonical subtypes of embryonic spinal INs (Goulding, 2009; Grillner and Jessell, 2009). Importantly, the different populations of INs are defined by the transcription factors they express during postmitotic differentiation and by unique placements in the spinal cord ventral horn. For example, Renshaw cells (RCs) and Ia inhibitory neurons (IaINs), two distinct subclasses of V1 inhibitory interneurons (V1-INs), both express the transcription factor engrailed-1 (En1). While characteristics such as expression of En1 and ipsilateral axon projection remain common to all V1-derived cells, RCs and IaINs occupy very different positions in the ventral spinal cord which determine the preferential inputs they receive (Benito-Gonzalez and Alvarez, 2012). For example, RCs are located close to the ventral root and receive mainly motor axon inputs while IaINs are located medially to the motor pools and in the trajectory of sensory afferent axons from muscle proprioceptors, which are their main input. In addition, many other functional and synaptic properties of V1-INs differ according to their distinct roles in motor circuits. Although the exact mechanism by which INs differentiate remains unknown, the work of the Alvarez lab has suggested that

V1-IN differentiation is reflective of the embryonic birthdate and the expression of specific transcription factors (Benito-Gonzalez and Alvarez, 2012). The so-called "early" group of V1-INs, which includes RCs, are born during embryonic days (E) 9.5-10.5 and express the transcription factor MafB. Other "late" groups, including IaINs, are generated from E11-E12.5 and express Foxp2.

Foxp2 is part of the Forkhead Box (Fox) family of transcription factors that is expressed during neural development in parts of the striatum, cortex, thalamus, and spinal cord (Lai et al., 2003). Specifically, Foxp2 is first expressed in the mouse embryonic spinal cord at E11. The number of INs that express Foxp2 increases until E13.5 and then begins to decrease during late embryonic and postnatal development (Morikawa et al. 2009). Foxp2 in the developing nervous system has been implicated in regulating neurite outgrowth (Vernes et al., 2011). Other studies of foxp2 orthologs in different vertebrate species have suggested its role in speech and language development (Zhang et al 2002). Despite the importance of this transcription factor, there has been very limited characterization of Foxp2-expressing spinal interneurons.

The largest subgroup of embryonic V1-INs expresses Foxp2 (Morikawa et al. 2009) and in adult many become IaINs (Benito-Gonzalez and Alvarez, 2012). To gain a better understanding of this subgroup, we used an intersectional genetic approach to indelibly label Foxp2-positive and Foxp2-negative V1-INs in the postnatal spinal cord. To label the whole En1-expressing V1 lineage we crossed animals carrying one En1-Cre allele (Sapir et al., 2004) with the Ai9 reporter mouse model; these reporter mice have a flox-STOP-flox-tdtomato expression cassette inserted in the R26 locus (Madisen et al., 2010). In animals resulting from this crossing Cre-lox recombination occurs specifically

in V1 En1 neurons inducing permanent expression of tdtomato in postnatal INs derived from V1 cells. Labeling the subpopulation of V1-INs that express Foxp2 was achieved with a "dual conditional" (DC) reporter animal in which EGFP expression is dependent upon the action of both Cre and Flp recombinases (French et al., 2007). To obtain these animals we performed cross breeding to introduce the En1-Cre and Foxp2-Flp alleles into animals that carry in the R26 locus one copy of the flox-stop-tdtomato reporter and one copy of the DC-EGFP reporter. In the resulting animals we expected all V1-INs that co-express En1 and Foxp2 during development to express EGFP, while all V1-INs should express tdtomato. However, preliminary results in postnatal mice revealed three populations of neurons: 1) cells that only express tdtomato; 2) cells that colocalize tdtomato and EGFP; and 3) cells that only express EGFP. These populations might be distinct groups of V1-INs with possibly different functional roles. However, we also needed to find an explanation for this labeling pattern.

Upon closer inspection of the Ai9 reporter construct, we noted that the whole tdtomato reporter cassette is flanked by FRT sites, and therefore it should be removed by Flp recombination in Foxp2 expressing cells and this could explain V1-Foxp2 INs with only EGFP and not tdtomato. However, a proportion of cells expressed both reporters. This observation led to two possible explanations for the labeling pattern. Given that tdTomato has a very long life in mammalian cells, one possibility is that the presence of tdtomato in EGFP cells is transient and dependent upon the time course of its degradation over postnatal time after removal of the tdtomato expression cassette in Foxp2 V1-INs . Under this hypothesis, the number of EGFP and tdtomato-positive cells is expected to decrease with postnatal age. Alternatively, it is possible that the different Foxp2-V1 cell

types result from differences in the timing of expression of Flp and Cre recombinases, such that the removal or not of the tdtomato gene is more or less efficient in different types of cells depending upon the relative timing of expression of cre and flp and/or the duration of flp expression. If this is the case, Foxp2 expression should have different characteristics in V1-INs that express EGFP only, when compared to V1-INs expressing both tdtomato and EGFP.

To test these hypotheses and further characterize the location, percentages and phenotypes of these three different types of V1-INs we used these transgenic animals to label EGFP and tdtomato V1 cell lineages in conjunction with immunohistochemistry for the transcription factor Foxp2 to monitor its expression in different V1-INs from embryo to adult (embryonic days 12 and 15, and postnatal days 0, 5, and 15, and 3-month adult). The resulting fluorescent preparations were imaged with confocal microscopy and the images analyzed in Neurolucida for cell plotting and quantification.

Methods

Animal Models

Several different transgenic animal models were used in this study (Figure 1). $En1^{cre/+}$and $Foxp2^{Flp/+}$ animals were provided by Martyn Goulding and Thomas Jessell, respectively. $R26^{td+/td+}$ and $R26^{tdTom/DC}$ animals were obtained from Jackson Laboratories. $En1^{cre/+}$:$R26^{td+/td+}$ females were crossed with $Foxp2^{Flp/+}$:$R26^{DC/DC}$ males to produce $En1^{Cre/+}$: $Foxp2^{Flp/+}$: $R26^{tdTom/DC}$ animals. We observed aberrant tdtomato labeling of cells outside the ventral horn when $En1^{cre/+}$:$R26^{td+/td+}$ males were crossed with $Foxp2^{Flp/+}$:$R26^{DC/DC}$ females, so we did not pair animals in this way. Thus, a cre-lox recombination system facilitated expression of the reporter gene tdTomato in all En1 expressing V1-INs. Cre expression from the En1 locus removes a floxed stop signal upstream of the tdtomato gene on the Rosa26 locus. Labeling of the intersectional population of Foxp2-expressing V1-INs with EGFP is conditional to the expression of both Cre and Flp recombinases (dual conditional, DC) from the En1 and Foxp2 loci, respectively. In DC reporter animals two STOP signals are present upstream of the EGFP gene on the Rosa26 locus: one flanked by FRT sites, and the other flanked by loxP sites. Cre and Flp-mediated recombination will remove these STOP signs and allow transcription of EGFP.

