The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord

Thomas Müller,[1,3] Katrin Anlag,[2,3] Hendrik Wildner,[1] Stefan Britsch,[1] Mathias Treier,[2,4] and Carmen Birchmeier[1,5]

[1]Max-Delbrück-Center for Molecular Medicine, 13122 Berlin, Germany; [2]European Molecular Biology Laboratory, 69117 Heidelberg, Germany

Neurons of the dorsal horn integrate and relay sensory information and arise during development in the dorsal spinal cord, the alar plate. Class A and B neurons emerge in the dorsal and ventral alar plate, differ in their dependence on roof plate signals for specification, and settle in the deep and superficial dorsal horn, respectively. We show here that the basic helix–loop–helix (bHLH) gene *Olig3* is expressed in progenitor cells that generate class A (dI1–dI3) neurons and that Olig3 is an important factor in the development of these neuronal cell types. In *Olig3* mutant mice, the development of class A neurons is impaired; dI1 neurons are generated in reduced numbers, whereas dI2 and dI3 neurons are misspecified and assume the identity of class B neurons. Conversely, *Olig3* represses the emergence of class B neurons in the chick spinal cord. We conclude that *Olig3* expression distinguishes the two major classes of progenitors in the dorsal spinal cord and determines the distinct specification program of class A neurons.

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Somatosensory information is processed by neurons in the dorsal horn of the spinal cord. Dorsal horn neurons integrate sensory information, relay it to the brainstem and thalamus, and modulate spinal cord reflexes (for review, see Gillespie and Walker 2001; Julius and Basbaum 2001). Physiological and anatomical studies indicate that many distinct dorsal neuron types exist that are ill defined on a molecular level (Rexed 1952; Brown 1982). The complex circuitry in which these sensory interneurons participate is established during development and depends on a spatially and temporally ordered appearance of neuron types. The cascade of molecular events that allows the specification of dorsal sensory interneurons is incompletely understood (see Goulding et al. 2002; Caspary and Anderson 2003; Helms and Johnson 2003). The previous systematic and elegant analysis of neural development in the ventral spinal cord has, however, revealed basic mechanisms used to create neural diversity, and provides a paradigm for dorsal spinal cord development.

The ventral horn of the spinal cord contains motoneurons and interneurons that coordinate motoneuron output. The majority of these neurons arises in the ventral spinal cord. The different neuron types are generated at stereotypic positions along the dorso–ventral axis from progenitors that possess positional information. Ventrally, positional information is provided by a graded Shh signal that directs the expression of patterning genes to restricted progenitor domains along the dorso–ventral axis (Briscoe and Ericson 1999; Briscoe et al. 1999). Most of these patterning genes encode homeodomain transcription factors, and their expression defines different stripes of progenitors from which the various post-mitotic neuron types and oligodendrocyte precursors arise (Briscoe et al. 2000; Jessell 2000; Briscoe and Ericson 2001; Rowitch et al. 2002). Although the expression of most patterning genes is extinguished in the emerging neurons and oligodendrocytes, they instruct these cells to express a particular set of transcription factors, which determines their further differentiation program (Chen et al. 2001; Moran-Rivard et al. 2001; Novitch et al. 2001; Vallstedt et al. 2001; Zhou et al. 2001; Gross et al. 2002; Lu et al. 2002; Muller et al. 2002; Qian et al. 2002; Takebayashi et al. 2002a; Zhou and Anderson 2002; Cheng et al. 2004; Ding et al. 2004).

Dorsal horn neurons arise during development in the dorsal part of the spinal cord, the alar plate, in two neurogenic waves. The first wave produces six neuron types that can be grouped into classes A and B, whereas the second wave produces two neuron types of the class B

[3]These authors contributed equally to this work.
Corresponding authors.
[4]E-MAIL treier@embl.de; FAX 49-6221-387-166.
[5]E-MAIL cbirch@mdc-berlin.de; FAX 49-30-9406-3765.
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(Gross et al. 2002; Muller et al. 2002). The expression of homeodomain factors distinguishes the various class A and B neuron types and instructs their differentiation (Gross et al. 2002; Muller et al. 2002; Cheng et al. 2004). Class A neurons are born in the dorsal alar plate and require roof plate signals for specification (Liem et al. 1997; Lee et al. 1998, 2000; Millonig et al. 2000; Muroyama et al. 2002). Post-mitotic neurons of the class A migrate in a characteristic stream into deep layers of the dorsal horn, where they are thought to participate in the processing of proprioceptive information (Bermingham et al. 2001). Class B neurons arise in the ventral alar plate and express Lbx1. Lbx1$^+$ neurons do not require dorsal or ventral signals for their specification and might therefore correspond to a default neuron type produced in the spinal cord. The majority of class B neurons matures to form the upper layers of the dorsal horn (Gross et al. 2002; Muller et al. 2002; Cheng et al. 2004).

Three subtypes of class A neurons, dI1–dI3, can be distinguished in the dorsal alar plate (Bermingham et al. 2001; Gowan et al. 2001; Gross et al. 2002; Muller et al. 2002). Homeobox genes that specify dI1–dI3 neurons and whose expression defines the specific d1–d3 progenitor domains have not been identified. Instead, roof-plate signals establish progenitor domains that express *Math1* and *Ngn1*, two basic helix–loop–helix (bHLH) factor genes that possess proneural activity and also function in the specification of class A neuronal subtypes (Lo et al. 1991; Helms and Johnson 1998). *Math1* is expressed in dI1 progenitors, and gain and loss-of-function experiments demonstrated that *Math1* is essential and sufficient for the generation of dI1 neurons in the alar plate (Bermingham et al. 2001; Nakada et al. 2004). *Ngn1* and *Ngn2* are expressed in dI2 progenitors, and dI2 neurons are not specified in *Ngn1* and *Ngn2* compound mutant mice (Gowan et al. 2001). A gene that imposes a class A character on neurons in the dorsal spinal cord has not been characterized.

The Olig subfamily of bHLH transcription factors was recently identified. *Olig2* is expressed in a progenitor domain of the ventral spinal cord that generates initially motoneurons and, subsequently, oligodendrocytes, and is required for the specification of both cell types (Novitch et al. 2001; Zhou et al. 2001; Lu et al. 2002; Takebayashi et al. 2002a; Zhou and Anderson 2002). In the brain, *Olig1* and *Olig2* coordinately instruct an oligodendrocytic fate. Misexpression experiments in the chick spinal cord indicate that *Olig2* and *Ngn2* cooperate to specify motoneuron identity and pan-neuronal properties, whereas *Olig2* and *Nkx2.2* impose together an oligodendrocytic fate. Temporal shifts in the expression of *Nkx2.2* and *Ngn2* in the Olig2$^+$ progenitor domain coordinate the consecutive generation of motoneurons and oligodendrocytes (Mizuguchi et al. 2001; Qi et al. 2001). The third member of the family, *Olig3*, is expressed in three small groups of post-mitotic neurons in the ventral, and in a broad progenitor domain of the dorsal spinal cord (Takebayashi et al. 2002b). We show here that *Olig3* marks the dorsal progenitors that generate class A neurons. In *Olig3* mutant mice, dI1 neurons are formed in reduced numbers, dI2 and dI3 neurons are not generated, instead, ectopic neurons of the class B appear in the dorsal alar plate. Our experiments demonstrate that *Olig3* is essential for the correct specification of class A neurons in the dorsal spinal cord and suppresses specification of class B neurons.

