A Hox Code Defines Spinocerebellar Neuron Subtype Regionalization

Highlights

- *Hox*-microRNA reporters allow in vivo characterization of spinocerebellar neurons
- *Hox9-Hox11* paralog expression defines axially restricted spinocerebellar neurons
- Hoxc9 activity is essential for neuron subtype identity at thoracic levels
- Molecular heterogeneity is evident within Clarke’s column

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In Brief

Coughlan et al. utilize fluorescent reporter mice to characterize the projection patterns of embryonic and adult spinocerebellar neurons (SCNs). Within the spinal cord, axially restricted SCNs are delineated by their unique repertoire of posterior *Hox*-cluster gene expression, and a requirement for Hox function in SCN subtype regionalization is demonstrated.
A Hox Code Defines Spinocerebellar Neuron Subtype Regionalization

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SUMMARY

Coordinated movement requires the integration of many sensory inputs including proprioception, the sense of relative body position and force associated with movement. Proprioceptive information is relayed to the cerebellum via spinocerebellar neurons, located in the spinal cord within a number of major neuronal columns or as various scattered populations. Despite the importance of proprioception to fluid movement, a molecular understanding of spinocerebellar relay interneurons is only beginning to be explored, with limited knowledge of molecular heterogeneity within and between columns. Using fluorescent reporter mice, neuronal tracing, and in situ hybridization, we identify widespread expression of Hox cluster genes within spinocerebellar neurons. We reveal a “Hox code” based on axial level and individual spinocerebellar column, which, at cervico-thoracic levels, is essential for subtype regionalization. Specifically, we show that Hoxc9 function is required in most, but not all, cells of the thoracic spinocerebellar column, Clarke’s column, revealing heterogeneity reliant on Hox signatures.

INTRODUCTION

The human body navigates movement with astonishing success, displaying locomotor actions that are fluid and coordinated. Proprioceptive sensory neurons (PSNs), various subclasses of which are located within muscles, tendons, and joints, convey information to the central nervous system regarding changes in muscle length and muscle force (Prosk and Gandevia, 2012). This input, along with the perception of skin deformation sensed by cutaneous receptors (Shambes et al., 1978), provides a constant awareness of the relative positioning of body components such as the limbs. In humans, proprioception declines with age (Goble et al., 2009) and in numerous disease states (Borchers et al., 2013; Conte et al., 2013), manifesting as irregular movement trajectory, exaggerated force, and postural instability (Goble et al., 2009). In mice, the ability to selectively ablate PSNs has revealed their essential contribution to locomotor pattern generation (Akay et al., 2014), maintenance of spinal alignment (Blecher et al., 2017a), and the realignment of long bones following fracture (Blecher et al., 2017b).

Peripheral information detected by PSNs is processed via both local and long-range neural circuits. Locally, PSNs from the muscle spindle (type Ia afferents) or the Golgi tendon organ (type Ib afferents) form monosynaptic or disynaptic connections, respectively, with α-motor neurons, which innervate the same, or functionally similar, muscle groups from which the sensory information arose (Bikoff et al., 2016; Mears and Frank, 1997; Ozaki and Snider, 1997). In contrast to this relatively well-characterized local spinal reflex circuit, the long-range circuits for higher-order integration prior to motor output modulation have received less attention, particularly in mice. Early studies in cat have shown that types Ia, Ib, and II muscle PSN afferents synapse on spinocerebellar (SC) neurons (SCNs) of the spinal cord that send axonal projections directly to the cerebellar cortex via spinocerebellar tracts (SCTs) (Aoyama et al., 1988; Edgley and Jankowska, 1988). The more recent topographic mapping of SC projections to the cerebellar cortex in mouse (Sengul et al., 2015), which is broadly consistent with extensive anatomical literature in other species (Grant and Xu, 1988; Grant et al., 1982; Matsushita and Hosoya, 1979), provides a comprehensive understanding of where in the mouse spinal cord SC neurons reside. This study defined five axially restricted populations spanning the cervical (central cervical nucleus [CeCV]), thoracic (dorsal nucleus, here referred to as Clarke’s column [CC]), lumbar (lumbar precerebellar nucleus [LPPrCb]), and sacral (sacral precerebellar nucleus [SPPrCb]) regions, and four scattered populations spread along the rostro-caudal (R-C; top to bottom) extent of the spinal cord within the deep dorsal horn (DDH) and laminae VI-VIII (Sengul et al., 2015). These populations are of mixed embryonic origins, with a small subset of CC cells and various scattered populations deriving from an Atoh1-positive...
lineage (Bermingham et al., 2001; Yuengert et al., 2015), others from a Neurog1-positive lineage (Sakai et al., 2012), and the remaining majority of SCNs of unknown origin. How these mixed-lineage populations are divided among the regional nuclei of the SC system is currently unknown.