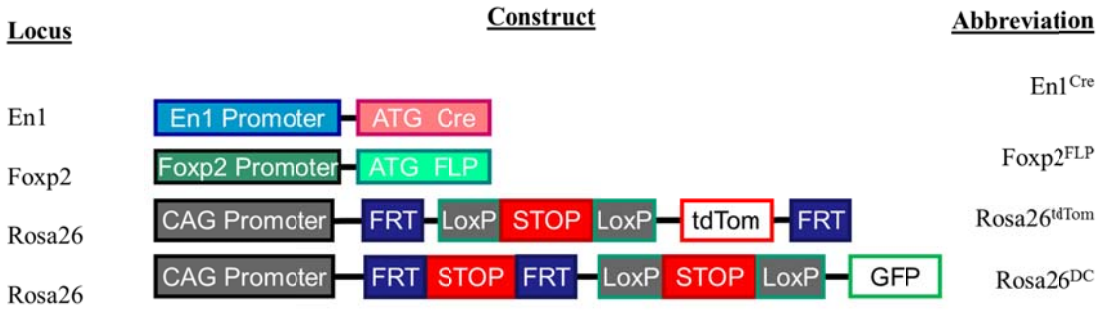

Figure 1. Schematic of the four transgenic alleles used in our mouse lines to specifically label populations of V1 INs according to their expression of Foxp2.

Animal and tissue preparation

All animal procedures were carried out according to NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Emory University (Atlanta, GA). Animals were anesthetized by a 100mg/kg intraperitoneal injection of Euthasol. Postnatal mice (P0, 5, 15, 30 and 3-month) were perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). Spinal cords from these mice were extracted and postfixed in 4% PFA in the same buffer overnight. The following day, cords were placed in a solution of 30% sucrose in 0.1M PB. One animal was analyzed at each age, except P5 in which two animals were analyzed.

In addition we analyzed one E11.5 and one E15.5embryo. To generate these embryos we set up two females for time pregnancies. Just before 7:00 pm (start of the dark cycle), male and female animals were paired. The timing of pregnancies was contingent upon visualization of a vaginal plug at 7:00 am the next day, at which point males and females were separated. A positive plug at 7.00 am was considered E0.5. At gestation times E11.5 and E15, timed pregnant female mice were sacrificed and the

embryos were harvested. Once embryos were extracted, they were photographed in a fluorescent dissecting microscope and the embryos were further staged according to external features and comparison with the Mouse Developmental Atlas. In particular, we looked at the developmental status of the forelimb and hindlimb. Embryos were then fixed in a solution of 4% PFA in 0.1 MPB overnight. The next day they were placed in 30% sucrose in 0.1M PB.

Tissue Sectioning and Processing

Fifty μm thick transverse sections were obtained from the postnatal spinal cords using a freezing-sliding microtome and serially collected. All postnatal spinal cord sections were processed free floating. Embryos were cut in a cryostat. Twenty micrometer thick transverse sections from embryonic spinal cords were obtained and collected on slides. Processing of embryonic tissue was therefore all done on slides.

Immunohistochemistry

To optimize visualization the EGFP and tdTomato reporter signals were amplified using immunohistochemistry for EFP and tdtomato. All sections were blocked with 10% normal donkey serum in 0.01M phosphate buffer saline (PBS) with 0.1% Triton-X-100. Sections were double immunostained with EGFP (raised in chicken) and DsRed (raised in rabbit) primary antibodies at 1:1000 dilutions in 0.01M PBS. P0, P5 and embryonic sections were additionally immunostained with a Foxp2 antibody (raised in goat, 1:1000 dilution in 0.01M PBS. The details of the tree primary antibodies are summarized in Table 1. Immunoreactive sites were revealed with species-specific donkey raised secondary antibodies (Jacskson ImmunoResearch) against chicken IgYs coupled fluorescein isothiocyanate (FITC), against rabbit IgGs coupled to cyanine 3 (Cy3) and

against goat IgGs coupled to cyanine 5 (Cy5). All secondary antibodies were diluted 1:100 in PBS. Fluorochromes were protected from fading by applying VectaShield mounting medium and a coverslip. Embryonic spinal cords were mounted with a Vectashield that contained DAPI for better resolution of cell nuclei and individual cells.

Table 1: Antibody Summary

Antibody Name	Type	Host Species	Dilution	Provider	Catalog Number	Lot Number
EGFP	Polyclonal	Chicken	1:1000	Aves	1020	0425FP07
DsRed	Polyclonal	Rabbit	1:1000	Clonetech	632496	1104060A
Foxp2	Polyclonal	Goat	1:500	Santa Cruz	21069	G2911

Image Acquisition

Images of postnatal spinal cord sections were collected at 20X magnification and images of embryonic spinal cords were collected using at 60X on an Olympus FV1000 confocal microscope. Lasers with wavelengths of 405nm, 488nm, 559nm and 635nm, were used to excite DAPI, FITC/EGFP, Cy3/tdtomato, and Cy5 fluorochromes, respectively. We used a motorized stage and automatic tiling to image the full spinal cord at this magnification.

Neurolucida Analysis

Tiled images from the confocal microscope were analyzed using Neurolucida software in order to plot and count cells in the ventral horns of processed tissue slices. Using confocal images in conjunction with Neurolucida allowed us to go through optical sections of the tissue slices. We outlined each spinal cord section, central canal, and in

postnatal animals, the grey matter border was also outlined. We went through each optical plane and used different markers to count the number of cells in V1-IN subpopulations defined by the combination of reporter protein expression and Foxp2 immunoreactivity.