Results

Olig3 marks progenitor cells of the dorsal spinal cord

The expression of the gene encoding the bHLH factor *Olig3* is detected around embryonic day 9 (E9) in the central nervous system of mice and can be observed in one broad domain of the dorsal and in three small domains of the ventral spinal cord (Takebayashi et al. 2002b). Our immunohistological analysis showed that Olig3 protein is present on all axial levels of the spinal cord in cells located in the ventricular zone of the dorsal alar plate at E10.5 and E12.5 (Fig. 1A,B). The dorsal Olig3$^+$ progenitor domain is broader at E10.5 than at

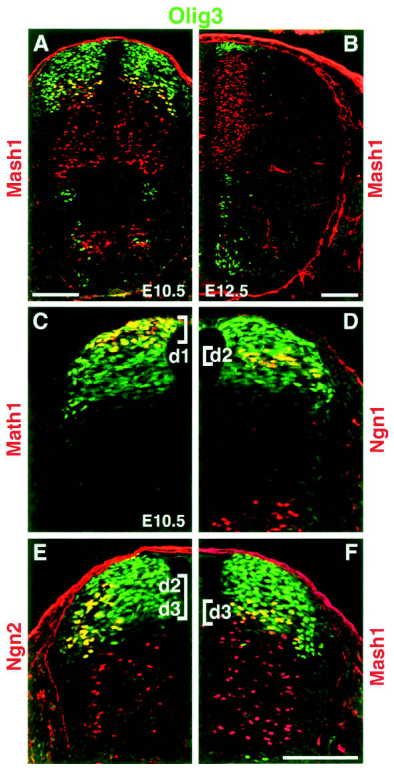

Figure 1. Olig3 marks a dorsal progenitor domain in the spinal cord. Immunofluorescence analysis of the developing mouse spinal cord using antibodies directed against Olig3 (green) at E10.5 (*A*,*C*–*F*) and E12.5 (*B*). In addition, Mash1- (*A*,*B*,*F*), Math1- (*C*), Ngn1- (*D*), and Ngn2- (*E*) expressing cells were visualized (red, the overlap with green appears yellow). Three distinct Olig3$^+$ progenitor domains (d1–d3) can be distinguished, which contain cells that coexpress Olig3 and either Math1 (d1), Ngn1 (d2), or Mash1 (d3). Ngn2 is present in cells of the d2 and d3 domains, and Mash1 is in d3 and in ventrally abutting progenitors (see also Fig. 3C for a summary). Bars, 100 μm.

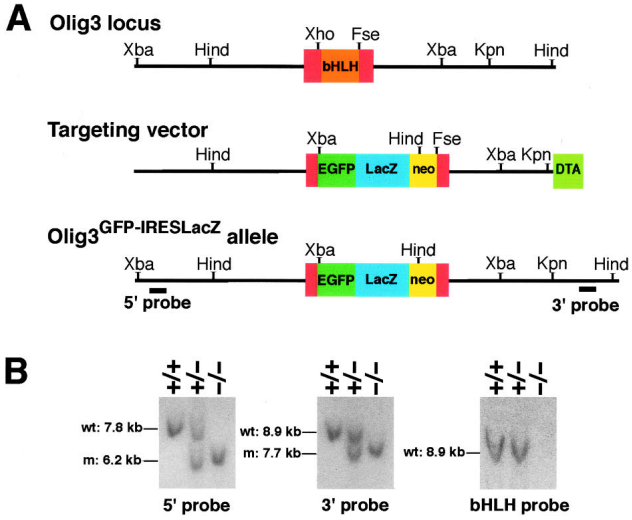

Figure 2. Strategy used to mutate the *Olig3* gene by homologous recombination. (*A*) Targeting strategy used to mutate the *Olig3* gene by homologous recombination in embryonic stem (ES) cells. At the *top*, the wild-type *Olig3* locus is shown that contains a single coding exon (red box) with the sequences encoding the bHLH domain (orange box). In the targeting vector, *Olig3* coding sequences between the XhoI and FseI restriction sites, which include the sequences encoding the bHLH domain, are replaced by an *EGFP–IRES–nlsLacZ* cassette (green/blue box) fused in frame to the 5′-coding sequence and a *Neomycin-resistance* cassette (yellow box). A *diphtheria toxin* (*DTA*, light green) cassette was included for negative selection. The mutant allele after homologous recombination is denoted $Olig3^{GILacZ}$. The locations of the sequences used as probes for verification of the homologous recombination event are indicated at the *bottom* by black bars. The cassettes are not shown in scale. (*B*) Southern blot analysis of genomic DNA digested with XbaI (*left*) and HindIII (*middle* and *right*) from different animals. As probes, 5′- and 3′-sequences as indicated in *A*, and a DNA fragment encoding the bHLH domain of *Olig3* were used. The genotypes of the animals are indicated.

E12.5. BrdU-labeling experiments and the location of dorsal Olig3$^+$ cells indicate that they correspond mainly to proliferating progenitor cells and that Olig3 is quickly down-regulated in post-mitotic neurons (Fig. 1; data not shown). We used additional bHLH proteins as markers to define Olig3$^+$ progenitors at E10.5. Olig3 is coexpressed with Math1 in the most dorsally located progenitor domain of the spinal cord (d1) (Fig. 1C). Ventrally abutting this domain are Ngn1$^+$ and Ngn2$^+$ progenitor cells, which coexpress Olig3 (d2 and d3) (Fig. 1D,E). Further ventrally, a broad domain is observed that contains Mash1$^+$ progenitors. Olig3 is expressed in a dorsal part of the Mash1$^+$ progenitor domain (d3) (Fig. 1F). Thus, three distinct progenitor domains can be defined at E10.5, in which Olig3 is detected and coexpressed with Math1, Ngn1, and Mash1 (see also Fig. 3C for a summary). At E12.5, the overlap of Mash1 and Olig3 expression is no longer detectable (Fig. 1B).

