Renshaw cell interneuron specialization is controlled by a temporally restricted transcription factor program

Floor J. Stam1,*, Timothy J. Hendricks1,*, Jingming Zhang1,*, Eric J. Geiman1, Cedric Francius2, Patricia A. Labosky3, Frederic Clotman2 and Martyn Goulding1,‡

SUMMARY
The spinal cord contains a diverse array of physiologically distinct interneuron cell types that subserve specialized roles in somatosensory perception and motor control. The mechanisms that generate these specialized interneuronal cell types from multipotent spinal progenitors are not known. In this study, we describe a temporally regulated transcriptional program that controls the differentiation of Renshaw cells (RCs), an anatomically and functionally discrete spinal interneuron subtype. We show that the selective activation of the Onecut transcription factors Oc1 and Oc2 during the first wave of V1 interneuron neurogenesis is a key step in the RC differentiation program. The development of RCs is additionally dependent on the forkhead transcription factor Foxd3, which is more broadly expressed in postmitotic V1 interneurons. Our demonstration that RCs are born, and activate Oc1 and Oc2 expression, in a narrow temporal window leads us to posit that neuronal diversity in the developing spinal cord is established by the composite actions of early spatial and temporal determinants.

KEY WORDS: Foxd3, Onecut, Renshaw cell, Interneuron specification, Spinal cord, Transcription factor, Mouse

INTRODUCTION
Recent studies have defined many of the developmental events that pattern neuronal cell types in the embryonic spinal cord (Briscoe and Ericson, 2001; Goulding et al., 2002; Dessaud et al., 2008). Of particular importance are the early transcriptional programs activated by sonic hedgehog (Shh) and BMP/TGF-β signaling that play instructive roles in subdividing the neural tube longitudinally into eleven primary progenitor domains (Jessell, 2000; Briscoe and Ericson, 2001; Goulding et al., 2002; Dessaud et al., 2008). These dorsoventrally restricted progenitor domains produce 12 early-born cardinal classes of neurons (motor neurons, V0-V3 interneurons and dI1-dI6 interneurons) along with two late-born cell types (dIL₅ and dIL₆ interneurons) (Goulding et al., 2002; Caspary and Anderson, 2003; Goulding, 2009). However, a considerable level of cell diversity exists in the differentiated progeny arising from these cardinal classes of embryonic neurons (Stepien and Arber, 2008; Goulding, 2009; Grillner and Jessell, 2009). Motor neurons that develop from the motor neuron progenitor (pMN) domain differentiate into branchiomotor, somatic and autonomic motor neuron cell types at different anterior-posterior (AP) levels in the hindbrain and spinal cord (Jessell, 2000; Dasen and Jessell, 2009). Moreover, somatic motor neurons within the lateral motor column (LMC) exhibit considerable specialization with respect to pool identity and subtype (Hollyday, 1980; Vanderhorst and Holstege, 1997). The interneuron cell types that arise from a single progenitor domain are also quite diverse. For example, V0 interneurons that derive from Dbx1 neurons differentiate as V0b, V0c and V0v interneurons (Moran-Rivard et al., 2001; Pierani et al., 2001; Lanuza et al., 2004; Zagoraiou et al., 2009). Likewise, the V1 class of inhibitory premotor interneurons produces multiple functional cell types (Goulding, 2009). The V1 interneurons are anatomically homogeneous when first generated (Saueressig et al., 1999), but subsequently differentiate into a range of inhibitory premotor interneuron cell types, including Renshaw cells (RCs) and putative reciprocal Ia inhibitory interneurons (Sapir et al., 2004; Alvarez et al., 2005).

The cellular and molecular processes that drive neuronal diversification within each of the aforementioned cardinal classes of embryonic spinal neurons remain largely unknown. Motor neurons acquire columnar and pool-specific identities in a progressive manner with intrinsic transcriptional programs guiding the sequential specification of generic and columnar motor neuron identity (Jessell, 2000; Dasen and Jessell, 2009). Extrinsic signals also act at a number of steps in the motor neuron differentiation program. For example, retinoic acid (RA) regulates the early development of LMC₁ motor neurons (Sockanathan and Jessell, 1998), whereas glial-derived neurotrophic factor (GDNF) and c-ret signaling regulate later events that determine axon pathfinding, motor pool identity and pool-specific patterns of sensory afferent connectivity (Haase et al., 2002; Livet et al., 2002; Kramer et al., 2006; Vrieseling and Arber, 2006). Activity and stochastic processes might also contribute to the specialization of motor neurons by controlling axon guidance and motor pool identity (Hanson and Landmesser, 2004; Dasen and Jessell, 2009). Much less is known about the mechanisms that govern the differentiation of specialized cell types from the broad interneuronal classes in the embryonic spinal cord. RCs that arise from V1 interneurons are an ideal system for exploring the mechanisms that generate neuronal diversity. RCs constitute a specialized spinal interneuron cell type, actions of which include the recurrent inhibition of motor neurons (Eccles et al., 1954; Windhorst 1996). RCs occupy a characteristic locale in the ventral horn, between motor neurons and the ventral white matter border, and they are uniquely defined by their co-expression of En1 and
the calcium-binding protein calbindin D28k (calbindin 1 – Mouse Genome Informatics) (Alvarez et al., 1997; Carr et al., 1998; Geiman et al., 2000; Sapir et al., 2004).

In this study, we show that that the first postmitotic V1 interneurons to be born are committed to an RC fate. Moreover, RC fate is determined by a temporal transcriptional program, in which the Ocl/O2, Foxd3 and MafB transcription factors have essential roles in establishing and maintaining the RC phenotype. We also find that RC specification and differentiation proceeds normally in the absence of neurotransmission and evoked neural activity, which argues that activity-dependent events play little or no role in the early development of RCs.

