Bcl11a is required for neuronal morphogenesis and sensory circuit formation in dorsal spinal cord development

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SUMMARY
Dorsal spinal cord neurons receive and integrate somatosensory information provided by neurons located in dorsal root ganglia. Here we demonstrate that dorsal spinal neurons require the Krüppel-C2H2 zinc-finger transcription factor Bcl11a for terminal differentiation and morphogenesis. The disrupted differentiation of dorsal spinal neurons observed in Bcl11a mutant mice interferes with their correct innervation by cutaneous sensory neurons. To understand the mechanism underlying the innervation deficit, we characterized changes in gene expression in the dorsal horn of Bcl11a mutants and identified dysregulated expression of the gene encoding secreted frizzled-related protein 3 (sFRP3, or Frzb). Frzb mutant mice show a deficit in the innervation of the spinal cord, suggesting that the dysregulated expression of Frzb can account in part for the phenotype of Bcl11a mutants. Thus, our genetic analysis of Bcl11a reveals essential functions of this transcription factor in neuronal morphogenesis and sensory wiring of the dorsal spinal cord and identifies Frzb, a component of the Wnt pathway, as a downstream acting molecule involved in this process.

KEY WORDS: Spinal cord, Transcription factor, Neuronal differentiation, Bcl11a (CTIP1), Mouse

INTRODUCTION
The ability of the nervous system to integrate and relay information relies on the coordinated development of neuronal circuits. An immense diversity of neuron types is created during development. Extrinsic and intrinsic mechanisms control their subsequent maturation and the establishment of functional connectivity. Connectivity relies on the presence of dendrites in postsynaptic neurons and on the cues that allow navigation of the axons of presynaptic neurons, both of which have to be precisely orchestrated to establish a functional neuronal circuitry (Marmigere and Ernfors, 2007; Dasen, 2009).

Somatosensory neurons and their target, the spinal cord, provide a model for the molecular analysis of neuronal circuit development (Vrieseling and Arber, 2006; Yoshioka et al., 2006; Pecho-Vrieseling et al., 2009). During development of the dorsal spinal cord, different neuron types are generated from defined progenitor domains and subsequently become positioned in a highly organized laminar structure, the dorsal horn (Goulding et al., 2002; Helms and Johnson, 2003). Somatosensory neurons with cell bodies in dorsal root ganglia (DRG) innervate these spinal neurons, and distinct sensory neuron types project to different spinal cord layers. For instance, nociceptive neurons project to the upper layers of the dorsal horn, whereas mechanosensory and proprioceptive neurons innervate deeper layers (Brown, 1981).

Transcriptional networks are essential for spatiotemporally correct neuronal differentiation and wiring in the spinal cord (reviewed by Dasen, 2009). For example, members of the Paired related, LIM homeobox or TLX families of transcription factors are involved in cell fate decisions, morphogenesis, positioning and wiring of dorsal spinal neurons (Chen et al., 2001; Qian et al., 2002; Cheng et al., 2004; Ding et al., 2004; Marmigere et al., 2006). The signals provided by dorsal horn neurons that guide sensory axons from DRG neurons to their spinal targets are incompletely defined. Several members of the Wnt pathway are expressed in the spinal cord. Wnts have been extensively demonstrated to possess axon guidance activity and to control neural circuit formation (reviewed by Salinas and Zou, 2008), making this pathway an attractive candidate regulator in the process of sensory wiring within the dorsal horn.

In a screen designed to identify novel candidate genes that control development of the somatosensory circuitry, we identified Bcl11a as a gene that is expressed in the dorsal horn of the spinal cord and in sensory neurons. Bcl11a (also known as Evi9, Ctip1) encodes a C2H2 zinc-finger transcription factor that acts as a transcriptional regulator through its interaction with COUP-TF proteins and through direct, sequence-dependent DNA binding (Avram et al., 2000). Bcl11a is expressed in lymphohematopoietic cells, in which it controls the development of B- and T-lymphocytes (Liu et al., 2003b) and the maintenance of mature erythroid cells (Sankaran et al., 2008; Sankaran et al., 2009). The close relative, Bcl11b, is required for differentiation of striatal neurons and the corticospinal tract (Arlotta et al., 2005; Arlotta et al., 2008). However, functions of Bcl11a in the somatosensory system have not been defined.

Here we show that Bcl11a is an important regulator of terminal neuronal differentiation and wiring. Mutation of Bcl11a in spinal neurons disrupts their maturation and morphogenesis,
which is accompanied by defective innervation of the dorsal horn by somatosensory neurons. Among the genes dependent on Bcl11a for expression, we identified the Wnt antagonist secreted frizzled-related protein 3 (sFRP3, or Frzb) as a transcriptional target. Interestingly, we observe similar innervation deficits in the dorsal spinal cord of Bcl11a and Frzb mutants. Our findings show that Bcl11a is essential for sensory circuit formation and implicate Frzb in one aspect of the changes observed in Bcl11a mutant mice.