Olig3 is quickly down-regulated in post-mitotic neurons of the dorsal spinal cord, which interferes with a direct analysis of the neuron types generated by Olig3$^+$ progenitors. GFP or β-galactosidase expressed from transgenic constructs have a longer half-life, which can be used to determine cell lineages in the spinal cord. To characterize the neuron types that are produced by Olig3$^+$ progenitors, we generated the $Olig3^{GILacZ}$ allele in which *Olig3* coding sequences were replaced by a *GFP–IRES–LacZ* cassette (Fig. 2). Comparison of the distribution of GFP, β-galactosidase, and Olig3 in heterozygous $Olig3^{GILacZ}$ embryos demonstrated similar expression domains of all three proteins in the spinal cord (Fig. 3A,B). However, subtle differences were apparent; for instance, Olig3 accumulated earlier than GFP and β-galactosidase in progenitors of the dorsal or in neuronal cells of the ventral spinal cord. In the dorsal spinal cord, Olig3 protein was present only in progenitors, whereas GFP and β-galactosidase were present in progenitors and persisted in post-mitotic neurons. We used the $Olig3^{GILacZ}$ allele to determine the neuron types that arose from dorsal Olig3$^+$ progenitors. In heterozygous $Olig3^{GILacZ}$ mice, we detected coexpression of β-galactosidase and Lhx2/9, indicating that Olig3$^+$ progenitors generate dI1 neurons (Fig. 3D). Ventrally abutting neurons coexpress GFP and Foxd3

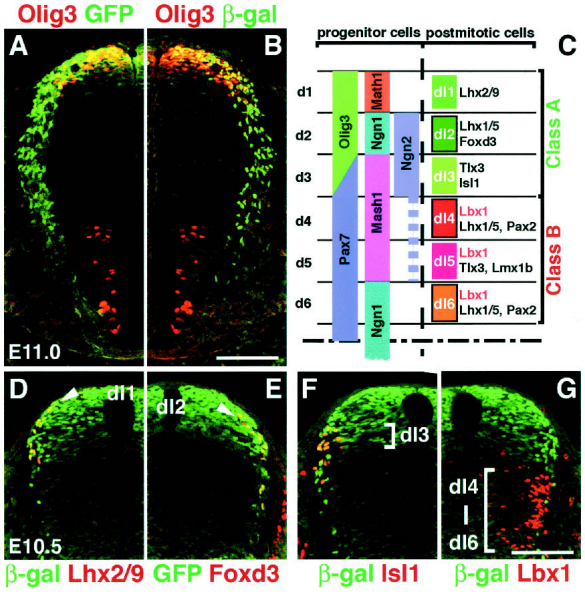

Figure 3. Olig3$^+$ progenitors give rise to the dorsal class A neurons. (*A*,*B*) Comparison of the distribution of Olig3 and of GFP and β-galactosidase produced from the $Olig3^{GILacZ}$ allele at E11.0. Immunohistological analysis using anti-Olig3 (red), anti-GFP and anti-β-galactosidase (β-gal) antibodies (green in *A* and *B*, respectively) shows overlapping expression in dorsal progenitors. Whereas Olig3 is confined to progenitors, GFP and β-galactosidase persist in post-mitotic neurons. (*C*) Schematic display of the six distinct dorsal neuronal subtypes (dI1–dI6) and of the progenitor domains (d1–d6) that produce these neurons. (*D–G*) Characterization of the neuronal subtypes that emerge from Olig3$^+$ progenitors using β-gal or GFP as lineage markers at E10.5. dI1–dI6 neurons are indicated and were detected using antibodies directed against Lhx2/9 (*D*), Foxd3 (*E*), Isl1 (*F*), and Lbx1 (red) (*G*). The distribution of β-gal or GFP is shown in green. Bars, 100 μm.

in heterozygous *Olig3*^{GILacZ} mice, which indicates that Olig3$^+$ progenitors give rise to dI2 neurons (Fig. 3E). Furthermore, neurons that coexpress β-galactosidase and Tlx3 (also known as Rnx) or β-galactosidase and Isl1 were observed in heterozygous *Olig3*^{GILacZ} mice (Fig. 3F; data not shown). Thus, Olig3$^+$ progenitors give rise to dI3 neurons. We conclude, therefore, that three neuron types, dI1–dI3, are generated from the Olig3$^+$ progenitor domain in the dorsal spinal cord (see Fig. 3C for a summary). These three neuron types constitute the class A of the dorsal spinal cord neurons. In heterozygous *Olig3*^{GILacZ} mice, we did not detect Lbx1$^+$ neurons that coexpress β-galactosidase (Fig. 3G). Olig3$^+$ progenitors do not, therefore, produce class B neurons.

Olig3 *is essential for the development of class A neurons*

The *Olig3*^{GILacZ} allele lacks sequences encoding the bHLH domain and the nuclear localization signal, and therefore, does not encode a functional transcription factor. We generated homozygous *Olig3*^{GILacZ} embryos to analyze the function of Olig3. Lhx2/9$^+$ neurons (dI1) arise in the most dorsal part of the alar plate and were present in reduced numbers in *Olig3*^{GILacZ}/*Olig3*^{GILacZ} embryos (Fig. 4A,B). We counted 50 ± 7 and 26 ± 5 Lhx2/9$^+$ neurons in control and *Olig3*^{GILacZ}/*Olig3*^{GILacZ} mice at E11.5, respectively. Foxd3$^+$ dI2 neurons are not detectable in *Olig3*^{GILacZ}/*Olig3*^{GILacZ} embryos (Fig. 4C,D). Furthermore, Tlx3$^+$/Isl1$^+$ dI3 neurons are absent in *Olig3*^{GILacZ}/*Olig3*^{GILacZ} embryos (Fig. 4E,F). Thus, dI1 neurons are formed in reduced numbers, whereas dI2 and dI3 neurons are not generated in *Olig3*^{GILacZ}/*Olig3*^{GILacZ} mice. Instead, an ectopic neuron type (dI2/3*) that expressed β-galactosidase together with Lhx1/5, Pax2, and Lbx1 arose in the dorsal alar plate of the *Olig3*^{GILacZ}/*Olig3*^{GILacZ} embryos (Fig. 4G–J). During normal development, dI4 neurons coexpress Lhx1/5, Pax2, and Lbx1. Therefore, class A neurons are not correctly specified in *Olig3* mutant mice; instead, a neuron type is produced that possesses the characteristics of dI4 neurons. The misspecified neurons of the class A assume therefore the characteristics of a class B neuron type.

The altered specification and differentiation of class A neurons also reflected itself in a change of their migration. We used GFP to follow the class A neurons in heterozygous and homozygous *Olig3*^{GILacZ} mice. Differences in migration were apparent at E11, and were more pronounced by E12.5 (Fig. 4K,L). In heterozygous *Olig3*^{GILacZ} mice, all GFP$^+$ cells assemble in a medial stream, migrate in a ventral direction, and the majority settles in deep layers of the dorsal horn (arrows in Fig. 4K indicate the migratory stream). Lateral positions of the dorsal spinal cord are filled with Lbx1$^+$ class B neurons, and GFP$^+$ cells cannot be observed at these sites (Fig. 4K; data not shown). The medial stream of migratory GFP$^+$ cells was also apparent in the homozygous *Olig3*^{GILacZ}/*Olig3*^{GILacZ} mice (see arrows in Fig. 4L). In addition, many scattered GFP$^+$ cells were detected in lateral positions of the dorsal spinal cord (see arrowheads in Fig. 4L). This indicates that the misspecified neurons present in the spinal cord of *Olig3*^{GILacZ}/*Olig3*^{GILacZ} embryos do not only attain the molecular characteristics of class B neurons, but can also settle at sites typically occupied by class B neurons.