The Hox transcription factors are fundamental regulators of development across bilateria, endowing cells with the positional information required for region-specific morphology and functionality (Wellik, 2009). In mouse and human, 39 Hox genes are subdivided into 13 paralogous groups (Hox1-13) based on evolutionary descent, with varying subsets of the 13 paralogs genomically clustered at four distinct loci (HoxA–D clusters). Transcription from each Hox cluster is, however, not limited to Hox protein-coding genes. A vast array of noncoding transcripts are also produced, including microRNAs and long-noncoding RNAs (reviewed in Casaca et al., 2018). Developmental expression of these noncoding RNAs is largely consistent with the ordered spatio-temporal activation of Hox gene expression that occurs from the 3′ to 5′ ends of each cluster (Mansfield and McGlinn, 2012; Mansfield et al., 2004; Wong et al., 2015). Moreover, important developmental functions have been ascribed to many of these Hox-embedded noncoding RNAs (reviewed in Casaca et al., 2018; Heimberg and McGlinn, 2012), including a striking homeotic role for the miR-196 family of microRNAs (McGlinn et al., 2009; Wong et al., 2015).

The central importance of Hox genes in imparting positional identity is particularly apparent in the developing nervous system, where cell-intrinsic mechanisms dictating migration and connectivity can change dramatically dependent on the Hox code of a neuron (Bechara et al., 2015; Gavalas et al., 1997; Hayashi et al., 2018; Mark et al., 1993; Oury et al., 2006; Studer et al., 1996; reviewed in Philippidou and Dasen, 2013). Prominent examples of this in the spinal cord are the generation of axially restricted motor neuron (MN) columns, which harbor neurons that project to broad muscle target regions (e.g., limb versus axial musculature), and the more restricted MN pools within these columns, which target specific muscles within a region. A combinatorial Hox code correlates with the axial restriction of column and pool subtypes and, moreover, functionally defines their appropriate connectivity (Dasen et al., 2003, 2005; reviewed in Philippidou and Dasen, 2013). The most striking example is seen following the loss of Hoxc9, where the trunk-specific hypaxial motor column is completely lost and the lateral motor column, normally specific to brachial and lumbar levels, subsequently extends along the entire intervening R–C axis (Jung et al., 2010). Pool identity is also affected in this and other Hox mouse knockouts, with neurons displaying random organization within a motor column, often projecting to more anterior muscle targets (Jung et al., 2010; Wu et al., 2008).

Here, we describe widespread expression of Hox-cluster genes throughout SC neurons of the thoracic, lumbar, and sacral regions. This expression is seen from early embryonic stages through to late adulthood, indicating their potential to function in the genesis and homeostasis of SC circuits. We find that the spatial regionalization of Hox code along the R–C axis parallels the anatomical subdivision of SC neurons within the spinal cord, and that loss of an individual Hox gene leads to a dramatic reduction of an axially restricted SC subtype. This work suggests a commonality in spinal cord patterning mechanisms across diverse neuron types and provides critical molecular tools that allow early and comprehensive visualization of SC development.

RESULTS

Posterior Hox-Cluster Reporter Expression Is Observed in the Adult and Developing Cerebellum

We have previously generated a mouse knockin reporter line that drives expression of EGFP from the endogenous miR-196a2 locus, genomically positioned between Hoxc9 and Hoxc10 (referred to as 196a2-eGFP) (Wong et al., 2015). Detailed characterization of this reporter line revealed unexpected EGFP protein within the adult and neonatal cerebellum, far outside the posteriorly biased pattern of expression one would expect from a gene located within the posterior HoxC cluster.

Analysis of 196a2-eGFP by immunofluorescence at postnatal day (p) 28 revealed a highly reproducible and restricted pattern of expression within the granule-cell layer of cerebellar lobules I–V, VIII, and IX (Figure 1A), forming rosettes characteristic of mossy fiber synaptic terminals, and clustering in parasagittal stripes as shown in a coronal section (Figure 1B; summarized in Figure 1C). The observed rosettes, lobule restriction, and parasagittal banding correspond closely with published literature on the terminal projection pattern of SC neurons in mouse (Reeber et al., 2011). One notable exception to this was reproducible EGFP expression on the ventral side of lobule IX in the most posterior region (Figure S1A), a site commonly associated with vestibular input, that we find no description of in any historical SC anterograde tracer literature. However, the lack of EGFP detection within vestibular nuclei (Figure S1B), together with the results of more recent single SC axon tracing (Luo et al., 2018), support our identification of EGFP at this site as originating from a SC projection. The absence of EGFP expression in cerebellar nuclei (Figure S1C) indicates these 196a2–expressing neurons do not send collaterals to this location, as has been observed for some SC neurons in rat (Matsushita and Gao, 1997; Matsushita 1999a, 1999b; Matsushita and Xiaoming, 1997; Matsushita and Yaginuma, 1993) and mouse (Luo et al., 2018). This pattern of EGFP expression was maintained from neonatal through to late adult life (Figures S1A and 1D–1F), with expression of EGFP observed in fibers of passage in two distinct tracts corresponding to the location of the ventral and dorsal SC tracts (VSCT and DSCT, respectively; Figure S1D), and the stereotypical parasagittal banding readily apparent at 1 week of age. At all stages, EGFP protein did not appear perinuclear within local cerebellar cells as determined by DAPI staining (data not shown), nor was EGFP mRNA detected by in situ hybridization within the cerebellum (data not shown), supporting the notion that 196a2-eGFP-expressing cells originate outside the cerebellum, with EGFP protein filling axonal projections and synaptic terminals. Parallel characterization of a second reporter line, miR-196a1-eGFP, which drives EGFP expression from the paralogous gene locus of the HoxB cluster, also revealed expression indicative of adult SC neurons (Figure S2).