MATERIALS AND METHODS

Animals

To generate a conditional knockout allele (Bcl11a<sup>lox</sup>) a neomycin resistance cassette was introduced at the 3’ end of exon 1 of the Bcl11a gene. Exon 1 and the neomycin resistance cassette were flanked by loxP sites using previously described strategies (Liu et al., 2003a). Upon Cre-mediated recombination, exon 1 and the neomycin resistance cassette were excised from the Bcl11a locus, resulting in a null allele. To obtain mice with tissue-specific ablation of the Bcl11a allele, Bcl11a<sup>lox</sup><sub>lox</sub> mice were crossed to either Deleter-Cre (Schwenk et al., 1995), Brn4-Cre (Berc-32 pedigree) (Wine-Lee et al., 2004) or Ht-PA-Cre (Pietri et al., 2003) transgenic mice. Homozygous mutants were compared with heterozygous littermates harboring a Cre allele. Frzb and Sox10 mutant mice were described previously (Brito et al., 2001; Lories et al., 2007). Mice were genotyped by PCR. All animal experiments were carried out in accordance with German law and were approved by the respective governmental authorities in Berlin, Göttingen and Tübingen.

In situ hybridization, antibodies and histology

For in situ hybridization, spinal cords were dissected from mouse embryos at E14.5-18.5, fixed in 4% PFA and embedded in OCT compound (Sakura). Hybridizations were performed with DIG-labeled riboprobes on 18 μm cryosections.

For immunofluorescence staining, tissue was fixed with 4% PFA in 0.1 M sodium phosphate buffer (pH 7.4). Cryosections (14 μm) were obtained from matched cervical spinal cords. Stained sections were examined on a Zeiss LSM510 or Leica SP5II confocal microscope.

The following antibodies were used: rabbit and guinea pig anti-Lbx1 (Thomas Mueller, Berlin), rabbit anti-Ebf1 (H. Wildner and C. Birchmeier, Berlin), guinea pig anti-Lmx1b (T. Jessel, New York), rabbit anti-TrkB (A. Reichardt, San Francisco), rabbit anti-CGRP (Chemicon), rabbit anti-parvalbumin (Chemicon), rabbit anti-aquaporin 1 (Chemicon), rabbit anti-AMAP2 (Chemicon), mouse monoclonal anti-Hu/C (Invitrogen), mouse monoclonal anti-tubulin (Sigma), and fluorophore-conjugated secondary antibodies (Dianova).

To generate an anti-Bcl11a antiserum, a 486 bp fragment of murine Bcl11a cDNA encoding amino acids 501-662 (NM_016707) was amplified by PCR and cloned into the bacterial expression vector pET-14b (Novagen), which provides the coding sequences for a His<sub>6</sub> tag. His<sub>6</sub>-Bcl11a was propagated in BL21(DE3) pLyS cells, affinity purified on TALON metal resin (BD Biosciences) and injected into rabbits and guinea pigs (Charles River).

For anterograde labeling of sensory axons, segments of the vertebrate column with the spinal cord and DRG in loco were dissected from mutant and control embryos and fixed overnight in 4% PFA. Dil crystals (Invitrogen) were placed directly onto DRG, matched for their axial levels. Dil-loaded tissues were incubated at 37°C for up to 5 days. Dil tracings were examined on 80 μm vibratome sections using a confocal microscope.

BrdU labeling of neurons and Golgi staining of spinal cord were carried out as described (Heimrich and Frotscher, 1991; Gross et al., 2002).

Cell counting

Cervical spinal cords matched for axial level from at least three mutant and control animals were sectioned serially (14 μm). Cell numbers were counted on every fourth section. A total of three sections were evaluated per animal. Values are presented as mean±s.e.m. Differences were considered significant at P<0.05 by Student’s t-test.

Neuron cultures

Primary cultures from dorsal spinal cord neurons were established according to a modified previously published protocol (Banker and Goslin, 1998). Dorsal spinal horns were dissected from control and mutant embryos at E18.5. Cells were plated on poly-lysine-coated slides at 6-8×10<sup>6</sup> cells/ml. Neurons derived from the superficial dorsal horn were identified by expression of Lmx1b and cultured for up to 14 days in serum-free medium in the presence of 10 ng/ml NGF. For Sholl analysis (Sholl, 1953), images of individual neurons were overlaid with a grid of concentric circles, and the length and number of neurite branches were determined at 2.5 μm intervals from the soma. Neuron cultures from three individual mutant and control animals and at least ten neurons per neuron culture were analyzed.

Electrophysiology

Whole-cell patch-clamp recordings of evoked excitatory postsynaptic currents (EPSCs) were performed as described (Liebel et al., 1997; Schmidt et al., 2007).

For slice preparation, cervical spinal cords from control and Bcl11a mutant animals were microdissected at E18.5 and embedded in agarose (2.5% SeaPlaque agarose, CAMBREX). Transverse slices (200 μm) were prepared on a vibratome. During recordings, slices were perfused with a bath solution of 125 mM NaCl, 4 mM KCl, 10 mM glucose, 1.25 mM Na<sub>H</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>.

Numerical data are reported as mean±s.e.m. Statistical analysis was performed using StatView (SAS Institute). Student’s t-test (two-tailed, unpaired; decay time, rise time, evoked EPSC) or χ<sup>2</sup> test (success rates, response rates) were used for statistical comparisons.