Results

The proportions of V1-Foxp2 interneurons that express EGFP alone or in combination with tdtomato do not change from birth to adult

We used dual conditional reporter animals to label the subpopulation of V1-INs that express Foxp2. These animals carry one En1-cre and one Foxp2-flp allele and therefore cells that express both transcription factors, at sometime during development, will remove FRT and Loxp flanked transcriptional STOPs and allow expression of EGFP from the dual conditional reporter cassette. In addition, in the same animal we have a single conditional tdtomato reporter transgene that is expressed in all cells expressing just En1-cre. This later cassette is also subject to flp recombinase and could be removed from cells that express Foxp2. Therefore Foxp2(+) V1-INs should be green (expressing EGFP) and Foxp2(-) V1-INs should be red (expressing tdtomato). However, in the postnatal spinal cords of these dual conditional animals we found three different types of cells. The two larger subgroups expressed tdtomato and EGFP exclusive of each other, but in addition a smaller subgroup co-expressed both fluorescent proteins (Figure 2). One possible explanation for such labeling pattern is that after removal in embryo of the tdtomato reporter cassette, the progressive degradation of tdtomato over postnatal time results in fewer EGFP-expressing cells coexpressing tdtomato as the age of the animal increases. To explore this possibility, we analyzed the percentages of only tdtomato-positive, only EGFP-positive, and tdtomato and EGFP double-positive cells in lower lumbar segments (L4 and L5) in P0, P5, P15, and 3-month adult animals (1 animal per age at P0, P15, and 3-month; 2 animals at P5; $n \geq 7$ ventral horns per animal). The quantitative results are shown in Figure 3. Across postnatal time, the proportion of each

of these V1-IN subpopulations to the entire V1 population remained very stable. Of the total V1-IN population, cells that express only tdtomato comprised 35-40 percent; only EGFP expressing cells comprised about 50 percent; and tdtomato and EGFP coexpressing cells comprised about 12-13 percent. Because we did not observe a decrease in the number of cells that colocalize tdtomato and EGFP with postnatal age, we conclude that the labeling pattern observed in our dual conditional is not dependent on the stability of the tdtomato protein over postnatal time after flp-mediated removal of the transgene. On the contrary, it reflects a stable pattern suggesting that flp recombination failed to remove tdtomato in cells that co-express tdtomato and EGFP at these postnatal ages.

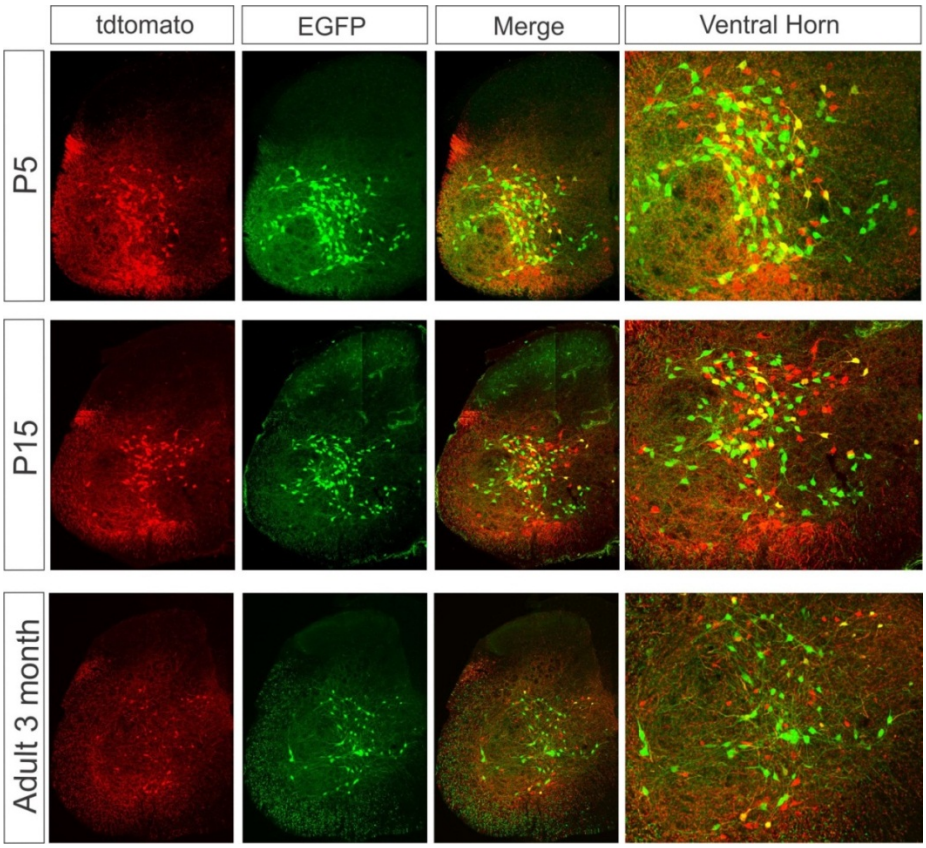

Figure 2. **Expression of tdtomato and EGFP in V1-IN subpopulations does not change from birth to adult.** Expression of the Foxp2 gene during some point in

development prompts EGFP expression. Most Foxp2-expressingV1-INs lack tdtomato postnatally, and are only green. A much smaller group of Foxp2-expressing V1-INs colocalizes EGFP and tdtomato and is therefore yellow. Cells that never express Foxp2 do not express EGFP and are red. The distribution and proportion of each V1 cell type to the total V1-IN population remains stable from birth to adulthood.

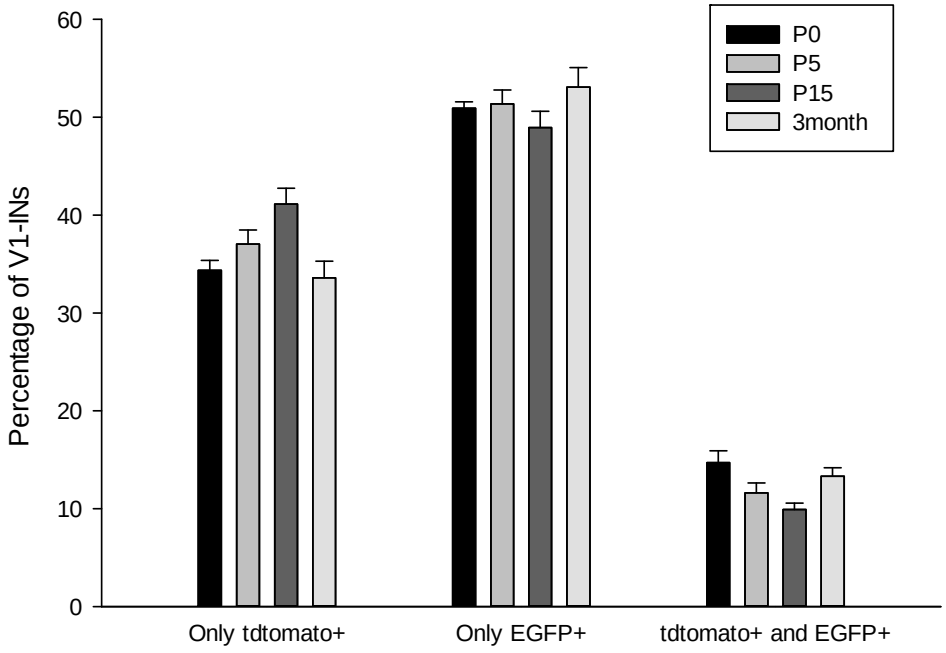

Figure 3. The percentages of V1-INs expressing different combinations of fluorescent proteins is stable at different postnatal ages. Each bar represent the average percentage estimated in 4 to 8 ventral horns collected form one animal at each age, except P5 where two animals were analyzed. Error bars indicate the standard error of the mean.