To investigate the potential origin of cerebellar 196a2-eGFP expression, we characterized reporter expression within the adult spinal cord. At p28, EGFP displayed region-specific expression in cell bodies along the R–C axis within, though not
Immunofluorescent detection of EGFP protein in adult and embryonic miR196a2-eGFP reporter mice.

(A) Sagittal section at p28 reveals lobule-restricted EGFP in the cerebellum.

(B) Coronal section at p28 reveals parasagittal banding of EGFP in the cerebellum, indicated by yellow arrowheads.

(C) Cartoon composite of EGFP expression along the A-P axis of the cerebellum, based on complete coronal series from three p28 animals.

(D and E) Cross sections of E12.5 (D) and E15.5 (E) embryos reveal axially restricted EGFP expression along the A-P axis. For each series, cartoon embryos indicate the axial level where sections were imaged (dotted line) and the right panel provides a magnified view of the neural tube. The red bracket indicates the location of cell bodies and the orange bracket indicates the location of SC tracts.

(F) Sagittal section of an E15.5 embryo reveals EGFP-positive cell bodies in the posterior embryo and EGFP-positive neural tracts extending rostrally into the developing brain. Boxes represent regions of interest where higher magnification images in the bottom panels were taken.

Scale bars in (A), (B), and (F), 500 μm; scale bars in (D) and (E), 100 μm. Roman numerals I-X indicate lobules of the cerebellum. Expression in all panels is representative of expression observed for ≥3 animals. See also Figures S1, S2, and S3.
exclusive to, regions previously defined as harboring SC neurons. Expression was visible from approximately thoracic (T) spinal segment T3, in large scattered neurons of laminae 5-7 and small neurons in lamina 1 (Figure S3). The number of neurons in the medial region increased more posteriorly, with a clustered population becoming apparent in the region of CC (Figure S3). Fiber bundles positive for EGFP were observed in the region of the ascending DSCT and VSCT along the entire R-C extent of the spinal cord, particularly evident at upper thoracic and cervical levels where EGFP-positive cell bodies were not present (Figure S3).

Knowledge of the molecular signatures defining collective and individual SC neurons is scarce, a fact that underpins the current lack of molecular tools to visualize ascending SC axonal projections during development. To determine whether our 196a2-eGFP reporter mouse can be used to visualize the genesis of SC circuitry, we characterized EGFP expression at embryonic stages corresponding to time points when SC axons begin to ascend toward (embryonic day 12.5 [E12.5]), or enter (E15.5), the cerebellar primordium. In the E12.5 neural tube, we observed a small number of medially located cell bodies defining the rostral limit of EGFP expression, with expression becoming less restricted caudally (Figure 1D). We observed ventrally biased fiber bundles up to the level of the forelimb/heart (Figure 1D). As development proceeded, these fiber bundles were seen at more rostral levels, and by E15.5 we could detect EGFP-positive tracts along the entire rostral spinal cord and hindbrain (Figures 1E and 1F) with a large majority of these fibers entering the cerebellar primordium (Figure 1F, inset 1). Together, the striking expression revealed by these reporter lines allow us to propose that Hox-cluster genes are expressed in SC neurons during both genesis and adult homeostasis.