Microarray analysis

Microarray experiments were performed according to MIAME standards (Brazma et al., 2001) (http://www.mged.org/Workgroups/MIAME/miame.html). For each time point analyzed (E14.5 and E16.5), dorsal spinal cords from ten age-matched Bcl11a mutant (Bcl11a<sup>lox</sup><sub>lox</sub>;Brn4-Cre) and ten control (Bcl11a<sup>lox</sup><sub>lox</sub>:Brn4-Cre) animals were used. Segments of the cervical spinal cord were dissected in ice-cold PBS and matched for axial level. To isolate their dorsal halves, we used the sulcus limitans as an anatomical landmark. After dissection, dorsal spinal cord segments were transferred to Trizol (Invitrogen). For RNA isolation, mutant and control tissue were pooled separately. cDNA synthesis and biotinylation of cRNA probes were carried out according to standard Affymetrix protocols with three technical replicates of mutant and control RNA, starting with 5 μg total RNA for each replicate. From each replicate, 15 μg of labeled cRNA were hybridized for 16 hours at 45°C to mouse Affymetrix MOE430 2.0 microarrays in a GeneChip hybridization oven 645. After hybridization, microarrays were washed and stained in a GeneChip fluidics station 450, and scanned on a GeneChip scanner 3000 7G. Raw data analysis was performed using Bioconductor software (www.bioconductor.org). Quality of hybridization results was assessed with the affyPLM Bioconductor tool. After GC-RMA normalization of data, log<sub>2</sub> signal intensity was used for value definition.

Quantitative real-time RT-PCR

Total RNA was prepared from dorsal spinal cord of controls and Bcl11a mutants using the RNeasy Mini kit (Qiagen). RNAs were reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and quantitative real-time PCR (qRT-PCR) was performed using the Quantitect SYBR Green PCR kit (Qiagen) in a LightCycler 480 system (Roche). The following oligonucleotides (5’-3’) were used: Frzb, GCTGAGAA-GTGGGAAGGATCG and AACTGTCGGCCTTTTTCTTTTG; Gapdh, CCAGACCTGAACGGAAG and TGCTGGTGAAGTGCCAGG. The relative copy number of Gapdh RNA was quantified and used for normalization. Data were analyzed using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

Chromatin immunoprecipitation (ChIP) on embryos

ChIP assays were performed on wild-type dorsal spinal cords collected from ten embryos at E14.5 using the EpitQuiK Tissue Chromatin Immunoprecipitation kit (Epigentek, Framingdale, NY, USA) including...
specific antibodies recognizing RNA polymerase II and mouse IgG as controls. Every ChIP was replicated at least three times. The precipitated DNA was analyzed by quantitative PCR using oligonucleotides (5'-3') that recognize the promoter regions of: Gapdh, AAGGCTGGTGCTGTGGAGAAAACGT and GTCCCTTTGCACATACATACTG; Frzb promoter region 1 (Frzba1), GAGCACGGGACTAGACCA and ACCTGGAGGACACTTGGATC; Frzb promoter region 2 (Frzba2), TTAGTTTTCAGGGACCTGG and TCTCTGTGGTGCTTGGACAC; Frzb promoter region 3 (Frzba3), ACCAATGCGAGGAGATTG and TCTCCCTAAGTGCCATTGAC; Drg11, TCCAAATGGCCTACTGCCTT and GGGACTGGCCAGAATTGATA. All primer sets were tested by PCR using input and genomic DNA (supplementary material Fig. S4). Promoter fragments for Frzb regions were selected using ensembl.org, avoiding known RNA polymerase II binding sites. The promoter fragment for Gapdh specifically flanks the TATA box of this gene (Iankova et al., 2006). ChIP-qPCR results were analyzed by determining the amount of precipitated DNA as a percentage of input DNA. Data of three independent ChIP assays were pooled, mean±s.e.m. was calculated and statistical analysis performed using Student’s t-test.

RESULTS

Expression and conditional mutation of Bcl11a in the spinal cord

In mice, at least three different isoforms, termed Evi9a, b and c, are generated from the Bcl11a gene (Nakamura et al., 2000). To analyze expression of Bcl11a, we generated antibodies against a 161 amino acid fragment of Bcl11a that is present in the Evi9a and b isoforms, but not in Evi9c (Fig. 1D-F; supplementary material Fig. S1), and performed in situ hybridizations with pan-specific (Fig. 1A-C) and isoform-specific (Fig. 1G-I) riboprobes. Bcl11a transcript and protein were first detected at E11.0 and E11.5, respectively, in the mantle zone of the most superficial dorsal spinal neural tube, and, albeit at much lower levels, within a few cells of the ventricular zone (Fig. 1A,D). With ongoing development, Bcl11a was expressed by the majority of postmitotic dorsal spinal neurons, and at E18 81.47±2.41% of cells expressing the pan-neuronal marker HuC/D co-express Bcl11a within the dorsal horn (Fig. 1B,C,E,F,K; supplementary material Fig. S1). All three isoforms of Bcl11a were detected in the spinal cord. Unlike Evi9b, Evi9a and Evi9c expression appeared in almost identical patterns restricted to the dorsal spinal cord (Fig. 1G-I). In addition, Bcl11a was expressed by the majority of primary sensory neurons in DRG, as well as in a few cells in the ventral neural tube, some of which co-express Isl1 and appear to represent differentiating motoneurons (Fig. 1D,E; data not shown).