**MATERIALS AND METHODS**

**Mice**

The following mice were bred to generate the appropriate genotypes for analysis: En1Cre (Sapir et al., 2004), R26Rloxstop-LacZ (Soriano, 1999), R26Rloxstop-flostop (Zhang et al., 2008), R26Rloxstop-cre (F.J.S. and M.G., unpublished), Ai14 (R26Rloxstop-tdTomato) (Madisen et al., 2010), Foxd3cre, floxstop (Teng et al., 2008), Oc1lox–/–;Oc2lox–/– (Clotman et al., 2005), Nes::Cre (Tremere et al., 1999), Ngn1::Cre (Quinones et al., 2010), Zoc (Zhang et al., 2008) and MafBFlox (Blanchi et al., 2003). PCR genotyping was undertaken with allele-specific primers for each strain. For timed pregnancies, midday on the day the vaginal plug was observed was designated as embryonic day (E) 0.5.

**Identification of genes enriched in V1 interneurons**

En1Cre mice were crossed with ZnG reporter mice (Zhang et al., 2008) to drive expression of nuclear EGFP in postmitotic V1 interneurons. Neural tubes (cervical to lumbar region) were dissected from E12.5 embryos in ice-cold Ca2+–Mg2+-free PBS. Tissue from three to six EGFP-positive embryos was collected from a single pregnant dam for each experiment, dissociated with trypsin/EDTA on ice followed by trituration to dissociate cells and passage through a 35-mm strainer. Cells were kept on ice and sorted directly into Trizol (Invitrogen). Fluorescence-activated cell sorting was performed using the BD FACSVantage SE Cell Sorter (DiVa Digital Electronics) and 5 μg/ml propidium iodide was used to exclude damaged cells. EGFP-positive and EGFP-negative cells were collected and biotin-labeled mRNA prepared using the MessageAMPlification Kit (Ambion). EGFP-positive and EGFP-negative mRNA was hybridized on Affymetrix 430 2.0 microarrays in triplicate. Microarray data were statistically significant when P<0.05. Data are expressed as mean ± s.d. unless otherwise stated.

**RESULTS**

A postmitotic transcription factor code delineates the RC differentiation program

To shed light on the mechanisms that guide the differentiation of V1 cells into adult interneuronal subtypes, including RCs, we undertook a search for genes that are enriched in differentiating V1 interneurons using Affymetrix gene arrays. This analysis was performed at E12.5 when V1 interneurons are in the midst of producing their differentiated progeny. High-priority candidate genes were analyzed further, and their expression in V1 cells was mapped by in situ hybridization and immunohistochemistry. Five transcription factors, Ocl, Oc2, Foxd3, Foxp2 and MafB, were selected for further analysis on the basis of their differential expression in RCs and other V1 interneuron subtypes.

The expression of Ocl, Oc2, Foxd3, Foxp2 and MafB in RCs was downregulated from E12.5 onwards, En1Cre;R26Rloxstop-cre mice were used to lineage trace their progeny. In these animals, RCs can be unambiguously identified by their characteristic position in the ventral horn and the expression of both GFP and calbindin (Geiman et al., 2000; Sapir et al., 2004). In E12.5 embryos, Ocl, Oc2 and MafB expression in V1-derived cells was largely restricted to RCs (Fig. 1A-F, arrowheads), although some MafB+ V1 interneurons

The following antibodies and dilutions were used in this study: chicken anti-β-gal 1:1000 (Abcam ab9361), rat anti-BrdU 1:250 (Immunologicals Direct, OBT0030), goat anti-calbindin D28k 1:200 (Santa Cruz Biotechnology, SC7691), rabbit anti-calbindin D28k 1:1000 (Swant, Switzerland, CB38), goat anti-Choline acetyltransferase 1:1000 (Chemicon, AB144P), mouse anti-En1 1:10 [Developmental Studies Hybridoma Bank (DHSB), 4G11], rabbit anti-En1 1:1000 (gift from A. Joyner, Sloan-Kettering, New York, NY, USA), rabbit anti-Foxd3 1:200 (Dottori et al., 2001), goat anti-Foxp2 1:1000 (Novus Biologicals, NB100-55411), chicken anti-GFPI 1:1000 (Aves Laboratories, GFP-1020), rabbit anti-GFP 1:1000 (Molecular Probes, A-11212), rabbit anti-Hb9 1:8000 (gift from Sam Pfaff, Salk Institute, La Jolla, CA, USA), goat anti-MaflB 1:200 (Santa Cruz Biotechnology, SC10022), rabbit anti-MafB 1:1000 (gift from Carmen Birchmeier, MDC, Berlin, Germany), rabbit anti Ocl 1:1000 (Santa Cruz Biotechnology, SC13050), rat anti-Oc2 1:200 (Clotman et al., 2005), rabbit anti-Pax2 1:200 (Zymed, 71-6000), mouse anti-Pax6 1:20 (DSHB, PAX6), goat anti-Vesicular Acetylcholine Transporter 1:1000 (BD Pharmingen, 556337).

Images were captured using a Zeiss LSM510 confocal microscope and assembled using Adobe Photoshop, Adobe Illustrator and Canvas software. For quantification of Foxd3 immunofluorescence, scans without saturated pixels in the Foxd3 channel were used. GFP, Oc2 positive nuclei were identified and signal intensity determined with ImageJ software.