miR-196a2-eGFP Is Dynamically Expressed across SC Neuronal Columns

We next investigated whether 196a2-eGFP was expressed throughout all major SC nuclei and scattered populations, or was restricted to subpopulations. To confirm that 196a2-eGFP is in fact localized to SC neurons, we performed dual immunofluorescent staining for EGFP alongside the vesicular glutamate transporter VGLUT2, which is strongly expressed in, though not exclusive to, SC terminals (Gebre et al., 2012). Indeed, all EGFP-expressing rosettes in the granule-cell layer of p28 animals co-labeled with VGLUT2 (Figures 2A–2E). Given the current paucity of molecular markers defining individual SC nuclei and scattered populations, we next performed retrograde tracing on 196a2-eGFP postnatal pups. A glycoprotein-deleted rabies virus expressing the mCherry fluorescent reporter (Rabies-∆G-mCherry; Wickersham et al., 2007) was injected into the p2 mouse (n = 1) or p4 mouse (n = 3) 196a2-eGFP cerebellum, and spinal cords were collected for analysis at p7 (Figure S4). Co-localization analysis of spinal cord sections revealed mCherry/EGFP double-positive cells within almost all previously defined SC populations, including CC, LBPr, and SPrC nuclei, and scattered cells within the DDH and BS (Figures 2G–2J; 2L, and 2L*; see STAR Methods). Exceptions to this included the CeCV nucleus, which falls outside the T3-Co3 expression domain of the 196a2-eGFP reporter and thus would not be expected to exhibit mCherry/EGFP dual positivity. Additionally, the LPPrC nucleus lacked any evidence of mCherry/EGFP dual positivity (Figures 2K, 2K*, and 2M). This latter observation was unexpected in light of confirmed EGFP expression in adjacent SC populations both rostral and caudal to the LPPrC nucleus (Figures 2H, 2H*, 2L, and 2L*), and even within LBPr and scattered SC cells of the same or similar axial levels (Figures 2I, 2I*, 2J, and 2J*). This supports the presence of specific molecular signatures across SC populations that do not rely solely on axial level, though additional support for this is required given the technical limitations of this tracer approach. Focusing on the proportion of mCherry-positive neurons which co-expressed EGFP, we observed considerable variation between the various SC populations. For example, while over 90% of mCherry-positive cells within CC and the SPrC nuclei co-expressed EGFP, most of the scattered populations showed approximately 50% co-expression (Figure 2M). Moreover, a gradient of mCherry/EGFP dual positivity was observed along the R-C extent of CC, ranging from ~30% at upper thoracic levels (T3-T7) compared to ~95% at lower levels (T8-T13) (Figure 2M). Together, these various approaches have allowed us to demonstrate expression of a Hox-cluster gene within SC neurons, with detailed characterization of 196a2-eGFP indicating heterogeneity within and between SC populations.

SC Neurons Display Population-Specific Repertoires of Hox Gene Expression

The miR-196 genes are genomically located in intergenic regions between Hox9 and Hox10 paralogs (Yekta et al., 2004), and their expression conforms to the overarching vertebrate Hox-cluster constraint of collinear temporal activation during embryogenesis (Wong et al., 2015). This prompted us to question whether posterior Hox (Hox9-11) genes are also expressed in this neuronal context. To date, however, there is limited information as to whether Hox9-11 gene expression is maintained in the developing spinal cord throughout the late embryonic and early postnatal stages critical to SC tract formation and circuit establishment. To this end, we performed a section in situ hybridization screen for the Hox9-11 genes and found expression of all 10 genes maintained in the neural tube/spinal cord from E12.5, when SC axons begin ascending rostrally, through to p7, when mature patterns of connectivity with the cerebellum have been established (Figure S5). To assess Hox gene expression specifically within SC neurons, we performed an identical p7 in situ screen following injection of the Rabies-∆G-mCherry retrograde tracer into the cerebellum of p4 wild-type (WT) pups (n = 4) (Figures 3 and S6A). Each Hox9-11 gene was expressed in at least three SC populations, and collectively, each SC population was positive for expression of at least seven Hox genes (data summarized in Figure 3C). Assessing from rostral to caudal, the CeCV nucleus is devoid of Hox9-11, being anterior to most of these genes’ expression domains and a location where we would predict Hox5-8 paralog expression. The subsequent major SC nuclei can be characterized by distinct Hox expression profiles: CC being Hox9 positive in the upper thoracic region, a Hox9/Hox10-positive lower CC and LPPrC, and a Hox9-11-positive SPrC signature. Widespread Hox9-11 expression was seen in...
Figure 2. miR196a2-eGFP Exhibits Heterogeneity within and across SC Neuron Sub-populations

(A–E) Coronal section of a p28 miR196a2-eGFP cerebellum, co-stained for EGFP (green) and VGLUT2 (red) proteins (n = 3/3 animals).

(A and B) Maximum projection images. The boxed region encompassing lobule III in (A) can be seen in higher magnification in (B). I-V = lobules of the cerebellum. Scale bar in (A), 1 mm.

(C–E) Z-projection view of the boxed region in (B). Visualization of EGFP (C), VGLUT2 (D), and a merge of the two (E) demonstrates co-labeling of these proteins at synaptic terminals in three dimensions. Scale bars in (C–E), 50 μm.

(F) A schematic of the retrograde tracer experimental strategy. Approximate locations of sections used for imaging in (G–L) are marked. C, cervical; T, thoracic; L, lumbar; S, sacral; and Co, coccygeal. (G–L) Cross section of p7 miR196a2-eGFP spinal cords following Rabies-ΔG-mCherry retrograde tracer injection at p4. Co-localization analysis of EGFP and mCherry was performed for each major SC nucleus and for various scattered populations from the thoracic level and below. Cartoon diagrams were drawn from adjacent sections stained for acetylcholine esterase. White boxes indicate the location of inset Z-projection views presented for EGFP expression (G0–G00) and merged EGFP and mCherry expression (G00–G000). EGFP expression was observed in each population except the LPrCb (K and K00). Scale bars in (G)–(L), 200 μm; scale bars in (G0–G00) and (G00–G000), 50 μm.