Mice with a null mutation in Bcl11a show defects in lymphopoiesis and die shortly after birth (Liu et al., 2003b). To define functions of Bcl11a in neurons and to distinguish functions in presynaptic primary sensory neurons from those in postsynaptic dorsal spinal neurons we employed the Cre/loxP system (Fig. 1L-N). Homozygous mutant offspring from floxed Bcl11a mice crossed to a deleter-Cre (Del-Cre) mouse strain (Schwenk et al., 1995) died within a few hours after birth and were indistinguishable from Bcl11a null mutants (Liu et al., 2003b). Bcl11a protein expression was undetectable in such mice (Fig. 1N). In situ hybridization with isoform-specific probes demonstrated the absence of Evi9c transcripts in the Bcl11a mutants (Fig. 1J). When floxed Bcl11a mice were crossed to Brn4-Cre mice that express Cre in neurons of the central but not of the peripheral nervous system (Wine-Lee et al., 2004), Bcl11a expression was eliminated from spinal neurons but not in primary sensory neurons within DRG (Fig. 1L). Crossing floxed Bcl11a mice to an Ht-PA-Cre mouse line, in which Cre is active in neural crest cells and their derivatives (Pietri et al., 2003; Yoshida et al., 2006), completely abolished Bcl11a expression in primary sensory neurons, whereas Bcl11a expression was preserved in most of the dorsal spinal neurons (Fig. 1M).

Bcl11a is required for terminal differentiation and morphogenesis of dorsal spinal neurons

Analysis of dorsal spinal neurons of Brn4-Cre;Bcl11a mice revealed changes in differentiation that were first apparent at E14.5. In control mice, neurons of the superficial dorsal horn express the transcription factors Lmx1b, Ebf1, Drg11 (Prxl1 – Mouse Genome Informatics) and Pax2, with a few Lbx1-positive cells intermingled (Fig. 2A,C,E; supplementary material Fig. S2). This region corresponds to layers I and II in the adult spinal cord and is innervated by cutaneous sensory afferents from DRG. During this period, Bcl11a is expressed by the majority of postmitotic neurons of the dorsal spinal horn, with Evi9a and Evi9c being more restricted to the superficial zone, and overlaps with Lmx1b...
expression (Fig. 1C,F,G,I; supplementary material Fig. S1). In Brn4-Cre;Bcl11a mutants the superficial zone was invariably compressed, with the positions of the deeper layers shifted superficially. Lmx1b+, Ebf1+ or Drg11+ nuclei within this zone appeared compacted when compared with controls (Fig. 2B,D,F; supplementary material Fig. S2A-D). To explore whether this phenotype is caused by changes in the size of specific neuron populations we determined the numbers of Toto+, NeuN+, Lbx1+ or Lmx1b+ cells within the dorsal horn. Total neuron numbers, as well as those of individual neuron populations, were unchanged in mutants (Fig. 2K). Pulse labeling with BrdU during late phase neurogenesis (Gross et al., 2002; Muller et al., 2002) revealed normal birth rates and migratory capacities of Bcl11a mutant dorsal spinal neurons (Fig. 3A-E). With ongoing development, neurons of the superficial dorsal horn express late differentiation markers, such as gastrin releasing peptide receptor (Grpr), galanin and Gria2 (Baccei and Fitzgerald, 2004; Sun and Chen, 2007; Xu et al., 2008). Expression of these genes was greatly reduced (Gria2) or undetectable (Grpr, galanin) in mutants (Fig. 2G-J; data not shown), suggesting that neurons within the superficial dorsal horn require Bcl11a for terminal differentiation.

The compaction of neurons within the superficial dorsal horn might be caused by a reduced neuropil. To test this, we defined the expression of the microtubule-associated protein MAP2 (Mtap2 – Mouse Genome Informatics) (Ding et al., 2004). Compared with controls, MAP2 expression was greatly reduced in the superficial dorsal horn of Bcl11a mutant mice (Fig. 4A,B). At E18.5, Golgi-stained neurons in the dorsal horn of controls frequently display complex neurite trees (Fig. 4C). In homozygous mutants, neurites were severely reduced, and remaining structures often appeared misshapen (Fig. 4D). To quantify neurite formation in spinal neurons, we cultured primary neurons from superficial dorsal horn tissues and carried out a Sholl analysis (Fig. 4E-G). Viability and plating efficiency of mutant and control spinal neurons were
Mean±s.e.m. *

Bcl11a and spinal cord

Fig. 4. Dorsal spinal neurons require Bcl11a for morphogenesis.
(A–D) Transverse sections of the superficial dorsal horn of control (A,C) and Del-Cre recombined Bcl11a mutant (B,D) mice. Note that in mutants, Lmx1b-expressing neurons are more densely packed (B). Neurite morphology of control (C) and Bcl11a mutant (D) neurons in the superficial dorsal horn at E18.5 was visualized by Golgi staining. 

(E,F) Primary neuron cultures derived from dorsal spinal cord tissues of E18.5 control (E) and Bcl11a mutant (F) animals stained with anti-tubulin antibodies. 