**Electrophysiology**

Renshaw cell-mediated inhibitory post-synaptic potentials (IPSPs) were recorded in motor neurons from E18.5 or postnatal day (P) 0 spinal motor neurons as previously described (Sapir et al., 2004), with some modifications. The membrane potential was maintained at ~70 mV by injection of a constant current. Recording pipettes were filled with a solution containing (in mM): 120 K-Glucanate, 30 KCl, 1 MgCl2, 10 HEPES, 4 ATP-Mg, 0.3 GTP, 10 phosphocreatine, and 0.2 EGTA (pH 7.3). To record IPSPs only, CNQX (20 μM) and APS (50 μM) were included in the recording chamber. QX-314 (500 μM) was also included in the intracellular solution to block antidromic action potentials. RCs were activated by electrical stimulation of the ventral horn. In some cases, the potentials induced by antidromic stimulation were confirmed as being inhibitory by bath application of strychnine (20 μM) and bicuculline (20 μM).

**Data analysis and statistics**

Student’s t-tests were performed for each data set. Results were considered statistically significant when P<0.05. Data are expressed as mean ± s.d. unless otherwise stated.
were detected outside of the RC area (Fig. 1E, arrow). Foxp2, which is expressed in many V1 interneurons (Fig. 1G-H, arrows), was not detected in RCs (Fig. 1G-I, arrowheads). The forkhead transcription factor Foxd3 is also expressed in V1 interneurons (Fig. 1) (Gross et al., 2002). In contrast to Oc1, Oc2, MafB and FoxP2, Foxd3 expression was broad and encompassed all newborn V1 interneurons. From E12.5 onwards, Foxd3 was differentially regulated within the developing V1 lineage, with its expression remaining high in RCs while being downregulated in other V1 interneurons (Fig. 1J-L; see supplementary material Fig. S1 for quantification).

**Early-born V1 interneurons differentiate as RCs**

The identification of cell-type specific markers led us to ask when RCs are generated during spinal cord development. Expression of Foxd3 and the RC-specific factors Oc1, Oc2 and MafB was examined at E9.5, E10.5 and E11.5. This is the time interval (E9-12) during which V1 interneurons are generated in the developing mouse spinal cord at E9.5 and E10.5 showing the onset of RC markers in newborn V1 interneurons. The V1 domain is indicated by square brackets. Dashed line in A-H indicates lateral edge of the neural tube. (A) V1 interneurons dorsal to the Hb9+ motor neurons express En1. (B) Foxd3 is expressed in newborn En1+ V1 cells (arrowhead). The presence of Foxd3+En1+ cells indicates that Foxd3 expression precedes that of En1 (arrow). (C) Newborn V1 interneurons (Foxd3+ cells) express Pax6. (D) Foxd3+ cells are not labeled 30 minutes after pulse labeling with BrdU, indicating that they are postmitotic. (E,F) Oc1 and Oc2 are co-expressed in newborn V1 interneurons (arrowheads). (G) Oc2+Foxid3+ (arrowhead) and Oc2+Foxid3+En1+ (asterisk) cells are present in the V1 domain at E9.5. (H) MafB is not expressed in En1+ V1 cells at E9.5. (I) Foxd3 (green) is broadly expressed in V1 interneurons at E10.5, many of which express En1 (red). Arrowhead, Foxd3+En1+ cell; asterisk, Foxd3+En1+ cell. (J) Oc2 is expressed in a lateral subpopulation of V1 interneurons (arrowhead), suggesting that the V1 cells that are born first express Oc2. (K) MafB (green) is also expressed in this lateral subpopulation of En1+ V1 interneurons (arrowheads, J,K). (L) This lateral subpopulation of En1+ cells (arrowhead) contains RCs that express calbindin (green).
During the initial phase of V1 interneuron generation (E9-9.5), we observed that all newborn V1 cells express either Oc2 or a combination of both Oc1 and Oc2 (Fig. 2E,F). The expression of Oc1 and Oc2 in V1 cells was seen to precede that of En1, suggesting that Oc1 and Oc2 are among the first cell type-specific transcription factors to be upregulated in newborn V1 interneurons. By contrast, MafB and calbindin expression in RCs was seen at later developmental times, beginning at E10-10.5 (Fig. 2K,L). These MafB+ and calbindin+ V1 cells were positioned laterally in the ventral horn. At E10.5, this V1 subdomain is marked by the expression of Oc2 (Fig. 2J), which is consistent with the idea that these MafB+calbindin+ cells represent early born Oc2+ V1 interneurons that are in the process of differentiating as RCs. We posit that the first V1 interneurons to be born activate Oc2 (and Oc1), MafB and calbindin in a sequential manner, before going on to differentiate as RCs.

To confirm that RCs are derived from the first cohort of postmitotic V1 neurons, we performed BrdU labeling studies in En1Cre;R26loxstop-HTB reporter mice. Pregnant females were pulsed once with BrdU at different time points of gestation between E9.0 and E11.5, and their spinal cords were examined at E12.5. When BrdU-labeled RCs (GFP+calbindin+ cells in the RC area; Fig. 3B,D,F,H, arrows) were counted (Fig. 3I), a clear temporal difference was seen in the birthdates of RCs and other V1 subtypes (green only) show no incorporation of BrdU. (C,D) Pulses at E10.0 label RCs along with the occasional non-RC V1 subtype (arrow, D). Arrowheads, triple-labeled cells. (E,F) At E10.5, a much larger fraction of non-RC V1 interneurons incorporate BrdU. Few RCs are born at this time (arrowhead). Note the large number of BrdU labeled V1 neurons in lamina VII (arrows). (G,H) BrdU pulses at E11 label non-RC V1 interneurons (H, arrows), but not RCs. (I) Quantification of BrdU labeling at each time point. The error bars indicate s.e.m. for three embryos. (J) Model showing the temporal generation of RCs and other V1 interneuron subtypes.