The adult pattern of EGFP and tdtomato expression is established between E11.5 and E15 and appears dependent on the dynamics of Foxp2 expression

To investigate when this pattern is first established we next analyzed two different embryonic ages. First, we studied five sections from one E15 animal. This age was

chosen because at this stage the neurogenesis of spinal interneurons has been completed (V1 neurogenesis occurs between E9.5 to E12.5, Benito-Gonzalez and Alvarez, 2012). The spinal cord at E15 is organized similarly to the postnatal spinal cord because all ventral horn neurons have already finished their migration and are placed in the locations that they also occupy in the P0 and adult spinal cords (Figure 4).

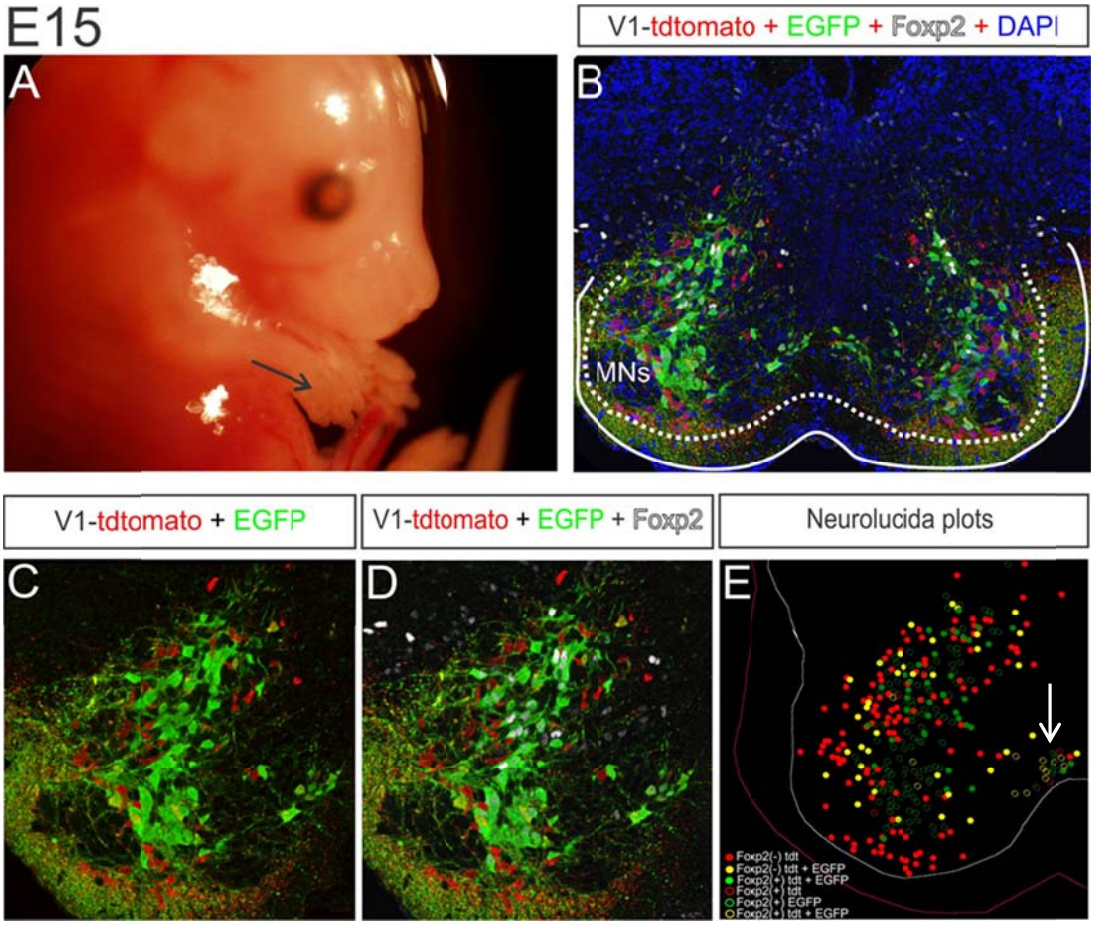

Figure 4. Reporter fluoresecnt proteins and Foxp2 expression in V1 INs at E15. A) Presence of separated fingers (indicated by an arrow) in addition to stage of eye development and whisker pad confirms the age of this animal as E15. **B**) Low magnification of a single confocal plane of transverse spinal cord section. Tdtomato expression is in red, EGFP expression is in green, Foxp2 expression is in white, and DAPI staining is shown in blue. MNs indicate the location of motor pools. Dashed line indicates gray-white matter border. Continuous line indicates the edge of the spinal cord. **C-D**) High magnification images of V1-INs expressing tdtomato (red cells), EGFP (green cells), or both (yellow cells) in a single confocal optical plane. Most cells that express

Foxp2 (shown in white in D) express EGFP only. **E**) Neurolucida plot showing different markers indicating Foxp2-positive V1-INs (open circles) and Foxp2-negative V1-INs (filled circles) in the entire confocal image stack. While most lateral Foxp2-IR V1-INs express EGFP only, we detected also a medial group (arrow) with variable pattern of expression of EGFP and tdtomato.

The percentages of V1-INs expressing tdtomato only, EGFP only or both proteins were respectively, 38, 47, and 15 percent, and therefore in the same range as in postnatal animals. This suggests that by E15 the expression pattern of these proteins has already been established, perhaps by an earlier mechanism occurring during V1 neurogenesis and related to the dynamics of Foxp2 expression.