Permissive and instructive Olig3 *functions in the specification of class A neurons*

Expression of Lbx1 marks class B neuronal subtypes. We previously showed that *Lbx1* is essential for the differentiation of class B neurons, and that class B neurons assume the molecular characteristics of class A neurons in *Lbx1*$^{-/-}$ mice. Misexpression of *Lbx1* in the chick spinal cord leads to changes in specification of class A neurons that are remarkably similar to those observed in *Olig3* mutant mice, i.e., reduction of the numbers of dI1 neurons, and misspecification of dI2 and dI3 neurons that assume the fate of a class B (dI4) neuronal subtype. An apparent antagonism thus exists between *Olig3* and *Lbx1*, and *Olig3* might exert its function primarily by

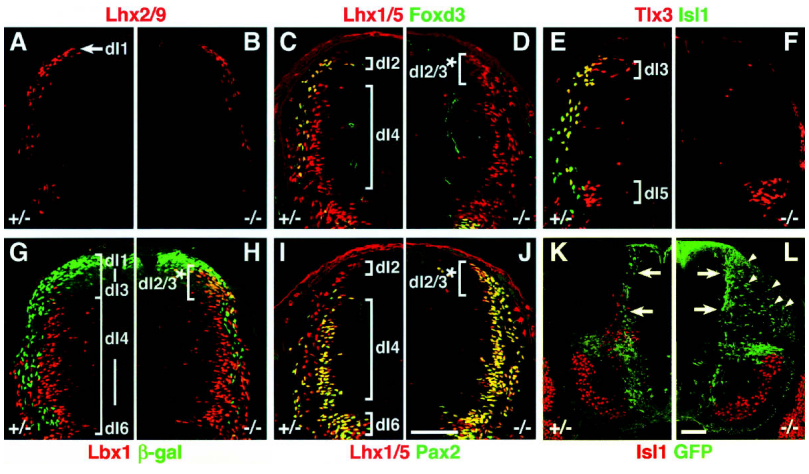

Figure 4. *Olig3* is essential for the correct specification of class A (dI1–dI3) neurons. The dorsal spinal cord of E11.0 (*A–J*) and E12.5 (*K,L*) embryos heterozygous (*A,C,E,G,I,K*) or homozygous (*B,D,F,H,J,L*) for the *Olig3*^{GILacZ} allele were analyzed using antibodies against Lhx2/9 (red) (*A,B*), Lhx1/5 (red) and Foxd3 (green) (*C,D*), Tlx3 (red) and Isl1 (green) (*E,F*), Lbx1 (red) and β-gal (green) (*G,H*), Lhx1/5 (red) and Pax2 (green) (*I,J*), and Isl1 (red) and GFP (green) (*K,L*). The arrow in *A* points to dI1 neurons. The brackets in *C*, *E*, *G*, and *I* mark the various indicated neuron types. In *D*, *H*, and *J*, the brackets mark the misspecified dI2/3* neurons. In *K* and *L*, the arrows point to the medial migratory stream of GFP$^+$ neurons, and the arrowheads indicate ectopically located GFP$^+$ neurons in *Olig3* mutant embryos. Bars, 100 μm.

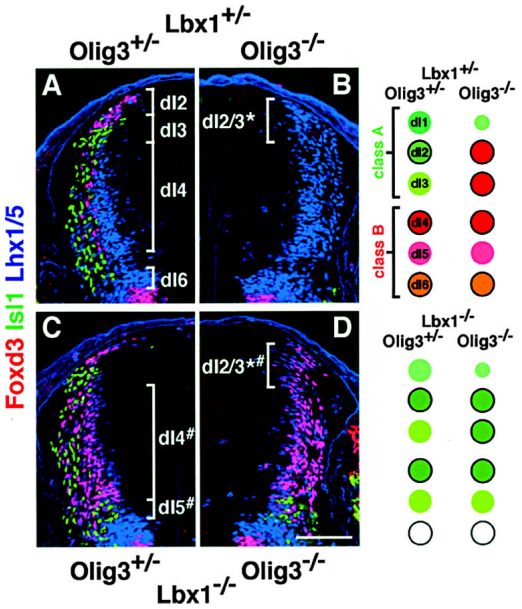

Figure 5. Permissive and instructive functions of *Olig3* revealed by the analysis of *Olig3*/*Lbx1* double-mutant mice. Specification of class A neuronal subtypes in $Lbx1^{+/-}$;$Olig3^{GILacZ/+}$ (*A*), $Lbx1^{+/-}$;$Olig3^{GILacZ/GILacZ}$ (*B*), $Lbx1^{-/-}$;$Olig3^{GILacZ/+}$ (*C*), and $Lbx1^{-/-}$;$Olig3^{GILacZ/GILacZ}$ (*D*) embryos at E11 was analyzed by immunohistology using antibodies directed against Foxd3 (red), Isl1 (green), and Lhx1/5 (blue). Foxd3 and Lhx1/5 double-positive neurons appear magenta. (*A*) Dorsal neuronal subtypes are indicated by brackets and are denoted dI2–dI6 in control mice. Misspecified neuronal subtypes observed in *Olig3* mutant mice are designated as dI2/3* (*B*), those observed in *Lbx1* mutant mice are indicated by dI4/5$^{\#}$ (*C*), and those present in Olig3/Lbx1 double-mutant mice are denoted as dI2/3$^{\star\#}$ (*D*). A summary of the neuronal subtypes generated in the alar plate of the various mutant mice is shown to the *right*.

suppressing the emergence of Lbx1^{+} class B neurons. We therefore investigated whether the deficits in neuronal specification of *Olig3* mutants were rescued in the absence of *Lbx1*. *Olig3*/*Lbx1* double-mutant mice were generated and used for an analysis of the development of class A neurons (Fig. 5). Lhx2/9^{+} dI1 neurons were formed in comparable numbers in *Olig3* and in *Olig3*/*Lbx1* double-mutant mice (data not shown). Foxd3^{+} dI2 neurons are not specified in *Olig3* mutants, and their generation was rescued in *Olig3*/*Lbx1* double-mutant embryos (Fig. 5B,D). However, the appearance of Tlx3^{+}/Isl1^{+} dI3 neurons was not rescued (see Fig. 5 for a summary). Therefore, *Olig3* does not solely suppress the appearance of Lbx1^{+} class B neurons, but provides additional instructive information necessary for the generation of the dI3 neuronal subtype.