**Oc1 and Oc2 are required for RC development**

The selective expression of Oc1 and Oc2 in newborn RCs prompted us to assess the role these two transcription factors play in RC specification and differentiation. In mice lacking either Oc1 or Oc2 alone, RC differentiation appeared to proceed normally (data not shown); however, in Oc1/Oc2 mutant embryos there was a clear loss of RCs at later developmental times (Fig. 4). Cells expressing the Oc2-dependent lacZ tracer and bearing the signature expression profile of RCs (MafB’calbindin’) were still present in the E10.5 Oc1/Oc2 double knockout (DKO) cord (Fig. 4A-F), although at slightly reduced numbers (Fig. 4Q). This reduction in MafB’calbindin’ cells in the E10.5 Oc1/Oc2 mutant spinal cord suggests that inactivation of Oc1/Oc2 might compromise the initial generation of RCs from V1 progenitors.

The reduction in the number of MafB’calbindin’ RCs in the Oc1/Oc2 DKO cord at E12.5 was far more pronounced (Fig. 4G-J). Whereas calbindin’MafB’ RCs are normally arrayed in a crescent-shaped domain ventrolateral to motor neurons at E12.5 (Fig. 4GJ, outlined RC area), cells expressing both of these markers were completely absent from the RC area in the Oc1/Oc2 DKO cord (Fig. 4H,J). The dense network of calbindin’ RC axon processes that are normally present in the ventrolateral funiculus of control animals...
were also missing in Oc1/Oc2 DKO mutant spinal cords (Fig. 4, cf. 4G and 4H, outlined RC area). Calbindin-expressing cells were occasionally detected at ectopic locations within the motor column (Fig. 4H, asterisk). Although these cells might be presumptive RCs that have not migrated to the RC area. (K-N) RCs in E16.5 control cords express Oc2, MafB and calbindin (K,M). Cells with a similar expression profile are missing in the Oc1/Oc2 DKO spinal cord (L,N). The sections shown in K and M match those in L and N, respectively. (O,P) Oc2-derived Foxd3high RCs are present in control cords (O, yellow), but not in the Oc1/Oc2 DKO cords (P). Arrowheads, Foxd3high RCs. Dashed line in K-P indicates RC area. (Q) Quantification of RC numbers in control and Oc1/Oc2 DKO mutants. Error bars represent s.e.m. *P≤0.05.

Fig. 4. Oc1 and Oc2 consolidate the Renshaw cell differentiation program. (A-J) Calbindin (Cb) and MafB expression in control (Oc1+/+;Oc2lacZ+/+) and Oc1/Oc2 DKO mutant (Oc1−/−;Oc2lacZ/lacZ) mouse embryos at E10.5 and E12.5. At E10.5, MafB and calbindin are expressed in presumptive RCs, which are located dorsal to the bilateral motor columns (arrowheads, A-F). At E12.5, cells expressing calbindin (red; G,H) and MafB (red; I,J) are missing from the RC area (indicated by the dashed line) in Oc1/Oc2 DKO embryos. Calbindin+ cells positioned within the developing motor columns were occasionally observed in Oc1/Oc2 DKO mutant cords (asterisk, H). These cells might be presumptive RCs that have not migrated to the RC area. (K-N) RCs in E16.5 control cords express Oc2, MafB and calbindin (K,M). Cells with a similar expression profile are missing in the Oc1/Oc2 DKO spinal cord (L,N). The sections shown in K and M match those in L and N, respectively. (O,P) Oc2-derived Foxd3high RCs are present in control cords (O, yellow), but not in the Oc1/Oc2 DKO cords (P). Arrowheads, Foxd3high RCs. Dashed line in K-P indicates RC area. (Q) Quantification of RC numbers in control and Oc1/Oc2 DKO mutants. Error bars represent s.e.m. *P≤0.05.

**RESEARCH ARTICLE**

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**DEVELOPMENT**

were also missing in Oc1/Oc2 DKO mutant spinal cords (Fig. 4, cf. 4G and 4H, outlined RC area). Calbindin-expressing cells were occasionally detected at ectopic locations within the motor column (Fig. 4H, asterisk). Although these cells might be RCs that are unable to migrate properly, their identity cannot be determined unambiguously. Nonetheless, even when we take these cells into account, the overall number of MafB’ calbindin’ cells in the E12.5 Oc1/Oc2 DKO spinal cord was greatly reduced compared with age-matched controls (Fig. 4Q). Examination of E16.5 Oc1/Oc2 DKO cords confirmed the loss of RCs seen at E12.5, with the RC area being largely devoid of MafB’ calbindin’ cells (Fig. 4N,Q). It therefore appears that although V1 cells can initiate the RC differentiation program in the absence of Oc1 and Oc2, albeit less efficiently, the RC differentiation program is not maintained and RCs are depleted from E12.5 onwards.

**Foxd3 functions in parallel to Oc1 and Oc2 to control RC differentiation**

The observation that Foxd3 is expressed in the newborn V1 cells as they emerge from the ventricular zone (Fig. 2), prompted us to ask whether Foxd3 is required to initiate and execute the RC differentiation program. Mouse embryos that lack Foxd3 die at ~E7 (Hanna et al., 2002), well before V1 interneurons are born and differentiate. To circumvent this, a Neurogenin-1::Cre (Ngn1::Cre) transgene was used in combination with a conditional (floxed) Foxd3f/f (Teng et al., 2008) to inactivate Foxd3 in V1 interneurons and their progenitors. When monitoring Foxd3 protein expression in Ngn1::Cre; Foxd3f/f embryos, we observed a complete loss of Foxd3 in the V1 domain (supplementary material Fig. S3), which indicates that the Foxd3 gene is efficiently inactivated in V1 progenitors.