Neurogenesis of Foxp2 V1-INs was previously reported between E11 and E12.5 (Benito-Gonzalez and Alvarez, 2012) and therefore we analyzed one embryo at E11.5 (Figure 5). At this age V1-INs are still being generated and there is a stream of migrating cells coming out from the progenitor area corresponding to the p1 domain. In these E11.5 animals, there were predominantly cells that expressed only tdtomato or co-expressed both tdtomato and EGFP, and almost none expressed only EGFP. Respectively, they represent 90, 9, and 0.01 percent of the entire V1 population. Moreover, a discrete spatial distribution of V1-INs with different combinations of reporter proteins and Foxp2 immunoreactivity was apparent at this age. We observed two distinct groups of cells expressing only tdtomato: one of these lies adjacent to the progenitor area and exhibits very strong Foxp2 immunoreactivity (IR), while the other group is an earlier born group that has already migrated ventrolaterally and does not express Foxp2. Similarly, there are also two distinct groups of tdtomato and EGFP coexpressing cells that can be divided based on Foxp2-IR and their position in the ventral horn. The larger group of tdtomato

and EGFP coexpressing cells streams from the progenitor area, is located dorsomedial to the V1-IN population, weakly expresses tdtomato, and is Foxp2-IR. In contrast a smaller group of coexpressing cells occupies a more ventrolateral position, strongly expresses tdtomato, and is not Foxp2-IR at E11.5. This later group may therefore have expressed Foxp2 at an earlier age. Given that according to Morikawa et al. (2009) and Benito-Gonzalez and Alvarez (2012) there is no Foxp2 expression in the neural tube at E10.5, Foxp2 expression this lateral cells must have been transient (24 hours or less). V1-INs generated at E11.5 (therefore they have not yet migrated away from the progenitor area) and expressing Foxp2 at the start of their differentiation could more effectively remove the tdtomato cassette and become green only cells.

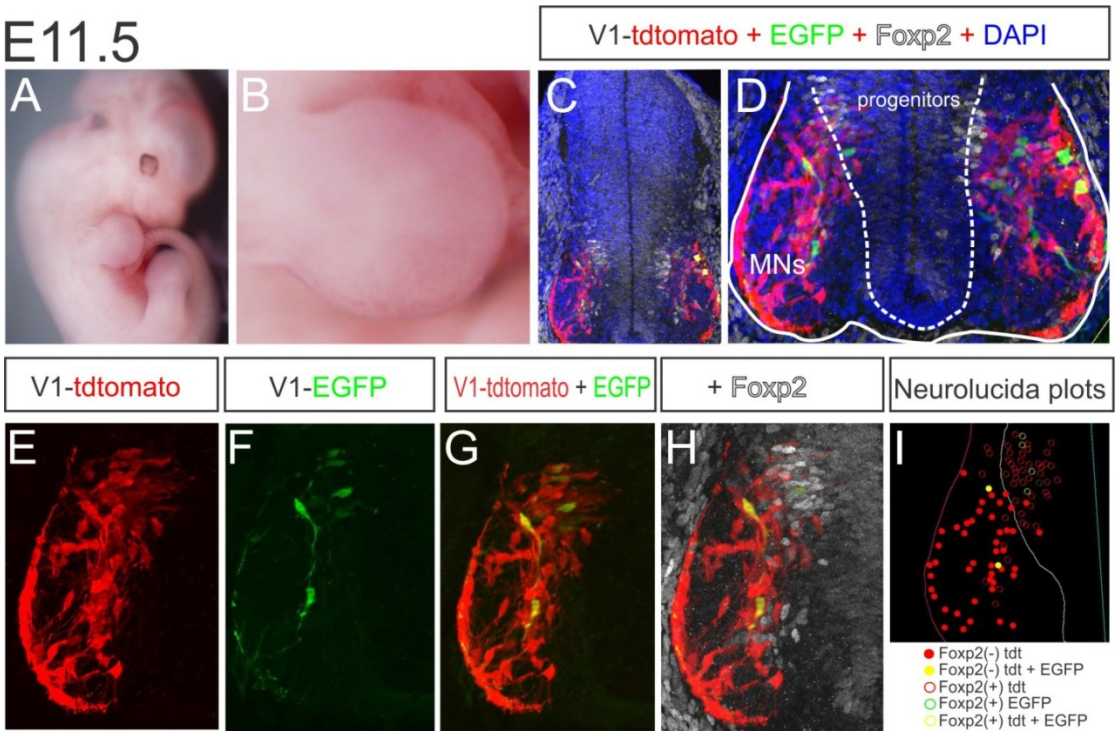

Figure 5. **Reporter fluoresecnt proteins and Foxp2 expression in V1-INs at E11.5.** ***A*** and ***B*** Embryos were staged according to anatomical features. Identification of paddle-shaped handplate confirmed the age as E11.5. ***C*** and ***D*** Low magnification confocal image of transverse spinal cord section. Tdtomato expression is shown in red, EGFP

expression is shown in green, Foxp2 expression is shown in white, and DAPI staining is shown in blue. In ***D***, the solid white line shows the outline of the ventral horn and the dashed white line demarcates the progenitor area. Motor neuron (MN) pools are surrounded by V1-INs. ***E-H*** High magnification images of a single ventral horn. Most V1-INs express tdtomato (***E***) at this age, but only a few express EGFP (**F**). Most of the cells that express EGFP coexpress tdtomato (yellow cells in ***G*** and ***H***). ***H*** V1-INs that are exiting the progenitor area are Foxp2-IR (white). ***I*** Neurolucida plots show outlines of the edge of the spinal cord (pink trace) and progenitor area (white trace). Different markers show the distribution of Foxp2-positive V1-INs (open circles) and Foxp2-negative V1-INs (filled circles). The different colors of the markers correspond to the cell types described in ***E-G***

Taken together, these results suggest that we may be capturing the time in which a population of tdtomato-expressing V1 cells exiting the progenitor area start down regulating tdtomato while upregulating Foxp2 expression. Those cells in the progenitor area that are Foxp2-IR, EGFP-positive, but only weakly express tdtomato may be in the middle of such a process, and by E15 may become cells that express EGFP but not tdtomato. The group of Foxp2-IR tdtomato-only cells in the progenitor area may be in the earlier stages of this conversion process in which FLP-mediated recombination of EGFP is still occurring and EGFP has not yet accumulated within the cells. This observation also suggests that in these cells En1 expression and cre recombination occurs prior to Foxp2 expression and FLP recombination.