Olig3 *is essential to maintain correct* Math1 *and* Ngn1/2 *expression in dorsal progenitors*

To investigate the mechanisms that cause the misspecification of class A neurons further, we analyzed gene expression in the progenitor domain of the $Olig3^{GILacZ}$/$Olig3^{GILacZ}$ mice. Compared with control embryos, the expression domain of *Math1* was reduced in size in the dorsal alar plate of $Olig3^{GILacZ}$/$Olig3^{GILacZ}$ mice at E10.5 (Fig. 6A,B). However, *Mash1* expression was similar in control and $Olig3^{GILacZ}$/$Olig3^{GILacZ}$ embryos (data not shown). Furthermore, the results of an in situ hybridization with mixed *Math1* and *Mash1* probes indicated that the dorsal border of the *Mash1* expression domain did not shift significantly in the spinal cord of $Olig3^{GILacZ}$/$Olig3^{GILacZ}$ mice at E11.5 (Fig. 6C,D). In addition, the expression of *Ngn1* and *Ngn2* in the dorsal alar plate was reduced at E10.5 (Fig. 6E–H). In contrast, *Pax6* and *Pax7*, as well as *Gsh1* and *Gsh2* were expressed in the alar plate in a similar manner in control and $Olig3^{GILacZ}$/$Olig3^{GILacZ}$ embryos (data not shown). *Lmx1a* expression marks the roof plate, whereas *Msx1* is expressed in cells in and near the roof plate. *Lmx1a* and *Msx1* expression were similar in control and $Olig3^{GILacZ}$/$Olig3^{GILacZ}$ embryos (Fig. 6I–L). BMP and Wnt signals pattern the dorsal spinal cord, and we therefore analyzed BMP and Wnt signalling in $Olig3^{GILacZ}$/$Olig3^{GILacZ}$ mice. The distribution of phospho-Smad1/5/8 and the expression of *conductin*/*Axin2*, which mark BMP and Wnt responding cells, were similar in control and $Olig3^{GILacZ}$/$Olig3^{GILacZ}$ embryos (Fig. 6M–P). Furthermore, the expression of *GDF7*, *BMP6*, *BMP7*, *Wnt1*,

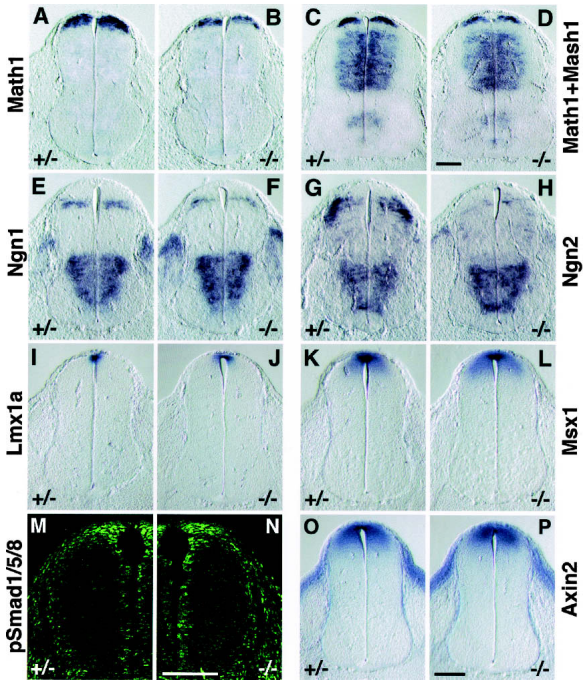

Figure 6. Gene expression in progenitors of the dorsal spinal cord of *Olig3* mutant embryos. The expression of various genes in the dorsal spinal cord was assessed by in situ hybridization at E10.5 (*A*,*B*,*E*–*P*) and E11.5 (*C*,*D*) of heterozygous (*A*,*C*,*E*,*G*,*I*,*K*,*O*) and homozygous (*B*,*D*,*F*,*H*,*J*,*L*,*P*) *Olig3* mutant embryos using probes specific for *Math1* (*A*,*B*), *Math1* and *Mash1* (*C*,*D*), *Ngn1* (*E*,*F*), *Ngn2* (*G*,*H*), *Lmx1a* (*I*,*J*), *Msx1* (*K*,*L*), and *Axin2*/*conductin* (*O*,*P*). The distribution of phospho-Smad1/5/8 was assessed by immunohistological analysis in heterozygous (*M*) and homozygous (*N*) *Olig3* mutant embryos. Bars, 100 µm.

Wnt3, *Wnt3a*, and *Wnt4* were similar in control and $Olig3^{GILacZ}/Olig3^{GILacZ}$ mice (data not shown). Thus, we found no evidence for a reduction in BMP or Wnt signaling in the dorsal spinal cord of *Olig3* mutant mice. Similarly, the expression of *Foxd3* and *Sox10*, which mark premigratory and migratory neural crest cells, were similar in control and $Olig3^{GILacZ}/Olig3^{GILacZ}$ mice (data not shown). We conclude therefore that *Olig3* is essential to maintain the correct expression of the bHLH factors *Math1*, *Ngn1*, and *Ngn2* in the dorsal alar plate of the developing spinal cord.

Factors of the bHLH family are known to possess proneural activity. We therefore determined the numbers of neurons generated in the dorsal alar plate of *Olig3* mutant mice using a BrdU-labeling protocol. Overall numbers of $GFP^+/BrdU^+/Kip1^+$ neurons were determined at E11.5, 28 h after BrdU injection. In hetero- and homozygous $Olig3^{GILacZ}$ embryos, we counted 86 ± 9 and 50 ± 7 cells, respectively, indicating that overall neurogenesis in the dorsal alar plate was reduced in the homozygous mutants.

Olig3 *antagonizes the differentiation of class B neurons*

We tested whether misexpression of *Olig3* interferes with development of spinal cord neurons (Fig. 7). Chick embryos were electroporated in-ovo with an expression construct that produces mouse Olig3. Electroporations were performed at HH16, and embryos were analyzed 30 h later to assess the generation of dorsal neurons that are born later than ventral neuron types in the spinal cord. On the electroporated side of the spinal cord, we observed an increase in numbers of $Isl1^+$ neurons in the alar plate, whereas the numbers of $Lbx1^+$ neurons were decreased (Fig. 7A,B, and see also C for the quantification of the results). Most of the ectopic $Isl1^+$ cells expressed Tlx3, but not Lmx1b and Lbx1, and appeared at the position where dI5 neurons are generated on the nonelectroporated control side of the spinal cord (Fig. 7A; data not shown). The overall numbers of $Tlx3^+$ neurons did not, however, change. The ectopic $Isl1^+/Tlx3^+$ neurons thus expressed the set of transcription factors characteristic for dI3 neurons, but appeared in the ventral alar plate. We conclude that *Olig3* misexpression induces *Isl1* and represses *Lbx1* and leads to the appearance of an ectopic class A neuronal subtype in the ventral alar plate, which is produced at the expense of class B neurons.