Inactivation of Foxd3 in V1 progenitors with Ngn1::Cre resulted in a marked loss of RCs (Fig. 5). In particular, the number of Oc2’ calbindin’ V1 interneurons at E10.5 was strongly reduced in Ngn1::Cre; Foxd3f/f embryos compared with age-matched control embryos (Fig. 5A,B,M), and the number of MafB-expressing cells in the V1 domain was also reduced (Fig. 5C,D). At E12.5, the loss of calbindin’ RCs was even more pronounced (Fig. 5E,F,M). Even though Oc2’ calbindin’ cells were occasionally observed in sections derived from Ngn1::Cre; Foxd3f/f cords (Fig. 5F, white arrowheads), these cells were typically located outside of the RC area (Fig. 5F, open arrowheads). MafB’ calbindin’ cells were also absent from the RC area (Fig. 5G,H) in the E12.5 Ngn1::Cre; Foxd3f/f cord. The depletion of identifiable RCs in
**Foxd3** mutant cords was even more pronounced at E16.5, where few, if any, calbindin+ cells were present in the RC area (Fig. 5I-M). From these data we conclude that Foxd3 activity functions during the early phase of V1 genesis (i.e. prior to E10.5) to induce expression of the RC-specific factors MafB and calbindin.

Although the Foxd3-dependent loss of MafB and calbindin expression points to an early loss of RCs (Fig. 5), we were still able to find cells that express Oc2 alone in the RC area at the border of lamina IX and the white matter (Fig. 5F,H, open arrowheads). This suggests that V1 cells possessing the migratory attributes of RCs are still present in the **Foxd3**-null cord, even though they no longer express MafB and calbindin. By E16.5, the RC area was found to be largely devoid of Oc2+ cells (Fig. 5I-M), indicating that Oc2 is either extinguished in these cells or the cells themselves are lost from the **Foxd3**-null cord. In experiments using an En1-dependent lineage tracer in combination with the **Foxd3**f/f allele (i.e. En1Cre;**Foxd3**f/f;R26loxstop-HTB) we observed a large reduction in GFP+ cell numbers in the RC area (data not shown). Whether this reduction reflects a loss in RCs is not clear, as En1 expression is itself regulated by Foxd3.

The observation that Oc2 (and Oc1) continue to be expressed in cells that have the migratory characteristics of RCs demonstrates **Foxd3** does not regulate Onecut transcription factor expression in RCs. This conclusion is consistent with the contemporaneous onset of Oc1/Oc2 and Foxd3 protein expression in newborn V1 cells (Fig. 2). The Oc1/Oc2 and Foxd3 regulatory pathways do, however, converge to regulate MafB and calbindin expression in differentiating RCs. Foxd3 is required for the initiation of MafB and calbindin expression in presumptive RCs, whereas Oc1 and Oc2 function at later developmental times to maintain MafB and calbindin expression in RCs.

Although Oc2+, MafB+ or calbindin+ cells were no longer found in the RC area at E16.5 or later, we could not rule out the possibility that RCs downregulate their markers but still remain functional. Physiologically, RCs are defined by their ability to mediate recurrent inhibition of motor neurons (Eccles et al., 1954; Windhorst, 1996). Therefore, we investigated whether RC potentials can be evoked in motor neurons from Ngn1::Cre;**Foxd3**f/f cords (Fig. 5N,O). As expected, whole cell intracellular recordings of motor neurons (n=8) from wild-type
E18.5/P0 spinal cords revealed robust inhibitory potentials following antidromic stimulation (Fig. 5N, control trace), that were blocked with strychnine and bicuculline. By contrast, inhibitory potentials were not elicited in motor neurons \( (n = 22) \) in E18.5/P0 \( \text{Ngn1::Cre; Foxd3}^{f/f} \) cords, (Fig. 5N,O), even at stimulus strengths twice those used in control experiments. This leads us to conclude that the recurrent RC-motor neuron circuit is no longer operative in the \( \text{Foxd3} \) mutant cord, and that this is most likely to be due to the aforementioned defect in RC differentiation.

\( \text{Foxd3} \) is broadly expressed in all V1 interneurons as they are being born and is maintained at high levels specifically in differentiating RCs at later times (Fig. 1; supplementary material Fig. S1). This raised the possibility that \( \text{Foxd3} \) activity might also be required in postmitotic V1 cells to execute and consolidate the RC differentiation program. To examine this issue more closely, we set out to inactivate \( \text{Foxd3} \) with a time delay. An \( \text{En1}^{\text{Cre}} \) knock-in allele was used for this purpose, as \( \text{En1} \) expression in V1 interneurons follows that of \( \text{Foxd3} \) (Fig. 2). This delay in Cre recombination results in the transient expression of \( \text{Foxd3} \) in newborn V1 cells in the \( \text{En1}^{\text{Cre}}; \text{Foxd3}^{f/g} \) cord (supplementary material Fig. S4A,B). When examining the spinal cords of \( \text{En1}^{\text{Cre}}; \text{Foxd3}^{f/g} \) mutants, we observed a marked reduction in the number of \( \text{MafB}^+ \) and calbindin\(^+ \) V1 cells in the RC area from E12.5 onwards (supplementary material Fig. S4C-G). Other transcription factors that are expressed in V1 interneurons, such as \( \text{Pax2} \) and \( \text{Foxp2} \), were less affected (supplementary material Fig. S4G). As was seen with \( \text{Ngn1::Cre} \)-mediated \( \text{Foxd3} \) inactivation, cells expressing \( \text{Oc2} \) were still present in the RC area at E12.5, indicating that a subset of V1 cells in the \( \text{En1}^{\text{Cre}}; \text{Foxd3}^{f/g} \) cord retain the migratory features of RCs. However, the depletion of \( \text{MafB} \) and calbindin expression in the RC area indicates that these cells do not maintain a differentiated RC phenotype. Taken together, these results indicate that RCs are particularly sensitive to the postmitotic loss of \( \text{Foxd3} \), and that \( \text{Foxd3} \) is necessary to maintain the RC developmental program after cell fate determination has occurred.