Foxp2 expression in late embryos and postnatal animals

The pattern of Foxp2-IR at E15 is significantly different from that at E11.5. In contrast to E11.5 in which 50% of V1-INs expressing tdtomato express also Foxp2-IR, at E15 only 1% of the cells that only express tdtomato are also Foxp2-IR. In fact, more than

90% of Foxp2-IR V1-INs at E15 are only EGFP-positive. Of these EGFP-only expressing cells, nearly 80 percent are Foxp2-IR. A smaller proportion—about 19 percent—of EGFP and tdtomato colocalizing cells are also Foxp2-IR. These observations could be explained if all Foxp2-IR V1-INs with only tdtomato expression at E11.5 correspond with new nascent V1-INs in the process of upregulating EGFP, downregulating tdtomato and migrating laterally. Thus, E11.5 newborn V1-INs with both reporter proteins and expressing FoxP2-IR could become EGFP-only cells that maintain FoxP2-IR at E15. In contrast, V1-INs already differentiated at E11.5 and located ventrolaterally close to the motor pools, express only tdtomato and almost never contain Foxp2-IR. These cells likely correspond with the population of V1-INs that at E15 also express tdtomato only and similarly lack Foxp2-IR. Finally, cells co-expressing tdtomato and EGFP fall in two categories, those with weak tdtomato usually express Foxp2-IR. In contrast, those with strong tdtomato expression almost never display Foxp2-IR. The first group might represent cells that just started expressing Foxp2 and are in the process of dowregulating tdtomato expression, while we do not have yet a definitive explanation for the second (see Discussion).

Interestingly, there is a medial group of V1-INs at E15 that express Foxp2-IR and contains a mix of tdtomato-only cells, EGFP-only cells and co-expressing cells with weak tdtomato. This pattern has similarities with the expression of these two reporter proteins in Foxp2-IR nascent cells at E11.5, suggesting that this group is at this age in the process of upregulating Foxp2 and switching reporter expression from tdtomato only, to co-expression and then EGFP only. Further observations are necessary at flanking ages, but if confirmed this group of cells might represent a group of cells that upregulate Foxp2

after differentiation and migration, by difference to the group described at E11.5 that upregulates Foxp2 just after neurogenesis and in the early stages of migration and differentiation.

To further investigate the fate of Foxp2-IR in these different populations of V1-INs, we tested Foxp2-IR in postnatal V1-INs. Similar to E15, none of the cells that express only tdtomato are Foxp2-IR at P0 and P5. V1-INs that only express EGFP seem to downregulate Foxp2 expression and at P0 and P5 we found Foxp2-IR in respectively 66 and 48 percent of all V1-INs expressing only EGFP. The pattern was similar whether they were located in the lateral or medial group of EGFP only cells. Furthermore, very few V1-INs coexpressing EGFP and tdtomato are Foxp2-IR at P0 (6%) and P5 (2%). In summary, Foxp2 expression in Foxp2 EGFP-only V1-INs slowly decreases from E15 to P5, while very few if any tdtomato expressing V1-INs are Foxp2-IR at postnatal ages

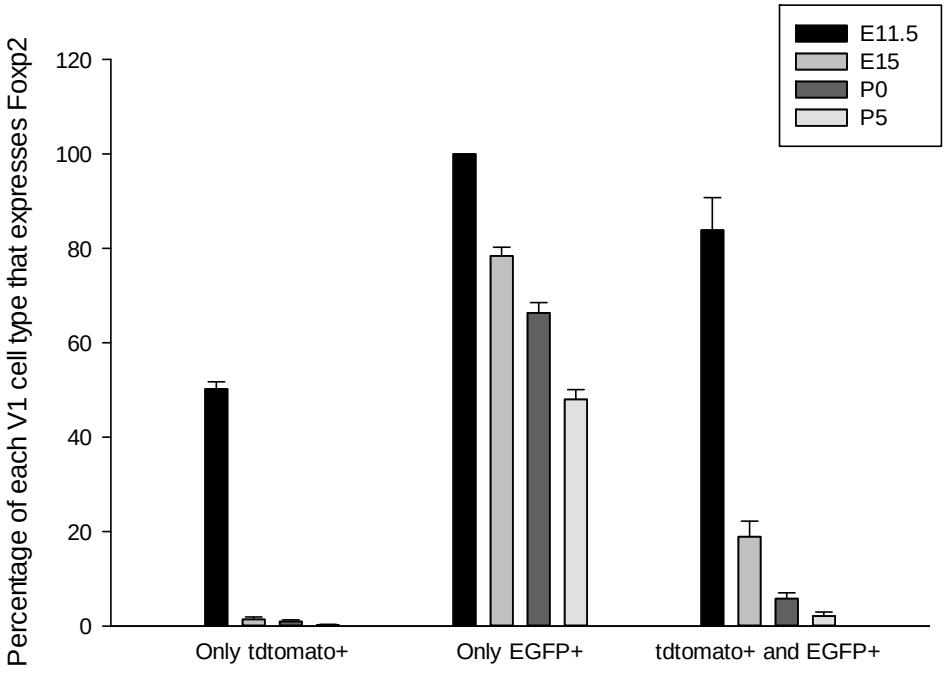

Figure 6. Foxp2 expression in V1-INs expressing different combinations of EGFP and tdtomato. Each bar represent the average percentage estimated in 4 to 8 ventral horns collected form one animal at each age, except P5 where two animals were analyzed. Error bars indicate the standard error of the mean. The large percentages of Foxp2 in tdtomato expressing cells at E11.5 correspond largely with newly generated cell located at the exit of the progenitor area. In contrast the majority cells expressing only EGFP have Foxp2-IR at E15 and then they downregulate this expression in postnatal time. At E11.5 all cells with EGFP only were Foxp2-IR but at this age these are few neurons (<5 per section). The tdtomato + EGFP expressing cells at different ages have different significance. AT E11.5 correspond with cells being newly generated and at E15 correspond to the medial group of interneurons. In most cases tdtomato was weak in these cells.

In summary, we were able to distinguish distinct cell types in the V1 population based on Foxp2-IR. We observed one group of cells that upregulates Foxp2 expression at early stages of its differentiation and before migration. We believe most of these cells become located laterally, close to the border with the lateral motor neuron columns. A second group corresponds with ventromedial V1-INs that upregulate Foxp2 expression at E15 and therefore later in development after completing their migration. Cells of both groups seem to express only EGFP at P0 and P5, but expressed tdtomato for a short time earlier in development. This suggests that in all cases En1 expression precedes Foxp2 upregulation. Another distinct group is made up of cells that never express Foxp2 and are only tdtomato-positive for life. Finally, a third group of V1-INs coexpress EGFP and tdtomato both at high levels. Only rarely could we detect Foxp2 expression in these cells and therefore it might be expressed only very transiently.