Sholl analysis of neurite length and arborization. Mean±s.e.m. *, P<0.05 (Student’s t-test). Scale bars: 20 μm in B; 30 μm in D; 40 μm in E,F.

Bcl11a is expressed in both presynaptic sensory neurons and postsynaptic spinal target neurons (Fig. 1). We next asked whether Bcl11a is required for correct wiring, and if so, on which site. Central axons of sensory neurons were labeled at E16.5 with DiI. In the superficial dorsal horns of Brn4-Cre;Bcl11a mutants, the density of Dil-positive fibers was greatly reduced and the remaining fibers appeared disorganized. Only a few axons crossing the midline or located in a dorsolateral region of the dorsal horn were detectable by DiI labeling in mutants (Fig. 5A,B). TrkA (Ntrk1 – Mouse Genome Informatics) -positive nociceptive fibers preferentially terminate in the superficial dorsal horn. Immunohistological analysis with antibodies against TrkA or aquaporin 1, a water channel protein that is expressed by small-diameter nociceptive fibers (Oshio et al., 2006), invariably revealed almost complete loss of such fibers in the dorsal horn of Brn4-Cre;Bcl11a mutants (Fig. 5C-F). Similar results were obtained with antibodies against CGRP (Calca – Mouse Genome Informatics), which marks a specific subset of peptidergic neurons terminating in the superficial dorsal horn (Fig. 5G,H). By contrast, parvalbumin-positive proprioceptive neurons projecting to the ventral spinal cord, where most neurons do not express Bcl11a (Fig. 1F,G,I), were unaffected by the mutation (Fig. 5I,J). Similar phenotypes were observed in spinal cords at E13.5 and E14.5, when cutaneous sensory afferents begin to grow into the dorsal horn (not shown). To exclude the possibility that the observed phenotype resulted from degeneration or aberrant differentiation of primary sensory neurons, we determined the numbers and apoptosis rates of TrkA-positive and parvalbumin-positive neurons within DRG. We did not observe any differences in apoptosis or in the size of individual neuron populations between Brn4-Cre;Bcl11a mutants and controls (Fig. 5K-P; data not shown).

To assess directly whether synaptic input from cutaneous sensory afferents to mutant dorsal spinal neurons was reduced, we recorded postsynaptic currents in dorsal horn neurons elicited by extracellular stimulation at the dorsal root entry zone (Fig. 5Q). Basic synaptic properties, such as amplitude, decay time constant (control: 3.86±0.70 mseconds, n=12; mutant: 3.55±0.41 mseconds, n=14) and rise time (control: 1.72±0.24 mseconds, n=12; mutant: 1.74±0.21 mseconds, n=14) were similar in mutant and control neurons (supplementary material Fig. S3A,B). In addition, paired-pulse properties, a commonly used criterion for presynaptic alterations, were unchanged in mutants (supplementary material Fig. S3C,D). However, the success rates of evoked excitatory postsynaptic currents (EPSCs) in dorsal horn neurons were significantly reduced (P<0.05) from 76.0% in controls to 52.4% in mutants (Fig. 5R).

When we analyzed Ht-Pt-Cre;Bcl11a mutants at E18.5, TrkA-positive and aquaporin 1-positive axon projections were unchanged (Fig. 6C,D; data not shown). In addition, the primordial layer architecture in Ht-Pt-Cre;Bcl11a mutants was indistinguishable from that of controls (Fig. 6A,B). Together, this indicates that Bcl11a is required in spinal target neurons, but is dispensable in presynaptic sensory neurons, for correct wiring and differentiation in the superficial dorsal horn.

The phenotype observed in Bcl11a mutant spinal cords does not exclude the possibility that impaired differentiation and morphogenesis of dorsal spinal neurons are caused indirectly by deficits in sensory innervation. To test this we analyzed Sox10 mutant mice. In such mice, primary sensory neurons are eliminated before E12.5 by apoptosis (Britsch et al., 2001), allowing an assessment of dorsal horn development in the absence of sensory input (Fig. 6G,H). In Sox10 mutants, expression of Lbx1, Lmx1b, Gria2 and Grpr, primordial layer architecture and neuron morphology as determined by Golgi and MAP2 staining were unchanged (Fig. 6E,F,I-N; data not shown). Thus, the defective

comparable. However, the overall number, length and branching frequency of neurites were significantly reduced in Bcl11a mutant neurons (Fig. 4G).

**Primary sensory neurons require postsynaptic Bcl11a expression for correct wiring**

Bcl11a is expressed in both presynaptic sensory neurons and postsynaptic spinal target neurons (Fig. 1). We next asked whether Bcl11a is required for correct wiring, and if so, on which site.
neuronal differentiation and morphogenesis as observed in the Bcl11a mutant dorsal horn is independent of defective sensory innervation.

Dysregulated expression of Frzb accounts in part for the disrupted innervation of the Bcl11a mutant spinal cord

To identify genes that are differentially expressed in Bcl11a mutants, we performed microarray analyses on dorsal spinal cord tissues of E14.5 and E16.5 embryos. Analysis of E16.5 embryos revealed that the expression of 20 and 121 genes was significantly downregulated and upregulated, respectively (change $P \leq 0.0001$; fold change $\leq -1.9$ and $\geq 1.9$; supplementary material Table S1). In accordance with a function in terminal differentiation (see above), Gria2 and Grpr were significantly downregulated in mutants at E16.5 but not E14.5 (Table 1; see Fig. 2).