(M) Variation is observed in the number of EGFP-positive cells, presented as a proportion of the total number of retrograde-labeled cells, across major SC nuclei and scattered populations. The mean of 3–4 animals is presented ± SEM. No miR196a2-eGFP expression was detected in CeCV or LPrCb nuclei. Clarke’s column (CC) is split into upper (T1–T7) and lower (T8–L3) portions to highlight the variation observed at different axial levels. The total numbers of cells counted for each population are as follows: CeCV = 17, CC (T1–T7) = 37, CC (T8–L3) = 168, LPrCb = 16, SPPrCb = 17, border cell = 18, and 8Sp = 17.

CeCV, central cervical nucleus; DDH, deep dorsal horn; LBPr, lumbar border cell; LPPrCb, lumbar precerebellar nucleus; PRPrCb, sacral precerebellar nucleus; 8Sp, lamina 8 cell. See also Figure S4.
Figure 3. Hox 9-11 Paralogs Molecularly Delineate SC Subpopulations

An in situ hybridization screen for expression of all Hox9-11 genes in the p7 wild-type (WT) spinal cord following Rabies-△G-mCherry retrograde tracer injection at p4 (n = 4).

(A) The in situ expression pattern of selected Hox mRNAs is presented, demonstrating individual cells co-labelled for a Hox mRNA and mCherry, with axial levels indicated on a cartoon summary of each major SC nucleus in mouse. The location of inset images is demarcated with a white dashed box.

(B) Summary heatmap cataloging the expression of each Hox9-11 gene (rows) within each major SC nucleus or scattered population (columns). The screen was performed on four animals; darker shades indicate the number of replicate animals where co-labelling was observed (see legend at the bottom).

Scale bars, 200 μm (entire spinal cord images) and 100 μm (zoomed in inset). See also Figure S5.
Figure 4. Region-Specific Loss of SC Neurons in Hoxc9⁻/⁻ Mutant Mice

(A) Tracing of cerebellar-projecting neurons following Fluoro-Gold (FG) injection into the adult cerebellum revealed a visible reduction of FG-positive cells in CC of Hoxc9⁻/⁻ animals compared to WT. Scale bars, 500 μm.

(B) Quantification of FG-positive cell number per section at defined axial regions along the spinal cord showed a statistically significant reduction in FG-positive cell number across three regions of CC in Hoxc9⁻/⁻ animals compared to WT, but no change in CeCV, LPrCb, or SPPrCb populations (multiple t tests ± SEM, two-

(legend continued on next page)
scattered populations, with the exception of DDH cells, where no expression of \textit{Hox10} paralogs was detected. Collectively, this analysis has allowed us to define the “Hox code” of major SC nuclei and scattered SC populations, with expression of more 5’ Hox genes demarcating progressively more posterior populations within the SC system.

**Hoxc9 Function Is Essential within a Large Subset of CC Neurons**

Functional analysis of \textit{Hox} genes, and of \textit{Hox}-embedded micro-RNAs such as miR-196, has been challenging due to redundancy between paralogs (McIntyre et al., 2007; Wellik and Capecchi, 2003; Wong et al., 2015). In the context of MN columnar organization, however, Hox9 activity alone is responsible for imparting thoracic identity and restricting limb-innervating lateral motor columns to the fore- and hindlimb levels (Dasen et al., 2003; Jung et al., 2010; Baek et al., 2017). Following detection of \textit{Hoxc9} expression in thoracic-level SC neurons of CC (Figure 3), we proceeded to investigate the effect of \textit{Hoxc9} removal by performing retrograde tracing experiments in \textit{Hoxc9}–/– and WT littermate mice. Fluoro-Gold (FG) tracer was stereotactically injected at six coordinates along the anterior-posterior extent of the cerebellum of 12-week-old mice, with the aim of providing comprehensive and reproducible coverage of the vermis. Following confirmation of reproducibility between animals (Figure S6B), we undertook counts of FG-labeled cells in four major SC populations: CeCV, CC, LPtCbl, and SPRCbl, with counts for CC conducted at three axial levels corresponding to the upper-, mid-, and lower-thoracic spinal cord segments (Figure 4). Comparison of \textit{Hoxc9}–/– and WT spinal cords (n = 4 animals per genotype) revealed no significant change in the number of FG-labeled neurons per section within the CeCV, LPtCbl, or SPRCbl populations (Figure 4B), consistent with their position outside of the known domain of \textit{Hoxc9} functional activity. In contrast, a significant reduction in FG-labeled neuron number within CC was observed in \textit{Hoxc9}–/– animals compared to WT at all three thoracic axial levels assessed (Figures 4A and 4B). Variation in the extent of tracer-positive cell reduction was observed dependent on axial level, with a maximum of ~75% reduction seen at lower thoracic regions (Figure 4B; p < 0.001). It is important to note that our results demonstrate a dramatic reduction in, but not complete abolition of, FG-labeled CC cells in \textit{Hoxc9}–/– mutants. Hox9 function is therefore only required in a subset of CC neurons, in contrast to its requirement for specification of the entirety of thoracic-specific preganglionic and hypaxial motor columns (Jung et al., 2010).