**MafB is required at late developmental times for RC survival**

The loss of RC \( \text{MafB} \) expression in \( \text{Oe1}/\text{Oe2} \) and \( \text{Foxd3} \) mutant mice prompted us to assess the function of \( \text{MafB} \) in developing RCs. \( \text{MafB} \) is known to restrict progenitor cell commitment and differentiation in a number of cellular contexts (Sieweke et al., 1996; Sadl et al., 2002), including the development of NK1R\(^+ \) neurons in the pre-Bötzinger complex (Blanchi et al., 2003). In examining spinal cords taken from E10.5-15.5 \( \text{MafB} \) mutant animals, we observed a normal complement of RCs (data not shown; Fig. 6). This suggests that \( \text{MafB} \) is not required for RC differentiation per se. E18.5 and P0 mutants did, however, exhibit a marked loss of calbindin-expressing cells in the RC area (Fig. 6A,B,I). A parallel but less pronounced reduction in the number of \( \text{Oc2}^+ \) RCs was also noted in these cords (Fig. 6K).
The reduction in calbindin+ RCs in the MafB mutant cords was even more pronounced at P0 (Fig. 6E-I), indicating RCs are progressively depleted from these cords at late gestational times. Unfortunately, we were not able to document any further loss of RCs in these cords at postnatal times, as MafB mutant pups are unable to breathe actively and die soon after birth (Blanchi et al., 2003).

Although the use of MafB<sup>GFP</sup> and En<sup>Cre</sup>;R26<sup>floxstop-TM</sup> reporters confirms the loss of RCs in the MafB mutant cord, efforts to detect apoptotic cells by TUNEL and Caspase 3 staining in the RC area were not successful (data not shown). However, we did observe an increase in the number of GFP-expressing V1 cells within the RC area that lacked calbindin expression (Fig. 6B,H,J), which suggests that calbindin is prematurely downregulated in RCs when MafB is absent. This downregulation of calbindin that occurs in the MafB mutant cord prompted us to ask whether RCs might be dying by an excitotoxic mechanism brought about by the loss of this calcium-buffering protein.

Examined, normal numbers of RCs were found throughout the spinal cord at P0 (data not shown). It is therefore unlikely that downregulation of calbindin alone causes the loss of RCs in MafB mutant animals. In a further test to see what contribution excitotoxicity makes to the loss of RCs in the MafB mutants, the MafB mutation was placed on a Nes::Cre;R26<sup>floxstop-TeNT</sup> background in order to abolish neural activity in the MafB mutant cord (Fig. 7) (Zhang et al., 2008). No rescue of RCs was observed, indicating that their depletion in the MafB mutants is unlikely to be due to an excitotoxic mechanism (data not shown).

Although RCs are reduced in the MafB mutant cord, it was not clear whether the remaining cells retain their ability to function as RCs. We therefore examined whether the recurrent inhibitory pathway between motor neurons and RCs is functionally intact in the MafB mutant cord. Whole-cell recordings from motor neurons at E18.5 revealed robust inhibitory potentials in the MafB mutant cord following antidromic stimulation of peripheral motor axons (Fig. 6K). Consequently, those RCs remaining in the MafB mutant

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Fig. 7. Renshaw cell development proceeds normally in the absence of neural activity. (A) Ventral root recordings from control (R26<sup>floxstop-TeNT</sup>) and Nes::Cre;R26<sup>floxstop-TeNT</sup> mouse embryos at E18.5 showing spontaneous burst of activity during a 2 minute period of recording. Bursts of activity (asterisks) are present in the control cord (upper traces), and their frequency is increased in the presence of strychnine. Nes::Cre;R26<sup>floxstop-TeNT</sup> cords show no complex burst activity, nor is activity enhanced by the addition of strychnine (lower traces). (B) Quantification of complex bursts in control (-Cre) and Nes::Cre;R26<sup>floxstop-TeNT</sup> (+Cre) cords. (C-F) Expression of calbindin (green), Oc2 (red; C,D) and MafB (red; E,F) in the RC area (indicated by dashed line) at E18.5. (G,H) Calbindin+ (green) nerve terminals contact the soma of VACH<sup>T</sup> motor neurons (arrowheads) in both control and activity-deficient spinal cords. (I,J) VACH<sup>T</sup> contacts are present on RCs (arrowheads). (K) Quantification of calbindin+ contacts on motor neurons and VACH<sup>T</sup> contacts on RCs. Error bars represent s.e.m.
are still able to provide recurrent inhibition to motor neurons. In summary, our results show that MafB does not control the formation of functional recurrent connections between RCs and motor neurons, but it is required for persistent calbindin expression in RCs and for RC viability.