The gene encoding the extracellular signaling molecule secreted frizzled-related protein 3 (Frzb) was most consistently downregulated according to our microarray analyses (Table 1; supplementary material Table S1). As members of the sFRP family can act as guidance cues for navigating axons (Rodriguez et al., 2005), we focused on this candidate for further analysis. In situ hybridization verified downregulation of Frzb in the Bcl11a mutant spinal cord (Fig. 7A,B), and Frzb mRNA was 5.6-fold downregulated in qRT-PCR of Bcl11a mutant dorsal horn tissue ($P<0.01$; Fig. 7C). Frzb expression was unchanged in Sox10 mutant dorsal horn, excluding downregulation of Frzb secondary to defective sensory innervation (Fig. 6O,P).

To test whether Frzb is directly regulated by Bcl11a, we performed ChIP on embryos; the specificity of the Bcl11a antibody was verified by western blot analysis (Jawaid et al., 2010). The Bcl11a antibody specifically precipitated two promoter fragments, corresponding to +623 to +849 (Frzb1) and –27 and +138 (Frzb2) relative to the transcription initiation site of Frzb (Fig. 7D,E). The amount of recovered Frzb1 and Frzb2 promoter DNA was significantly higher than with control IgG (Frzb1, Bcl11a antibody 0.233±0.02% and IgG 0.14±0.032% of input DNA; Frzb2, Bcl11a antibody 0.307±0.047% and IgG 0.12±0.03% of input DNA; $P<0.05$; Fig. 7D,E). No specific binding of Bcl11a to a distant fragment comprising –786 to –1008 was detected (Frzb3, Bcl11a antibody 0.009±0.0005% and IgG 0.03±0.02% of input DNA; Fig. 7D,E).

Fig. 5. Sensory axon projections in the Bcl11a mutant dorsal spinal horn. (A-J) Transverse sections of the dorsal horn of control (A,C,E,G,I) and Brn4-Cre recombinant Bcl11a mutant (B,D,F,H,J) mice at E16.5 (A,B) and E18.5 (C-J). Sensory axons were traced with DiI (A,B). Axons crossing the midline are marked (arrowheads in A,B). Residual fibers are disorganized (arrows in A,B). A similar phenotype is detectable with anti-CGRP staining (arrows in G,H). (C-J) Immunohistological analysis of sensory axons projecting into the spinal cord with antibodies against TrkA (C,D), aquaporin 1 (E,F), CGRP (G,H) and parvalbumin (I,J). (K-P) Immunohistological detection (K-N) and quantification (O,P) of TrkA-positive (K,L,O) and parvalbumin-positive (M,N,P) sensory neurons in DRG. Means±e.m. n.s., not significant. (Q,R) Electrophysiological analysis of evoked excitatory postsynaptic currents (eEPSCs) from dorsal horn neurons of control and Brn4-Cre recombinant Bcl11a mutants at E18.5. *, $P<0.05$ (Fisher’s exact test). rec, recording pipette; stim, stimulation pipette; dsn, dorsal spinal neuron; drez, dorsal root entry zone; drg, dorsal root ganglion; csa, central sensory axon. Scale bars: 50 μm.
To validate the ChIP assays, an antibody against RNA polymerase II and a PCR strategy amplifying a Gapdh promoter fragment that flanks the TATA box were used (Iankova et al., 2006). We recovered significantly more Gapdh promoter DNA using the antibody directed against RNA polymerase II than with the IgG control; no significant enrichment of Gapdh promoter DNA was obtained with Bcl11a antibodies (Gapdh, RNA polymerase II antibody 0.51±0.038% and IgG 0.1±0.05% of input DNA, P<0.05; Bcl11a antibody 0.04±0.02% of input DNA; Fig. 7D,E). In addition, we used primers amplifying a promoter sequence of Drg11, which is expressed in dorsal horn neurons but is unaffected by the Bcl11a mutation (supplementary material Fig. S2). No significant recovery of Drg11 promoter DNA was obtained with Bcl11a antibodies (Gapdh, RNA polymerase II antibody 0.51±0.038% and IgG 0.1±0.05% of input DNA, P<0.05; Bcl11a antibody 0.04±0.02% of input DNA; Fig. 7D,E). In addition, we used primers amplifying a promoter sequence of Drg11, which is expressed in dorsal horn neurons but is unaffected by the Bcl11a mutation (supplementary material Fig. S2). No significant recovery of Drg11 promoter DNA was observed with Bcl11a antibodies when compared with the IgG control (Fig. 7E). Neither the Frzb1-3 nor the Drg11 fragments used for ChIP contain a TATA box or other known RNA polymerase II binding sites. Accordingly, no significant precipitation of these fragments was observed with RNA polymerase II antibodies (Fig. 7E).