To further investigate retrograde tracing results, we assessed the expression of glial cell line-derived neurotrophic factor (Gdnf). \textit{Gdnf} expression has been used as a marker of CC cell bodies in the spinal cord (Hantman and Jessell, 2010), though it is currently not known what proportion of CC cells express Gdnf, nor the upstream factor(s) driving expression within these cells. Comparison of \textit{Hoxc9}–/– and WT spinal cord sections at E18.5 (Figure 5A; n = 3/3 animals per genotype) and E15.5 (Figure S7A; n = 3/3 animals per genotype) revealed a complete loss of \textit{Gdnf} in mutant embryos. \textit{Gdnf} expression in the kidney remained unperturbed in \textit{Hoxc9}–/– embryos (Figure S7B), a site where Hox11 paralogs are known to directly regulate \textit{Gdnf} expression (Gong et al., 2007), indicating cell-type specificity and flexibility in Hox-dependent upstream regulation. Together, our work has revealed an essential role for Hoxc9 in a large majority of CC cells, and its requirement for the expression of \textit{Gdnf} in this context.

**Hoxc9 Regulates Region-Specific Identity of SC Neurons**

Concomitant with the reduction of FG-positive cells within CC cells in \textit{Hoxc9}–/– embryos, we observed ectopic FG-positive cells outside of CC at the same axial levels, particularly in laminae VI and VII (Figure 4A). The location of these cells was similar to WT retrograde tracer-positive cells immediately rostral of CC but caudal to the major CeCV nucleus that we (Figure S7E) and others have observed (Sengul et al., 2015). This suggests potential expansion of a currently ill-defined brachial-level SC population, which we predict would express a brachial \textit{Hox5-8} code, into more caudal levels. Hoxc9 is known to delineate the posterior boundary of brachial \textit{Hox} expression and cell identity within ventral MNs and interneurons (Dasen et al., 2003, 2005; Sweeney et al., 2018), though the cell-type exclusivity of this regulation is currently not known. Analysis of \textit{Hoxa5} and \textit{Hoxc6} expression in E15.5 (Figures 5A–5H) and E18.5 neural tubes (Figures S7C and S7D) shows widespread de-repression of these genes across much of the \textit{Hoxc9}–/– thoracic spinal cord when compared to WT, suggesting multiple motor and sensory neural networks are likely to be coordinately mis-patterned in Hoxc9 mutants (Baek et al., 2017; Sweeney et al., 2018). With specific focus on SC neurons, we next assessed the expression of \textit{Hoxc6} in adult \textit{Hoxc9}–/– and WT spinal cords following FG retrograde tracer injection. At cervical levels, we observe FG\textit{Hoxc6} double-positive cells in both WT and Hoxc9–/– animals in both the CeCV nucleus (Figures 5I, S7E, and S7F), \textit{Hoxc6} expression was extinguished in WT thoracic-level SC neurons (Figures 5K, 5L, 5M, and 5N), while we continued to observe FG\textit{Hoxc6} double-positive cells within laminae VI-VII of the mid- and lower-thoracic regions (Figures 5L, 5M, 5N, and 5P), of \textit{Hoxc9}–/– animals. It is important to note, that the increase in FG-positive (i.e., cerebellar projecting) cells observed outside of CC in \textit{Hoxc9}–/– animals does not directly correlate with the dramatic decrease in FG-positive cells within CC (Figure 4A). This suggests that while some CC cells change to a more rostral SC fate and settling position...
following removal of Hoxc9, a substantial proportion of Hoxc9-devoid CC cells undergo alternative consequences.

**DISCUSSION**

PSNs provide ongoing information to the central nervous system regarding changes in muscle length, muscle tension, and joint position that is essential for postural stability and controlled movement. These PSNs integrate within both local spinal reflexes and long-range circuits mediated by SC neurons, though the relative contribution of each circuit to motor output modulation remains unclear. This is largely due to the limited molecular understanding of SC circuitry, which has hampered identification of how axially restricted SC populations are established in the early embryo, and has consequently limited the generation of molecular-based tools with which to interrogate the origin, development, and function of this system. Here, we have demonstrated extensive and coordinated expression of Hox-cluster genes within SC neurons (summarized in Figure 6) and the requirement for Hox function in establishing regional specification of SC neuronal columns.

**Coordinated Hox-Cluster Expression Is Observed in SC Neurons**

To date, there is no evidence that posterior Hox-cluster expressing neurons are located or function within the brain. As such, our identification of miR-196a2-eGFP fluorescence within the cerebellum was unexpected. The possibility that posterior Hox-cluster expressing neurons project axons directly to the brain, in some cases traversing almost the entire length of the body, has until now been overlooked. This is because visualization of posterior Hox mRNAs/Hox proteins has concentrated on the cytoplasm/nucleus of soma at posterior axial locations. The use of a fluorescent reporter was critical in allowing us to visualize distal SC neuronal structures and define axonal projection patterns in time and space. We demonstrate expression of miR-196a2-eGFP in rostrally migrating axonal tracts as early as E12.5 and can follow development of this system at all stages. miR-196a2-eGFP expression in this system was constant throughout the entire course of development and adult life, and thus this resource could prove invaluable in revealing the contribution, and timing, of SC dysfunction in various genetic models of disease, such as the SC ataxias.