We next tested whether Olig3 functions as a transcriptional repressor or as an activator by fusing the bHLH domain of Olig3 to the repressor domain of *Drosophila Engrailed* ($Olig3^{EnR}$) and the transactivation domain of *Herpes simplex* VP16 ($Olig3^{VP16}$). Electroporation of $Olig3^{EnR}$ into the chick spinal cord caused similar qualitative and quantitative changes in neuronal specification as observed after expression of *Olig3*, i.e., an increase in the numbers of $Isl1^+/Tlx3^+$ neurons, and a decrease in $Lbx1^+$ neurons (Fig. 7D–F). In contrast, electroporation of $Olig3^{VP16}$ suppressed the emergence of $Isl1^+/Tlx3^+$ and

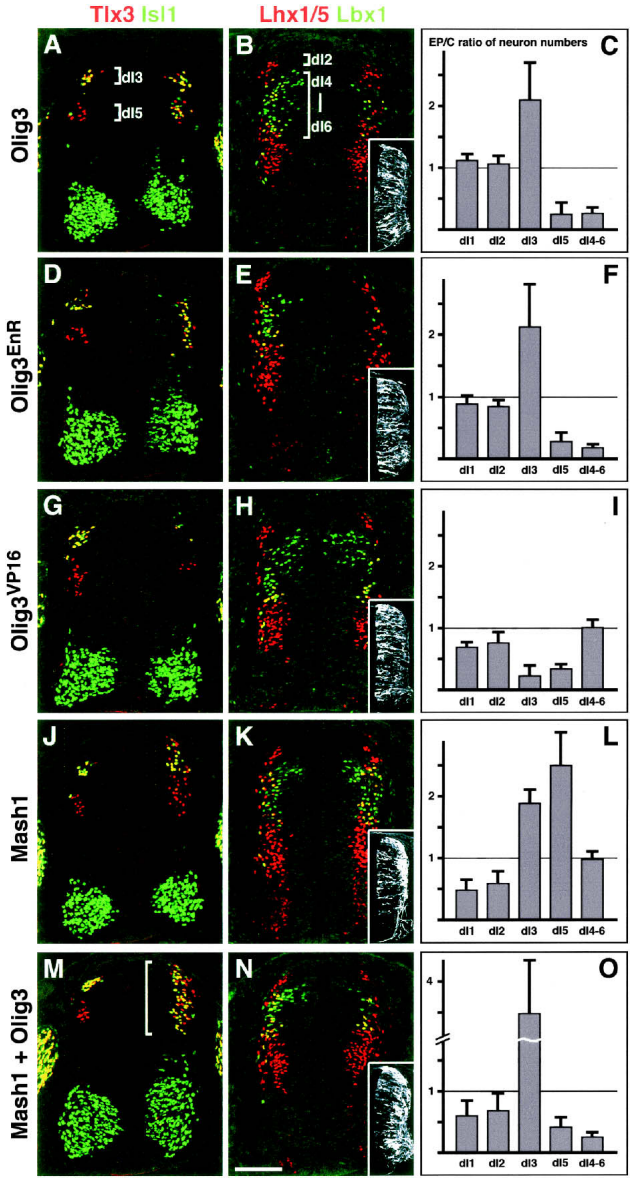

Figure 7. *Olig3* acts as a transcriptional repressor and cooperates with *Mash1* to instruct the dI3 neuronal subtype. Chick spinal cords were electroporated on the right side with mouse *Olig3* (*A*,*B*), $Olig3^{EnR}$ (*D*,*E*), $Olig3^{VP16}$ (*G*,*H*), *Mash1* (*J*,*K*), *Olig3* and *Mash1* (*M*,*N*), and the effect on the development of neurons was assessed using antibodies against Tlx3 (red) and Isl1 (green) (*A*,*D*,*G*,*J*,*M*), and Lhx1/5 (red) and Lbx1 (green) (*B*,*E*,*H*,*K*,*N*). The *insets* in *B*, *E*, *H*, *K*, and *N* show the expression of the coelectroporated GFP in the right side of the spinal cord. The brackets in *A* indicate dI3 and dI5 neurons, and the brackets in *B* point to dI2 and dI4–dI6 neurons. The bracket in *M* highlights the increased numbers of dI3 neurons. The quantification of the neuronal subtypes generated in the electroporated and the control side of the spinal cord are shown in *C*, *F*, *I*, *L*, and *O*. For this, numbers for various neuronal subtypes were counted; the data are displayed as the ratio of neuron numbers generated on the electroporated side and the control side. A ratio of 1 indicates no effect, a value <1 indicates a suppression, and a value >1 indicates an induction of a particular neuronal subtype. Bar, 100 μm.

Lmx1b$^+$/Tlx3$^+$ neurons, but did not affect the generation of Lbx1$^+$ neurons (Fig. 7G–I). In specification of dorsal neurons, Olig3 thus acts as a transcriptional repressor.

Loss-of-function experiments indicate that *Olig3* provides instructive information for specification of dI3 neurons. Misexpression of *Olig3* induced the appearance of Isl1$^+$ dI3 neurons mainly at one site along the dorso–ventral axis, the position at which dI5 neurons arise in normal development. This indicates that *Olig3* cooperates with other factors to instruct the specification of dI3 neurons. *Olig3* and *Mash1* are coexpressed in the progenitor domain that generates dI3 neurons, and we therefore misexpressed *Mash1* alone or together with *Olig3*. Misexpression of *Mash1* did not affect the overall number of Lbx1$^+$ neurons generated. However, *Mash1* induced Tlx3 on the electroporated side of the spinal cord (Fig. 7J–L). In the dorsal alar plate, ectopic Tlx3$^+$ neurons were Isl1$^+$. In the ventral alar plate, the ectopic Tlx3$^+$ neurons coexpressed Lmx1, but not Isl1 (Fig. 7J; data not shown). Thus, misexpression of *Mash1* induces Tlx3 and leads to the appearance of ectopic neurons that express the homeodomain factor codes of dI3 and dI5 neurons in the dorsal and ventral alar plate, respectively. Misexpression of both *Olig3* and *Mash1* strongly increased the numbers of Isl1$^+$/Tlx3$^+$ dI3 neurons in the dorsal spinal cord and suppressed *Lbx1*, *Lmx1b*, and *Lhx1/5* (Fig. 7M–O; data not shown). *Olig3* and *Mash1* therefore cooperate to impose a dI3 fate, and thus, a class A character on neurons in the alar plate. *Olig3* and *Olig3^{EnR}* affected neuronal specification in a similar manner in such coelectroporation experiments. It should be noted that Mash1 and Olig3 coelectroporation induced dI3 neurons in a pronounced manner in the dorsal, but not the ventral spinal cord, indicating that further positional information is important for specification of dI3 neurons.

Discussion

Two classes of neurons, class A and B, are generated in the dorsal spinal cord. We show here that the bHLH factor Olig3 marks the progenitors that give rise to class A neurons. Class A neurons consist of three neuronal subtypes (dI1–dI3) that arise in the dorsal alar plate, and require signals provided by the roof plate for specification. Class B neurons (dI4–dI6) express Lbx1 and can emerge independently of dorsal signals in the ventral alar plate. Our analysis demonstrates that *Olig3* is an important determinant for specifying class A neurons (see Fig. 8). In *Olig3* mutant mice, dI1 neurons are generated in reduced numbers, and dI2 and dI3 are not formed. Instead, neurons are generated that express homeodomain factors typical of class B neurons and that settle at aberrant positions in the dorsal spinal cord. Our analyses indicate that an important aspect of *Olig3* function is the suppression of Lbx1, and thus, the appearance of class B neurons. However, *Olig3* also provides instructive information essential for specification of one particular class A neuronal subtype, the dI3 neurons.