**RCs differentiate in the absence of evoked neurotransmission**

Studies in the *Xenopus* and zebrafish larval cord have described roles for activity in regulating the neurotransmitter and transcription factor profiles of embryonic spinal neurons (Borodinsky et al., 2004; McDearmid et al., 2006); however, the contribution that activity makes to the differentiation of spinal neurons in other vertebrate animals, including mammals, remains unclear. In light of this ambiguity, we investigated whether RC differentiation is altered when the tetanus toxin light chain fragment (TeNT) is expressed throughout the embryonic cord (Fig. 7). We first confirmed that there is little or no spontaneous activity in Nes::Cre;R26Rfoxstop-TeNT spinal cords by recording ventral root activity in the isolated spinal cord preparation (Hanson and Landmesser, 2003; Zhang et al., 2008). Spontaneous bursts of complex burst activity were completely lost from these spinal cords, even when synaptic inhibition was blocked with strychnine (Fig. 7A,B). These results are in accordance with our previous demonstration that evoked rhythmic activity is abrogated in the Nes::Cre;R26Rfoxstop-TeNT spinal cord (Zhang et al., 2008). Close examination of the expression patterns of a number of RC markers in the Nes::Cre;R26Rfoxstop-TeNT spinal cord revealed no differences in the location and number of RCs or RC markers between E18.5 Nes::Cre;R26Rfoxstop-TeNT spinal cords and age-matched controls (Fig. 7C-F). This suggests that the program of RC differentiation is able to proceed normally in the absence of spontaneous or evoked neural activity.

We then investigated whether RCs still receive and make synaptic contacts with motor neurons. When cholinergic synaptic contacts that are derived from motor neurons were examined, there was no change in the overall number of vesicular acetylcholine transporter (VACHt+) boutons or their distribution on the soma and proximal dendrites of RCs (Fig. 7G,H,K). Likewise, calbindin+ RC contacts were found on motor neurons in normal numbers (Fig. 7I-K). It therefore appears that motor neurons and RCs still form selective anatomical contacts with each other in the absence of neuronal activity in the cord. As the current system for inactivating neurons only allows us to express TeNT constitutively, we cannot assess the electrophysiological properties of these synaptic contacts. Nonetheless, our data do show that neuronal activity is not required during development for RCs and motor neurons to recognize each other and form ‘structural’ synaptic connections.

**DISCUSSION**

This study reveals the existence of a temporal developmental program that controls the genesis of RCs from V1 progenitors. In so doing, it provides novel insights into the mechanistic logic that generates interneuronal diversity in the vertebrate nervous system. First, it demonstrates that a genetically hardwired program controls the production of RCs within the V1 class. Second, it identifies a number of key transcriptional determinants that control the RC differentiation program. Third, it assesses the contribution that neuronal activity makes to the differentiated phenotype of RCs. Lastly, our results suggest that V1 interneurons are pre-determined with respect to their specialized identity at the time of their terminal mitosis.

**A temporal developmental program controls Renshaw cell differentiation**

Our expression analysis and birthdating of embryonic V1 interneurons reveals a strict timing mechanism that underlies the specification of V1 subtypes. RCs are the first V1 interneurons to be born with other V1-derived subpopulations appearing at later times. Interestingly, sequential production of molecularly distinct interneuron subtypes is also observed for dI6 and V2 spinal interneurons (M.G., unpublished). This leads us to posit that temporal coding represents a fundamental mechanism that operates in embryonic neural populations to generate neuronal diversity in the vertebrate spinal cord.

Developmental timing mechanisms also regulate neuronal progenitor competence elsewhere in the CNS, most notably in the cortex and eye (Marquardt and Gruss, 2002; Molyneaux et al., 2007; Okano and Temple, 2009). In these instances, however, particular neuronal subtypes are generated in broadly overlapping cohorts, such that different cell types are often produced simultaneously (Marquardt and Gruss, 2002; Miyoshi et al., 2007). By contrast, RCs are generated within a relatively narrow developmental time frame from the V1 progenitor domain, and for part of this time appear to be the only cell type produced. Consequently, the timing of RC differentiation is suggestive of neuronal diversity being generated by stepwise changes in progenitor potential rather than the graded changes in competence that operate in the forebrain and retina (Rapaport et al., 2004; Agathocleous and Harris, 2009). It remains to be determined whether the mechanisms that generate neuronal diversity in the embryonic spinal cord are akin to temporal feedforward/feedback transcriptional pathways that control neurogenesis in the *Drosophila* ventral nerve cord (Pearson and Doe, 2004; Baumgardt et al., 2009).

**Oc1/Oc2, MafB and Foxd3 act as cell-type specific regulators of neural development**

This study identifies Oc1/Oc2, Foxd3 and MafB as factors that control and maintain RC fate assignment in postmitotic V1 interneurons. Oc1/Oc2 and Foxd3 are required during the early phase of RC development, whereas MafB functions at later times to maintain RCs. All four factors have been implicated in the regulation of cellular differentiation. Oc1 and Oc2 are known for their role in liver development, where expression is required both for migration of early hepatoblasts and for the proper segregation of these progenitors into the hepatocytic and biliary cell lineages (Clotman et al., 2005; Margagliotti et al., 2007). In the pancreas, Oc1 promotes multipotent progenitor cells to enter the endocrine lineage (Zhang et al., 2009). Oc1 and Oc2 are also expressed in developing motor neurons. Initially, Oc1 is expressed in all newborn motor neurons, whereas at later times it is restricted to subsets of neurons in each of the somatic motor columns (Francius et al., 2010). This expression profile suggests that Oc1 might play a role in motor neuron subtype specialization that is similar to the one that we observe for Oc1/Oc2 in RCs.