We have also shown that expression within SC neurons is not limited to miR-196a family members, but encompasses all Hox genes tested to date. This SC “Hox code” parallels remarkably closely what has been observed for axially restricted MN columns. For example, the appearance of CC coincides with that of the preganglionic and hypaxial motor columns. Whether the specification of individual SC and MN progenitors utilize shared mechanisms remains to be determined since the majority of SC progenitors are yet to be identified. Our work has revealed the maintenance of posterior Hox-cluster gene expression in the neural tube and spinal cord well beyond the early- to mid-embryonic stages. This suggests a broader role for Hox genes in the development of the nervous system, which may extend beyond the early axial commitments of SC and MN progenitors.

**Figure 5. Thoracic SC Neurons Display Cervical Hox Identity in Hoxc9−/− Embryos and Adult Mice**

(A–H) In situ hybridization analysis of Hoxa5 (A, B, E, and F) and Hoxc6 (C, D, G, and H) in the E15.5 neural tube reveals expansion of these genes into the thoracic region of Hoxc9−/− embryos (B, F, D, and H) when compared to WT (A, E, C, and G) (n = 3/3). Scale bars, 100 μm.

(I–N) Retrograde tracing using Fluoro-Gold (FG) paired with in situ hybridization for Hoxc6 on WT (I, K, and M) and Hoxc9−/− (J, L, and N) adult spinal cords (n = 2/2). At cervical levels, FG-traced cells express Hoxc6 in both WT (I and K) and Hoxc9−/− (J and L) animals. The WT spinal cord was devoid of Hoxc6 at mid- (K and K') and lower-thoracic (M and M') levels while in Hoxc9−/− animals, FG/Hoxc6 double-positive cells can be seen extending into mid- (L and L') and lower- (N and N') thoracic levels. White boxes indicate the region of higher magnification presented in adjacent panels. Scale bars, 100 μm.

See also Figures S7E and S7F.
mid-gestation time points where Hox function is often assessed. The robust expression of both Hox-embedded microRNAs, and of all Hox9-11 paralogs, until postnatal day 7 indicates their potential role in all stages of SC circuitry formation, from progenitor specification through to rostral axonal migration, circuit establishment, and pruning. Moreover, the maintenance of 196a2-eGFP (Figure S1) and Hoxc6 (Figure 5) expression in adult SC neurons is consistent with the expression of anterior Hox genes in adult precerebellar nuclei of the brainstem (Hutlet et al., 2016). Together this may suggest an unappreciated role for Hox-cluster gene function in precerebellar network homeostasis and potentially in degenerative processes. While it is expected that the functional output of these Hox-cluster genes in SC neurons will be through transcriptional or post-transcriptional regulation within soma of the spinal cord, one cannot rule out the possibility that Hox-embedded microRNAs act in the distal axon and pre-synaptic nerve terminal (Kaplan et al., 2013), or that the homeo-domain-containing proteins themselves may be secreted and act non-cell autonomously within the cerebellum (Bardine et al., 2014; Prochiantz and Di Nardo, 2015). Indeed, given the complete absence of posterior Hox-cluster expression within neuronal cell bodies of the cerebellum, this system may serve as an excellent model to investigate such mechanisms.

**Hox Function Is Required to Axially Restrict SC Populations**

Here, we have used miR-196a2-eGFP as an active-locus reporter of SC neurons during development and in the adult. The functional requirement for miR-196 activity within this network remains to be investigated. However, the detection of both miR-196a paralogs within SC neurons, and the confirmation of SC expression of Hoxa9 and Hoxa10 genes that genomically surround the miR-196b locus, suggest that miR-196 double- or triple-knockout analysis is likely to be required to detect cellular and/or phenotypic alteration of this neural network. In contrast, we observed a marked loss of cerebellar-projecting neurons from CC, and an expansion of Hoxc6-positive laminae V-VII SC neurons normally restricted to brachial levels, following the removal of a single Hox gene, Hoxc9 (Figures 3 and 5). Our data are broadly consistent with that observed for thoracic-level MNs (Dasen et al., 2003, 2005; Jung et al., 2010) and spinal interneurons (Sweeney et al., 2018), whereby Hoxc9 alone is required to repress a brachial Hox code, thereby delineating axially appropriate neural connectivity. An important point of difference of our work to this well-defined model is that the marked loss of cerebellar-projecting SC neurons in Hoxc9-/- animals does not appear to be solely accounted for by a fate switch to a more rostral SC population. In contrast to MN columns that continuously cover the R-C extent of the spinal cord, SC neurons exhibit regional fluctuations. SC neurons of the upper cervical region from C1 to C5 cluster medially in the CeCV nucleus. The region between this and the CC nucleus that emerges around T1/T2 is less well defined, with scattered SC cells identified within laminae V-VII (Sengul et al., 2015). The lack of well-defined SC nucleus at this location potentially reflects a degree of divergence of ascending pathways between forelimb and hindlimb/trunk PSNs, since axons of forelimb PSNs have been shown to project directly to the external cuneate nucleus of the medulla without synapsing in the spinal cord as part of the spinocuneocerebellar tract (Abrahams and Swett, 1986; Nyberg and Blomqvist, 1982, 1984). As such, a complete fate change of CC neurons to an immediately rostral population in Hoxc9-/- mice is more difficult to reconcile than for the MN system. The fate of ‘‘lost’’ CC neurons in Hoxc9-/- mice remains to be explored, though numerous possibilities exist. A failure of progenitor specification is possible, though not strongly supported by previous Hox literature. Alternatively, these neurons may fail to differentiate from a neuronal precursor population, since Hoxc9 has been shown to induce neuronal differentiation in neuroblastoma (Mao et al., 2011; Wang et al., 2013, 2014). Hoxc9 may regulate neuronal survival, given the essential requirement for Hoxc9 in inducing SC expression of the neurotrophic growth factor Gdnf during late embryogenesis (Figure 4). Finally, progenitors may be diverted to an as yet unknown fate, and project axons to regions other than the cerebellum, thus precluding labeling by the retrograde tracer.