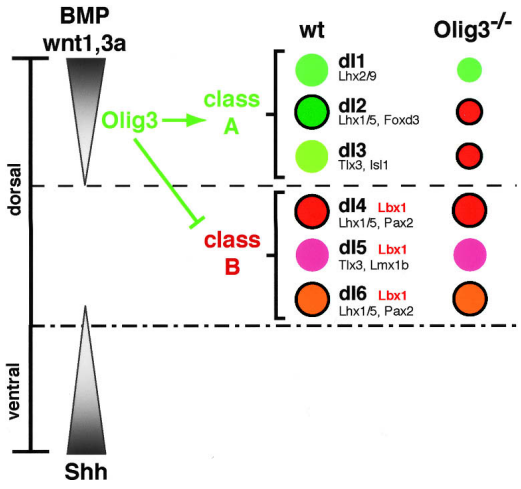

Figure 8. Olig3 and the specification of neurons in the dorsal spinal cord. Schematic diagram of dorsal spinal cord neurons and of the function of Olig3 in their development. BMP, Wnt, and Shh, secreted factors that pattern the dorsal and ventral progenitor domains, are indicated at *left*. The class A (green) and B (red) neuronal subtypes generated in the dorsal spinal cord are indicated. The positions along the dorso–ventral axis at which these neurons arise are shown, as well as the homeodomain factor code they express in wild-type mice. In *Olig3* mutant mice, class A neurons assume abnormal molecular characteristics, and express homeodomain factor combinations typical of class B neurons (as indicated by color code).

The roles of Olig3 *in the development of class A neuronal subtypes*

Three class A neuronal subtypes, dI1–dI3, exist that require *Olig3* for their correct development. The generation of dI1 neurons is only mildly affected by the mutation of *Olig3*, and such neurons are formed, albeit at reduced numbers. *Math1* is an important determinant in the specification of dI1 neurons; dI1 neurons are not formed in *Math1*$^{-/-}$ mice, and misexpression of *Math1* in the chick spinal cord suffices to generate ectopic dI1 neurons (Bermingham et al. 2001; Nakada et al. 2004). In the dorsal spinal cord of *Olig3* mutant mice, Math1 is expressed, but the expression domain is reduced in size. This is in accordance with the emergence of reduced numbers of dI1 neurons. Thus, *Olig3* appears to affect the generation of dI1 neurons by maintaining correct *Math1* expression in the dorsal progenitor domain.

dI2 and dI3 neurons are not specified in *Olig3* mutant mice, and a Lbx1$^+$ neuronal subtype of the class B is generated instead in the dorsal alar plate. Misexpression of *Lbx1* in the chick spinal cord and the mutation of *Olig3* cause similar changes in neuronal specification (Gross et al. 2002; Muller et al. 2002). We therefore tested whether Olig3 exerts its role solely by suppressing *Lbx1* expression in post-mitotic neurons. If this were the case, changes in specification of dI2 and dI3 neurons present in *Olig3* mutant mice should be reverted by the mutation of *Lbx1*. Analysis of *Olig3*/*Lbx1* double-mutant mice demonstrates that this is indeed the case for

the specification of dI2, but not of dI3 neurons. We conclude that during the generation of dI2 neurons, *Olig3* is required, but does not act in an instructive manner; *Olig3* exerts its function by suppressing the expression of *Lbx1*. In contrast, the function of *Olig3* in development of dI3 neurons is more complex, since dI3 neurons are formed neither in *Olig3* nor *Olig3*/*Lbx1* double-mutant mice. *Olig3* appears therefore to provide instructive information for the specification of dI3 neurons.

Overexpression experiments in the chick also support the dual role of Olig3 in specification of class A neurons. Olig3 suppresses *Lbx1* expression, a prerequisite for the specification of class A neurons. Furthermore, Olig3 induces the appearance of $Isl1^+$/$Tlx3^+$ neurons in the ventral alar plate, a reflection of its instructive function during specification of dI3 neurons. In such electroporation experiments, *Olig3* is expressed throughout the spinal cord, but induces *Isl1* expression mainly in neurons generated in the dI5 domain, indicating that it cooperates with other factors in specification of dI3 neurons. Electroporation experiments indicate that Mash1 can provide such a cooperative activity, since Olig3 and Mash1 effectively induce dI3 neurons in the entire alar plate. In such Mash1 and Olig3 electroporation experiments, a pronounced induction of dI3 neurons is observed in the dorsal, but not the ventral spinal cord, indicating that further positional information is important for specification of dorsal neuron subtypes. Endogenous Mash1 is present in progenitors of dI4 neurons, but this endogenous protein appears to be unable to cooperate effectively with Olig3. Endogenous Mash1 could be present in insufficient quantity, or might be inhibited by other mechanisms. dI3 neurons are produced at significantly reduced numbers in *Mash1* mutant mice (H. Wildner, unpubl.) and are not specified in *Olig3* mutants. Olig3 can thus cooperate with Mash1, and, potentially, with another factor to specify dI3 neurons in vivo. As development proceeds, the $Olig3^+$ domain retracts and does not overlap with Mash1 at E12.5. This coincides with the production of class B neurons from the entire Mash1 domain in the alar plate of the spinal cord.

The expression of *Math1*, *Ngn1*, and *Ngn2* was not appropriately maintained in *Olig3* mutant mice. *Math1* and *Ngn1/2* do not only function in neuronal specification, but also have proneural activity, forcing progenitors to exit the cell cycle and initiate neuronal differentiation (Lo et al. 1991). The reduced expression of these genes might contribute to the observed reduction in neurogenesis in the alar plate of *Olig3* mutant mice. Despite the fact that *Olig3* encodes a bHLH factor, its proneural activity in electroporation experiments is low compared with *Mash1*. For instance, 24 h after electroporation of *Olig3*, we observed many cells in the progenitor zone that ectopically expressed the factor, proliferated, and had incorporated BrdU. In contrast, cells that ectopically expressed Mash1 were located in the mantle zone and had left the cell cycle (data not shown).

Is Olig3 *a patterning gene?*

The ventral spinal cord provides a well-studied example of the molecular mechanisms used for patterning of neural progenitors and for neural specification (Jessell 2000; Shirasaki and Pfaff 2002). Shh, expressed initially in the notochord and subsequently in the floor plate, acts in a graded manner to regulate the expression of a set of patterning genes in neural progenitors of the ventral spinal cord. Many of these patterning genes encode homeodomain transcription factors and fall into two categories, which are either repressed or induced by Shh. Cross-repressive interactions between these two types of factors refine and maintain distinct stripes of progenitor domains that give rise to different neural cell types (Briscoe et al. 2000). Typically, mutations of such patterning genes lead to the expanded expression of a neighboring opposing factor into an inappropriate domain, and result in the generation of misspecified neurons from the altered progenitor domain (Briscoe et al. 1999; Sander et al. 2000; Pierani et al. 2001; Vallstedt et al. 2001). *Olig2* is closely related to *Olig3* and acts in a similar manner as classical patterning genes, i.e., its mutation leads to the spatially expanded expression of homeobox genes and to an altered specification of neural cell types (Lu et al. 2002; Takebayashi et al. 2002a; Zhou and Anderson 2002). Furthermore, *Olig2* misexpression suppresses the expression of homeobox factors like *Irx3* in spinal cord progenitors (Zhou et al. 2000; Mizuguchi et al. 2001; Novitch et al. 2001). Also, genes that encode bHLH factors like *Math1* or *Ngn1/2* can act in a remarkably similar manner to the homeobox patterning genes, i.e., *Math1* and *Ngn1/2* are expressed in progenitors, they are essential for specification of post-mitotic dI1 and dI2 neurons, respectively, and they cross-repress the expression of each other (Gowan et al. 2001; Nakada et al. 2004). Olig3 might act in a similar manner and might repress the expression of genes essential to specify class B neurons in the dorsal spinal cord. We tested genes like *Mash1*, *Irx3*, *Pax6*, *Pax7*, *Gsh1*, and *Gsh2* that are expressed in progenitors of class B neurons. However, in *Olig3* mutant mice, we did not identify a gene whose expression expanded inappropriately into the progenitor domain that gives rise to class A neurons. This might reflect the fact that important patterning genes in the dorsal spinal cord remain to be discovered. Alternatively, Olig3 might not suppress transcription of an unknown patterning gene, but might use other mechanisms to interfere with the function of gene(s) important for the specification of class B neurons.