Analyzing the DNA sequence of the precipitated Frzb promoter region 1 revealed putative Bcl11a binding sites GGCCGG (starting at +824) and GGCCGC (starting at +796), both of which display high similarity to the previously described consensus binding motif of Bcl11a (Avram et al., 2002; Chen et al., 2009). Furthermore, a transgenic Frzb promoter fragment (indicated in Fig. 7D) lacking region 1 has been shown to recapitulate endogenous Frzb expression in mice, except in neural tube (Tylzanowski et al., 2004), supporting the assertion that interaction of Bcl11a protein with Frzb promoter region 1 is functional in neurons.

To assess whether Frzb is required for sensory innervation of the dorsal horn, we analyzed Frzb mutant mice (Lories et al., 2007) (Fig. 8A,B). In the dorsal horn of Frzb mutants we observed a reduction in TrkA and aquaporin 1 but not in parvalbumin expression (Fig. 8E-J). The reduction in the innervation of TrkA+ and aquaporin 1+ sensory neurons was less pronounced than observed in Bcl11a mutants. Dil labeling of primary sensory axons revealed reduced and disorganized sensory innervation of the dorsal horn of Frzb mutants (Fig. 8C,D). Frzb is also expressed in some sensory neurons (Fig. 8K,L). In mutants, the numbers and distribution of TrkA+ and parvalbumin+ neurons within DRG were normal (Fig. 8M-R). The primordial layer architecture, differentiation and morphology of dorsal spinal neurons were unchanged in Frzb mutants as assessed by expression of Lbx1, Lmx1b, Grpr and Gria2 and by Golgi and MAP2 staining (supplementary material Fig. S5A-J). We conclude that the dysregulated Frzb expression can in part account for the disrupted sensory innervation of the Bcl11a mutant spinal cord.

**DISCUSSION**

The dorsal spinal cord processes somatosensory information and relays it to higher brain centers. In this study, we provide genetic evidence that the zinc-finger transcription factor Bcl11a is essential for the correct sensory innervation of the dorsal spinal cord. This function of Bcl11a is in part mediated by its role in the transcriptional control of Frzb.

**Bcl11a is required for sensory circuit formation**

Small-diameter cutaneous sensory afferents are greatly reduced in the dorsal horn of Bcl11a mutant mice. Similar phenotypes have been reported for mice with a mutation of the transcription factors Lmx1b, Drg11 or Tlx3/Tlx1 (Chen et al., 2001; Qian et al., 2002; Ding et al., 2004). These genes are expressed by the same neuron type and overlap in part with the expression of Bcl11a in the dorsal horn (supplementary material Fig. S1), raising the question of whether Bcl11a exerts part of its functions through a shared genetic program. However, the expression patterns of Lmx1b and Drg11...
as well as the number of Lmx1b-positive dorsal neurons were unchanged in Bcl11a mutants. Whereas we observed defects in the terminal differentiation of dorsal neurons in Bcl11a mutants, changes in neuron type composition and neuronal fate have been detected in the dorsal horns of Lmx1b and Tlx3/Tlx1 mutant animals (Qian et al., 2002; Cheng et al., 2004; Ding et al., 2004).

Bcl11a is expressed in presynaptic sensory neurons as well as in their postsynaptic spinal targets. We ablated Bcl11a selectively in pre- and postsynaptic neurons to determine the relative contribution of pre- versus postsynaptic expression of Bcl11a to sensory circuit formation. Genetic data presented in this study demonstrate that expression of Bcl11a in postsynaptic dorsal spinal neurons is required for the ingrowth of sensory afferents and for providing synaptic input to their targets. By contrast, Bcl11a expression in primary sensory neurons is dispensable for this process. This does not, however, exclude additional, as yet undetermined functions of Bcl11a in primary sensory neurons.

Somatosensory afferents are not equally affected by the mutation of Bcl11a in spinal neurons. Although sensory afferents expressing TrkA, aquaporin 1 or CGRP are strongly reduced in the superficial dorsal horn, we do not detect major defects in proprioceptive fibers traveling to the ventral spinal cord. One explanation for this could be that only a few of their target neurons within the ventral spinal cord normally express Bcl11a. Thus, somatosensory neurons might differ in their dependence on Bcl11a. In addition, we detected all three major isoforms of Bcl11a, i.e. Evi9a, b and c, as being differentially expressed in the spinal cord. Whereas Evi9b is expressed throughout the spinal cord, Evi9a and c are predominantly expressed in the dorsal horn. Previous studies demonstrated that Evi9 isoforms differ in their subcellular localization and biological activity (Nakamura et al., 2000; Kuo et al., 2009), raising the possibility that ingrowth of sensory afferents to the dorsal horn might depend on Evi9a and/or c selectively.

In addition to the innervation deficit, neurons of the dorsal spinal cord of Bcl11a mutants displayed disrupted morphogenesis and terminal differentiation. This raised the question of whether dorsal horn neurons require sensory innervation for correct maturation. However, Sox110 mutants lack sensory neurons by E12.5 but do not display major deficits in the differentiation and morphogenesis of development.
dorsal spinal neurons. Thus, the disrupted morphogenesis and differentiation of dorsal horn neurons in Bcl11a mutants is not caused by the deficit in sensory innervation.