The substantial changes to CC in Hoxc9-/- mice could reasonably be expected to result in overt locomotor or behavioral defects. In our hands, the majority of Hoxc9-/- mice die at birth, particularly when bred onto a C57B6 background. For those Hoxc9-/- mice that survive to adulthood, many display a hunched back (Suemori et al., 1995; our own observations), potentially representing peripubertal scoliosis, which has been identified following loss of Er81-positive PSNs (Blecher et al., 2017a), though alternative explanations such as loss of hypaxial motor column (Jung et al., 2010) may contribute to or underlie this phenotype. Similarly, the analysis of additional Hox gene contributions to SC function may, in many cases, be confounded by MN phenotypes, such as the hindlimb paralysis observed in Hoxc10/d10 mutant animals (Wu et al., 2008).

**Molecular Heterogeneity of SC Circuitry**

Our work demonstrates divergent Hox signatures that can discriminate axially restricted SC populations (summarized in Figure 6). Moreover, we observe molecular heterogeneity across the R-C extent of CC consistent with colinear Hox-cluster activation: a Hoxc9-positive upper region, an increasing proportion of mir-196a2-positive cells caudally, and Hoxc10-positive cells restricted to the lower regions. As CC neurons are heterogeneous in their inputs (Hantman and Jessell, 2010; Shrestha et al., 2012), it is possible that this heterogeneity in gene expression defines neurons of specific modality, for example, 196a2-eGFP expression in CC cells that receive hindlimb but not trunk proprioceptive input.

The extensive anatomical literature relating to SC neurons is based largely on tracer technology and lacks the precision of modern molecular tools. As a consequence, there is limited understanding of how SC circuitry is established in the developing embryo. While one progenitor population has been identified (Yuengert et al., 2015), the diversity of progenitor populations within and between SC populations is not known. Our work indicates that a large portion of CC cells are derived from a Hoxc9-positive progenitor, which during late embryogenesis requires Hoxc9 to activate expression of Gdnf. In parallel, a separate
Atoh-1 descendent population constitutes a further 10% of CC, with 0.5% of these positive for vGlut1 (Yuengert et al., 2015). Atoh-1 function is not required for the expression of Gdnf (Yuengert et al., 2015), further delineating these two populations (CC heterogeneity summarized in Figure 6). Whether Atoh-1 descendents constitute all remaining CC cells in Hoxc9−/− mice (Figure 4), or whether there are additional, molecularly diverse, CC subpopulations, remains to be determined.

Topography of SC Projections

A great deal of research on the cerebellum has been focused on its complex molecular parcellation, and the association between this organization and convergence of inputs from several CNS regions and cell-types based on their function (Apps and Hawkes, 2009; Hesslow, 1994; Odeh et al., 2005). Mouse mutants of Engrailed1 and Engrailed2 show widespread disruption of molecular organization in the Purkinje cells of the cerebellum (Sillitoe et al., 2008), with corresponding alterations in the projection pattern of SC neurons to these regions (Sillitoe et al., 2010). However, the molecular networks existing within SC neurons themselves, which allow appropriate connectivity with the diverse pool of Purkinje cells and thus formation of a topographic map within the cerebellar cortex, are yet to be determined. Similarly, there is evidence for topographic positioning of ascending SC tracts in the spinal white matter dependent on the axial level of input (Xu and Grant, 2005), though again with limited molecular understanding. The presence of an axial-specific Hox code in SC neurons could thus provide a parsimonious explanation for the control of topographic regionalization of these neurons, both in their ascending tracts and their projection to appropriate cerebellar target regions, and provide a means with which to dissect these key questions in future.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.10.048.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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