Materials and methods

Mouse strains and chick in ovo electroporation

A BAC clone containing *Olig3* was isolated from a 129-mouse BAC library (Research Genetics). Genomic 7.8-kb XbaI and 8.9-kb HindIII fragments containing the *Olig3* gene were subcloned. The targeting vector contains a 5.3-kb 5′-homologous arm generated by PCR (primer–XhoI fragment) and a 3.9-kb

3′-homologous arm (FseI–KpnI fragment). The DNA between the XhoI and FseI sites includes sequences encoding the bHLH domain and was deleted. The remaining N-terminal sequence was fused in frame with an EGFP–IRES–nlsLacZ cassette (provided by Tom Jessell, Columbia University, New York), and is followed by a neo^r cassette. The MC1-diphtheria toxin A (DTA) cassette was placed at the 3′ end of the vector and was used for negative selection. E14 ES cell colonies that had incorporated the targeting vector into their genome were selected by G418, and analyzed for homologous recombination events by Southern analysis. We injected blastocysts and identified chimeras that transmitted the mutant *Olig3* gene, *Olig3*^{GILacZ}, as reported (Schmidt et al. 2004). Routine genotyping was performed by PCR, and occasionally, genotypes were verified by Southern hybridization.

The full-length mouse *Olig3* coding sequence and fusion constructs that encode the Olig3 bHLH domain were inserted into the pCS2-MT$_6$ and RCAS(B) vectors. Mouse *Olig3* sequences encoding the bHLH domain (amino acids 70–161, counting from the first in-frame ATG) were fused to the *Herpes simplex* VP16 transactivation domain (Triezenberg et al. 1988) or the *Drosophila* Engrailed repressor domain (Smith and Jaynes 1996). The *Mash1* coding sequence was cloned into the RCAS(A) vector. These vectors were electroporated unilaterally into the spinal cord of stage HH16 chick embryos using a T820 electrosquareporator (BTX, Inc.), and the electroporated embryos were analyzed 30 h later. This allowed the assessment of neuronal specification in the dorsal spinal cord, because at the time of analysis, substantial numbers of dorsal neurons were present. Using electroporation at these late stages, we observed no major effects on the generation of motoneurons, presumably because these are born before dorsal neurogenesis occurs. pEGFP-c1 (Clontech) was coelectroporated, and embryos that did not express *EGFP* dorsally were excluded from the analysis. The effects observed after electroporation of *Olig3* expressed by the use of the pCS2-MT$_6$ and RCAS(B) vectors were similar, but subtle differences were apparent that might reflect the production of different amounts of protein. In particular, induction of Isl1$^+$/Tlx$^+$ dI3 neurons in the dI5 domain by misexpression of *Olig3* alone was more pronounced using the RCAS(B) vector. In contrast, after coelectroporation of *Mash1* and *Olig3*, the induction of ectopic dI3 neurons in the entire alar plate was more pronounced when the pCS2-MT$_6$ vector was used. For the experiments shown in Figure 7, we used the Olig3 RCAS(B) vector. The effects of the electroporated factors on neuronal specification were quantified as follows: The numbers of Lhx2/9$^+$ (dI1), dorsal Lhx1/5$^+$/Lbx1$^-$ (dI2), Isl1$^+$/Tlx3$^+$ (dI3), Isl1$^-$/Tlx3$^+$ (dI5), and Lbx1$^+$ (dI4–dI6) neurons in the dorsal alar plate were determined on the electroporated and the contralateral control side of embryos. A minimum of 10 sections from at least three independently electroporated embryos were counted. For BrdU-labeling experiments, 50 µL of 10 mM BrdU in PBS was applied to chick embryos in-ovo 24 h after electroporation. Embryos were incubated for 1 h at 38°C before dissection.

Generation of anti-Olig3 and Tlx3 antisera

Using RT–PCR, coding sequences for *Olig3* and *Tlx3* were amplified and inserted into the bacterial expression vector pET14b (Novagen), which provided coding sequences for a His$_6$-tag. His$_6$-Olig3, and His$_6$-Tlx3 were produced in the bacterial strain BL21(DE3)pLysS. Proteins were affinity purified on TALON metal resin (Clontech) and injected into rabbits and guinea pigs (Sequence Laboratories).

In situ hybridization, immunofluorescence, BrdU labeling, and histology

For in situ hybridization, embryonic tissues were embedded into OCT compound (Sakura) and cryosectioned. Hybridizations were performed with DIG-labeled riboprobes. We obtained many plasmids for generation of probes from other laboratories, as indicated in Acknowledgments. Whole-mount in situ hybridization was performed as described previously (Brohmann et al. 2000).

Immunofluorescence staining was performed on 12-µm cryosections of mouse embryos fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The following antibodies were used: rabbit and guinea pig anti-Lbx1; rabbit anti-Ngn1 (Jane Johnson, University of Texas Southwestern Medical Center, Dallas, TX); mouse anti-Mash1 and anti-Ngn2 (David Anderson, California Institute of Technology, Pasadena, CA); rabbit anti-Foxd3 (Martyn Goulding [Salk Institute, La Jolla, CA] and Dietmar Zechner, [MDC, Berlin, Germany]); goat (Biogenesis) and rabbit (Cappel) anti-β-gal; fluorophore-conjugated secondary antibodies (Dianova), mouse anti-p27^{Kip1} (BD Transduction Laboratories), rabbit anti-phospho-Smad1/5/8 (Cell Signaling), monoclonal anti-Lhx1/5, anti-Pax6, and anti-Pax7 (Developmental Studies Hybridoma Bank, University of Iowa). In addition, guinea pig anti-Isl1, guinea pig anti-Lmx1b, rabbit anti-Lhx2/9, and rabbit anti-Math1 were obtained from Tom Jessell and collaborators.

For BrdU-labeling experiments in mice, BrdU (Sigma; 75 µg/g body weight) was injected intraperitoneally at E10.5. Embryos were isolated 2 or 28 h after injection. Following neuron type-specific antibody staining, incorporated BrdU was detected with mouse (Sigma) and rat anti-BrdU mAbs (Oxford Biotechnology). Neuron numbers were counted on confocal images of sections from at least three distinct animals for each genotype.

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