Dendritic morphology and the presence of a particular transmitter receptor repertoire are important determinants of the number and type of presynaptic partners of a neuron (Hausser et al., 2000). Ingrowth of sensory afferents precedes terminal differentiation of spinal neurons (Ding et al., 2004). Thus, defective target differentiation, as observed in Bcl11a mutants, is unlikely to be the only cause for the deficit in dorsal horn innervation. We propose instead that Bcl11a regulates the expression of signals that guide somatosensory axons to their spinal targets. We identified such a guidance factor, Frzb, which was downregulated in the dorsal horn of Bcl11a mutants. The family of sFRPs function either as extracellular inhibitors of Wnt signaling or they can act independently of Wnt ligands. Wnt signals regulate multiple steps during the assembly of neural circuits (reviewed by Bovolenta et al., 2006; Salinas and Zou, 2008). In particular, sFRP1 acts as a guidance factor for retinal axons (Rodriguez et al., 2005). In addition, Wnts act as anterior-posterior guidance cues for commissural axons in the spinal cord, a function that is regulated by sFRPs (Lyuksyutova et al., 2003; Domanitskaya et al., 2010). Furthermore, neurite outgrowth of somatosensory neurons is controlled by the Wnt receptor Ryk (Lu et al., 2004).

We show that the disrupted Frzb expression is in part responsible for the deficit in sensory innervation of the dorsal horn of Bcl11a mutants. In particular, we observe reduced and disorganized cutaneous sensory innervation in the dorsal horn of Frzb mutants. Changes in the somatosensory system had not been noted previously in Frzb mutants (Lories et al., 2007). However, sensory innervation deficits in Frzb mutants are milder than those in Bcl11a mutants, and the differentiation and morphogenesis of dorsal horn neurons appeared unaffected. This indicates that, in addition to Frzb, other genes exist that control neuronal development and are expressed in a Bcl11a-dependent manner.

Several lines of evidence suggest that Frzb is a direct target of Bcl11a: (1) Frzb was downregulated at early and late time points (E14.5 and E16.5) during development of the dorsal spinal cord of Bcl11a mutants, and Frzb expression did not depend on sensory innervation and was unchanged in Sox10 mutants; (2) two distinct promoter fragments of Frzb are precipitated by ChIP with a Bcl11a-specific antibody; and (3) consensus binding sites for Bcl11a are present in the Frzb1 fragment. These experiments thus support a direct role of Bcl11a in the control of Frzb. Bcl11a was originally described as a COUP-TF-interacting protein (CTIP1). Mice with a targeted deletion of COUP-TF1 (Nr2f1–Mouse Genome Informatics) display aberrant projections of cranial nerve axons (Qui et al., 1997). COUP-TF1 is expressed in the developing spinal cord, and it is thus possible that Bcl11a cooperates with COUP-TF1 to regulate the differentiation of spinal neurons and sensory circuit formation.

A role for Bcl11a in neuronal morphogenesis

An important aspect of Bcl11a function in the dorsal horn is its role in neuronal morphogenesis. In the absence of Bcl11a, dorsal spinal neurons initiate neuritogenesis but form hypoplastic neurites with severely reduced branching complexity in vivo. A similar phenotype is observed when dorsal spinal neurons of Bcl11a mutants are cultured. It was previously reported that knockout of Bcl11a-L (which corresponds to Evi9a in mice) in cultured rat

Fig. 8. Sensory axon projections in the Frzb mutant dorsal spinal horn. (A-J) Transverse sections of the dorsal horn of control (A,C,E,G,I) and Frzb mutant (B,D,F,H,J) mice at E16.5 (A-D) and E18.5 (E-J) hybridized with probes specific for Frzb (A,B) or stained with antibodies against TrkA (E,F), aquaporin 1 (G,H) or parvalbumin (I,J). Sensory axons entering the dorsal horn were traced with DiI (C,D). Axons crossing the midline are marked by arrowheads (C,D). (K-P) Transverse sections of DRG of controls (K,M,O) and Frzb mutants (L,N,P) hybridized with Frzb-specific probes (K,L) or stained with antibodies against TrkA (M,N) and parvalbumin (O,P). (Q,R) Quantitative analysis of sensory neurons in DRG. Mean ± s.e.m. n.s., not significant. Scale bars: 100 μm in J; 50 μm in P.
hippocampal neurons results in enhanced axonal branching and dendrite outgrowth, an effect that was proposed to be mediated by transcriptional control of Dcc and Map1b (Kuo et al., 2009). We did not detect changes in Dcc and Map1b (Map1b – Mouse Genome Informatics) expression in the spinal cord of Bcl11a mutant mice (data not shown). In our mutants, all three isoforms of Bcl11a were ablated from the genome. Evi9a and Evi9b are the major isoforms expressed in the murine hippocampus during development (Sebastian Karl and S.B., unpublished). Thus, the increase in neurite branching after knockdown of Evi9a might reflect residual Evi9b functions in rat hippocampal neurons in vitro, suggesting that the biological activities of Bcl11a are isoform specific (Nakamura et al., 2000; Kuo et al., 2009); they might also be neuron type specific.

In summary, we provide here a genetic characterization of the functions of the transcription factor Bcl11a in neuronal differentiation and the formation of sensory circuits. By the identification of Frzb, we link the transcriptional function of Bcl11a to its role in the guidance of sensory neurons.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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