V1-INs expressing different combinations of reporter proteins occupy different locations in the ventral horn

We identified three unique groups of cells with different patterns of tdtomato and EGFP expression. These cells also exhibit different patterns of Foxp2-IR during embryonic and early postnatal development. To gain some insight into their possible functions we analyzed their exact localization in the adult spinal cord. Motor pools controlling axial musculature form a medial motor column while those innervating the limbs are located in a lateral motor column. Within the lateral motor columns the motor pools innervating different musculature of the leg are organized such that motor neurons innervating the more distal muscles (for example those in the foot) are located more dorsally and the more ventral motor pools innervate the more proximal musculature (for example hip). To understand the relationship of each group of V1-INs with the location of different motor neurons in the spinal cord and the muscle groups they control, we analyzed the dorsoventral and mediolateral position of V1-INs in the adult ventral horn. For this purpose we constructed Neurolucida plots, as shown in Figure 7, of the distribution of V1-INs with different combinations of fluorescent proteins at lower lumbar levels (Lumbar 4 and 5).

V1-INs expressing only tdtomato are dispersed throughout all regions of Lamina VII with two prominent groups. One is in the very ventral region close to the white matter border and in the area were motor axons exit and form ventral roots, another group distributes through the whole medio-lateral extent at the most dorsal edge of the distribution of V1-INs. Based on the work of Benito-Gonzalez and Alvarez (2012), we

know that V1 INs in these regions include dorsal and ventral groups of MafB positive V1-INs and that the ventral group are Renshaw cells. We confirmed that most ventral tdtomato only cells in this new animal model are calbindin positive, a marker of Renshaw cells (data not presented). Cells that coexpress tdtomato and EGFP are also dispersed widely throughout the ventral horn but at lower density than tdtomato cells. These cells may be similarly involved with the control of either extremities or axial musculature.

The majority of V1-INs expressing only EGFP are tightly clustered laterally at the border between Lamina VII and lateral Lamina IX and occupy the whole dorso-ventral extension of Lamina VII between dorsal and ventral MafB-expressing V1-INs. These cells are therefore tightly related to limb motor pools. In contrast, the ventro-medial group of EGFP only V1-INs is located just above the medial motor column in an area that might occupy medial regions of Lamina VII or Lamina VIII. This suggests that they may be involved with the control of axial musculature.

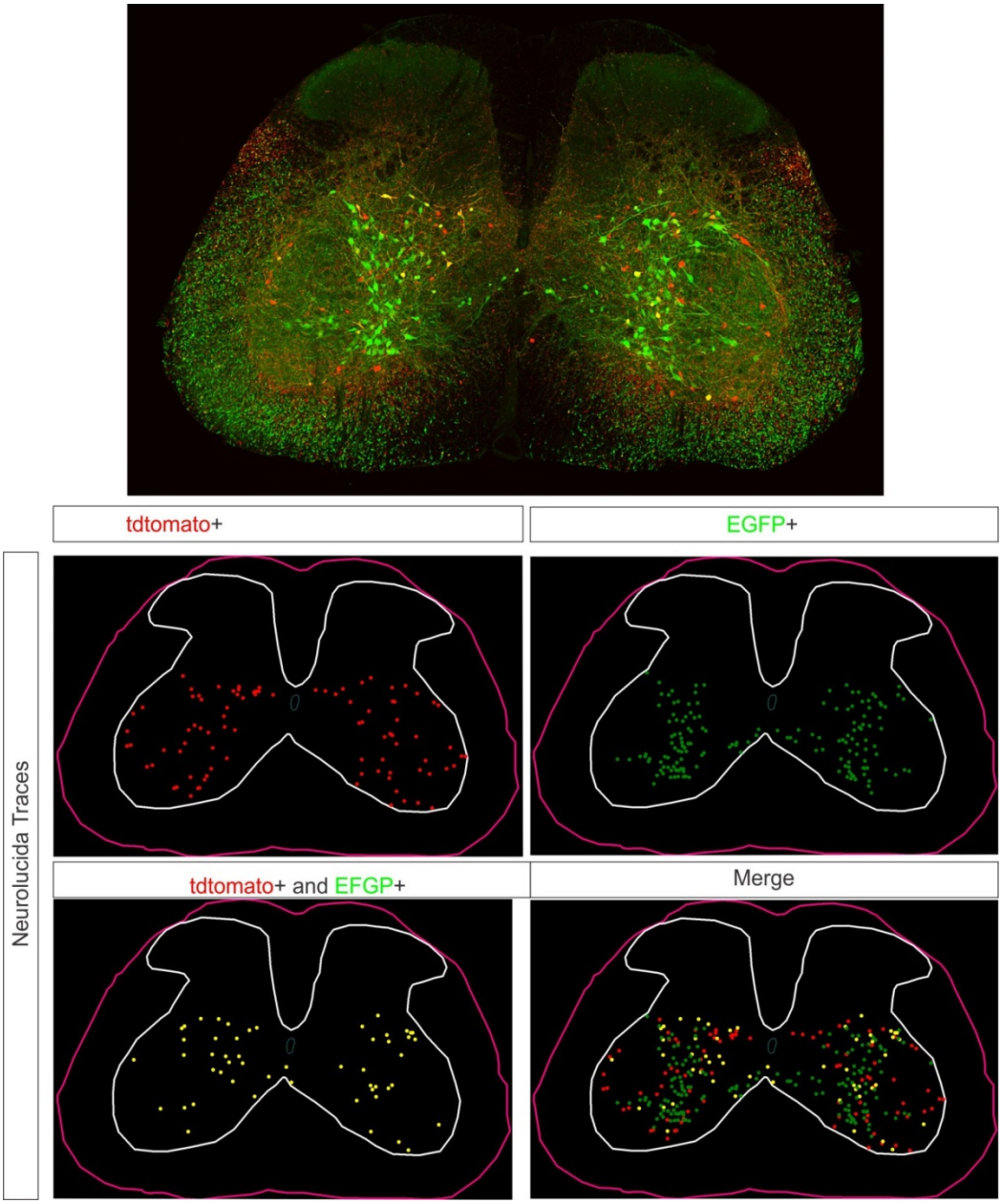

Figure 7. Distribution of V1-INs with different expression of fluorescent proteins. Top image corresponds to a superimposition of all confocal planes through a 50 µm thick section of the lower lumbar spinal cord. It shows the distribution of V1-INs and their axons expressing different combinations of tdtomato and EGFP. Lower panels correspond to cell plots of the image presented above. Similar analyses were done in 4 adult spinal cords.