The function of MafB in the CNS has been explored in two contexts. Loss of MafB expression in the hindbrain of Kreisler mice leads to the loss of rhombomeres 5 and 6 (McKay et al., 1994; Eichmann et al., 1997). MafB is also expressed in postmitotic neurons of the pre-Bötzinger complex, which controls breathing, and is required for the proper formation and function of this structure (Blanchi et al., 2003). Our results indicate that it plays a similar role in Renshaw cells, although the molecular mechanisms by which MafB deficiency causes defects in NK1R+ pre-Bötzinger neurons and RCs remain to be elucidated. In the
developing nervous system, Foxd3 regulates neural crest multipotency and self-renewal (Mundell and Labosky, 2011). Overexpression of Foxd3 in the chick spinal cord inhibits neuronal differentiation and leads to excess production of neural crest cells (Dottori et al., 2001). In this study, we show that Foxd3 plays an additional role in the differentiation of postmitotic V1 neurons. Foxd3 is also expressed in postmitotic dI2 interneurons in the dorsal cord (Gross et al., 2002); however, its role in dI2 interneuron development has not been investigated. In summary, our results reveal new roles for Oc1/Oc2 and MafB in the development of RCs, and they assign a novel role to Foxd3 in postmitotic neurons by demonstrating that it functions as a key regulator of RC differentiation.

**Activity versus intrinsic determinants: relative contribution to Renshaw cell specification**

The role that neuronal activity plays in the specification of spinal neurons and their assembly into functional circuits is still controversial. Whereas studies in *Xenopus* and zebrafish suggest early roles in regulating neuronal differentiation, transcription factor expression and neurotransmitter synthesis (Borodinsky et al., 2004; McDearmid et al., 2006; Marek et al., 2010), analyses in higher vertebrates point to later roles in axon pathfinding and synapse formation/maturity (De Marco Garcia et al., 2011; Hanson and Landmesser, 2004; Blankenship and Feller, 2010). In examining this issue in RCs, we found that *Nes::Cre;R26*loxstop-lacZ/Tm1 animals express a normal profile of RC differentiation markers, demonstrating that large parts of the RC differentiation program proceed normally in the absence of spontaneous activity.

Cholinergic and GABAergic transmission contribute to spontaneous activity in the cord (O’Donovan et al., 1998; Milner and Landmesser, 1999; Myers et al., 2005), and the avian equivalents of RCs, R-interneurons, are active at early stages (Wenner and O’Donovan, 2001). These observations point to a role for activity in shaping the circuits in which RCs participate. Our finding that Renshaw cells still form contacts, which are characteristic of early synaptic connections, with motor neurons and are contacted by motor axon collaterals (Fig. 7), indicates that the cellular processes controlling RC target recognition and selection still operate in the absence of neural activity. However, it is less clear whether RCs retain their selective pattern of functional connections with homonymous and synergistic motor neurons in the absence of neural activity (Windhorst, 1996).

Consequently, there remains a distinct possibility that activity contributes to the refinement of RC-motor neuron connections, which would be consistent with the demonstrated roles of activity elsewhere in the developing nervous system (Blankenship and Feller, 2010).

**Initial cell fate specification of V1 progenitors**

Although our studies indicate that the RC differentiation program is initiated in V1 progenitors, the instructive signals that activate this program have not been identified. The Notch ligand Jag1 is transiently expressed in V1 progenitors (Matise and Joyner, 1997) and, although Notch signaling is a crucial regulator of neurogenesis (Pierfelice et al., 2011), our analyses of both presenilin and Jagged1 knockout spinal cords (supplementary material Fig. S5), along with the studies of Marklund et al. (Marklund et al., 2010) and Ramos et al. (Ramos et al., 2010) largely rule out Notch signaling in V1 subtype generation. This stands in contrast to V2 neurogenesis for which Dll4-dependent Notch signaling in the p2 domain regulates the choice between V2a and V2b interneuron cell fates (Del Barrio et al., 2007; Peng et al., 2007).

Other candidate regulators that are expressed in the V1 progenitor domain are the Pax6 and Nkx6-2 transcription factors, both of which control the expression of En1 in V1 interneurons (Burrill et al., 1997; Ericson et al., 1997; Osumi et al., 1997; Vallstedt et al., 2001). Although Pax6 functions to establish the p1 domain and control V1 interneuron specification, it does not regulate V1 subtype diversity (Sapir et al., 2004) (T.J.H. and M.G., unpublished). Nkx6-2, which is selectively expressed in V1 progenitors between E9 and E9.5, was reported to be necessary for V1 interneuron development (Vallstedt et al., 2001). Although we confirmed that En1+ neurons are absent from the cord at rostral levels in the *Nkx6-2* mutant, calbindin+ RC and Foxp2+ V1 interneurons are generated normally, with no appreciable alteration in cell numbers for both populations (supplementary material Fig. S6). We posit that Nkx6-2 regulates the expression of En1, rather than functioning as a temporal determinant of V1 identity. En1, in turn, controls V1 axon guidance and target selection, but not the development of V1 subtypes (Saueressig et al., 1999; Sapir et al., 2004) (Fig. 8).

In summary, this study reveals the existence of a temporal program that generates neuronal diversity within the spatially defined V1 class of spinal interneurons. Similar sequential patterns of interneuron subtype generation are seen in other spinal interneuron classes, raising the possibility that the combination of spatial and temporal determinants might be the pre-eminent mechanism for generating neuronal diversity in the spinal cord.

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