Discussion

We used a dual conditional lineage tracing system to genetically label Foxp2(-) and Foxp2(+) V1 cell lineages with different combinations of tdtomato and EGFP. Using this model, we identified three unique subgroups of V1-INs—cells that express only tdtomato, only EGFP, or both. The first group corresponds to those cells that never express Foxp2 while the latter two groups are Foxp2(+) V1 cell lineages. To investigate whether this pattern of reporter protein expression is dependent on the stability of the tdtomato reporter cassette, we analyzed animals of various postnatal ages and found that this pattern is maintained from P0 to adulthood. Moreover, analyses of an E15 animal revealed that the postnatal pattern of reporter protein expression is already present at this age. We then hypothesized that the pattern of tdtomato and EGFP expression in postnatal animals may be related to the dynamics of Foxp2 expression during earlier embryonic development. Indeed, in our analysis of an E11.5 animal, the timing of Foxp2 expression seemed to be related to the establishment of the pattern of reporter protein expression. At this age, we observed a group of tdtomato-only cells and another group of coexpressing cells that were not Foxp2-IR and had already migrated away from the progenitor area. Interestingly, we also observed many Foxp2-IR cells that may be in the process of upregulating both Foxp2 and EGFP expression and concurrently metabolizing tdtomato, suggesting that these cells later become EGFP-only cells. Finally, we analyzed the dorsoventral and mediolateral placements of each V1 cell type identified in our study to propose possible functions of each of these groups.

Our data agree with the report by Benito-Gonzalez and Alvarez (2012) that between 20-35% of all V1-INs are Foxp2-IR at P0. However, because we have used a

lineage tracing method in which EGFP expression labels cells that expressed Foxp2 at any point prior to sacrificing the animal, we have identified many more cells that stem from a Foxp2(+) V1 cell lineage than previously reported. With our genetic lineage labeling we estimate that approximately 65% of all V1-INs are Foxp2 expressing cells at some point during development. The temporal dispersion of Foxp2 expression in which some cells express Foxp2 very early and transiently during development and other cells express Foxp2 later and or maintain it for longer time, accounts for the discrepancy between our counts of EGFP expressing V1-INs and the percentage of Foxp2-IR cells reported by Benito-Gonzalez and Alvarez (2012) in postnatal animals or at any embryonic time by Morikawa et al. (2009). Furthermore, in accordance with the work of Benito-Gonzalez and Alvarez (2012) and Morikawa et al. (2009), our results suggest that the onset of Foxp2 upregulation in V1-INs begins between E10.5 and E11.5.

At E11.5, we observed a small group of cells that coexpress tdtomato and EGFP, but are not Foxp2-IR. Because EGFP expression indicates a previous upregulation of Foxp2, we propose that these cells must constitute a group that very transiently expresses Foxp2 between E10.5 and E11.5. Interestingly these cells retain tdtomato expression despite expressing Foxp2 and thus flp recombinase. The main difference that we can account between these cells and the Foxp2-IR group that is being generated at E11.5 and largely become EGFP only cells, is that in these latter cells Foxp2 expression is retained in many cells, frequently through early postnatal development. It is possible that the phenotypic differences between cells that co-express EGFP and tdtomato with those that do not colocalize tdtomato may be due to differences in the length and/or strength of Foxp2 expression. If this was the case it would suggest that FLP recombination of the

FRT sites that flank a STOP upstream of EGFP in the Rosa26^{DC} construct is more efficient than FLP recombination of FRT sites that flank the entire tdtomato reporter cassette. Therefore, double-fluorescent cells may express tdtomato because they retain the tdtomato reporter cassette, while EGFP-only cells do not. Whether cells that express both reporter proteins constitute a unique group of V1-INs or are more related to one of the groups that express tdtomato or EGFP exclusive of each other remains unclear. To investigate this group, it would be necessary to monitor Foxp2-IR of V1-INs in E11 or possibly even E10.5 animals in order to confirm transient Foxp2 expression in cells with high co-expression of both tdtomato and EGFP. Additionally, identification of a transcription factor or other markers that is unique to this particular group would also help to distinguish these cells from other V1-INs and increase confidence that they are a unquiet subgroup with distinct properties. Alternatively our hypothesis might be incorrect and some other unknown genomic arrangement might be responsible for co-expression of EGFP and tdtomato is some V1-INs. One potential future direction of this work includes analyzing V1-INs in animals between the ages of E11.5 and E15. In this way, it may be possible to confirm when nascent Foxp2-IR tdtomato-only cells may be switching reporter expression from tdtomato only, to co-expression of both reporters, and then to EGFP only. Yet another potential future experiment would be to treat animals with an axonal transport blocker such as colchicine to control for the possibility that not all the cell bodies that express reporters are being counted. However, because we observed the adult pattern of reporter protein expression at E15—a time at which axons have not yet fully developed—it is unlikely that reporter proteins are being shunted down away from the soma.

We analyzed the spatial distribution of the different V1-IN subpopulations identified in this study to better understand their spatial relationships with different motor pools and might provide some clues about possible functions. Cells co-expressing EGFP and tdtomato represent just over 10% of the V1-INs and are distributed throughout the ventral horn. Their significance is at present unclear, as explained before and their widespread distribution does not offer any further insight. Therefore we focus the discussion on the cells that alternatively express either tdtomato or EGFP. We observed that cells that express only tdtomato were dispersed throughout the ventral horn and overlap the distribution of EGFP only cells at the dorsal and ventral edges of the V1-IN distribution. From a previous report by Benito-Gonzalez and Alvarez (2012), we know that the ventral-most of this group of V1-INs are Renshaw Cells (RCs). RCs provide recurrent inhibition to motor neurons and their position correlates with their known higher actions on proximal musculature compared to the very distal flexor muscles (Alvarez and Fyffe, 2012). In addition, we identified a group of tdtomato-only cells that have a very dorsal position in the ventral horn and correspond to some of the MafB-expressing V1-INs described previously by Benito-Gonzalez and Alvarez (2012). At present we don't know their exact functional significance. In contrast, EGFP-only cells extend medially across the dorsoventral axis and are densely packed at the border of Lamina IX. This region is usually regarded as the location of IaINs that mediate reciprocal inhibition between antagonist muscles and from the work of Benito-Gonzalez, we know that some of these EGFP-only cells are V1-derived IaINs, so it is possible that many of these EGFP-only cells are providing reciprocal inhibition to motor neurons of the limb musculature. In addition, there also a medial group of EGFP-only V1-INs

located above the medial motor columns. These cells could inhibit motor neurons innervating axial musculature. Future studies should investigate the connections of each of these V1-IN groups.

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