Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons

Amy W. Helms[1], James Battiste[1], R. Michael Henke[1], Yuji Nakada[1], Nicolas Simplicio[2], Francois Guillemot[2] and Jane E. Johnson[1,*]

[1]Center for Basic Neuroscience, UT Southwestern Medical Center, Dallas, TX 75390, USA
[2]Division of Molecular Neurobiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
*Author for correspondence (e-mail: Jane.Johnson@UTSouthwestern.edu)

Accepted 12 April 2005

Development 132, 2709-2719
Published by The Company of Biologists 2005
doi:10.1242/dev.01859

Summary

The dorsal spinal cord contains a diverse array of neurons that connect sensory input from the periphery to spinal cord motoneurons and brain. During development, six dorsal neuronal populations (dI1-dI6) have been defined by expression of homeodomain factors and position in the dorsoventral axis. The bHLH transcription factors Mash1 and Ngn2 have distinct roles in specification of these neurons. Mash1 is necessary and sufficient for generation of most dI3 and all dI5 neurons. Unexpectedly, dI4 neurons are derived from cells expressing low levels or no Mash1, and this population increases in the Mash1 mutant. Ngn2 is not required for any specific neuronal cell type but appears to modulate the composition of neurons that form. In the absence of Ngn2, there is an increase in the number of dI3 and dI5 neurons, in contrast to the effects produced by activity of Mash1. Mash1 is epistatic to Ngn2, and, unlike the relationship between other neural bHLH factors, cross-repression of expression is not detected. Thus, bHLH factors, particularly Mash1 and related family members Math1 and Ngn1, provide a code for generating neuronal diversity in the dorsal spinal cord with Ngn2 serving to modulate the number of neurons in each population formed.

Key words: Spinal cord development, Dorsal horn, bHLH, Neuronal specification, Mouse, Atoh1, Neurog2, Ascl1

Introduction

The circuitry of the nervous system depends on complex connections of diverse neuronal cell types. Thus, defining the mechanisms that regulate neuronal diversity is fundamental for understanding how the nervous system functions. Specification of distinct neuronal sub-types begins as early as the neural tube stage when differences in progenitor cells in the ventricular zone can be detected by differences in gene expression patterns (Briscoe et al., 2000; Caspary and Anderson, 2003; Helms and Johnson, 2003; Jessell, 2000). As the neural tube develops, proliferating cells exit the cell cycle, and the newly born neurons migrate laterally from the ventricular zone and begin to exhibit their specific neuronal identities. There is a complex interplay between extrinsic and intrinsic factors that mediate the precise timing of cell-cycle exit, cell migration and initiation of cellular properties specific to newborn neurons. It has been demonstrated in the spinal neural tube that the acquisition of identity involves a gradient of extracellular signals that set up a combinational code of transcription factors (Briscoe et al., 2000; Jessell, 2000). In this study, the role of two transcription factors, Mash1 and Ngn2, in the specification of neurons in the dorsal spinal cord is examined.

Neurons in the mouse dorsal spinal cord are largely born between embryonic day 10 and 14 (E10-E14). These neurons provide the network that connects and modulates sensory input from the periphery to the spinal cord and brain. Currently there are six early (dI1-dI6) and two late (dIL^{A} and dIL^{B}) dorsal neuron types defined by birthdate, position in the dorsoventral axis, and distinct homeodomain (HD) transcription factor markers (Caspary and Anderson, 2003; Helms and Johnson, 2003). The most dorsal of these, dI1-dI3, are dependent on roof plate signals, whereas dI4-dI6 and dIL$^{A/B}$ form independently of these signals and are distinguished by the presence of the HD factor Lbx1 (Gross et al., 2002; Lee et al., 2000; Müller et al., 2002). The combinations of HD factors in early stages of neuronal differentiation have been crucial for defining these distinct dorsal interneuron populations. Much less is known about how these neurons contribute to the overall circuitry of the spinal cord (Cheng et al., 2004; Lanuza et al., 2004). Mice mutant for specific HD factors, such as Lbx1 and Tlx1/3, have been used to link the fate of some of these populations to a GABAergic or glutamatergic-specific neuronal type at later stages (Cheng et al., 2004; Gross et al., 2002; Müller et al., 2002).

Progenitor populations located in the ventricular zone of the neural tube can also be classified by transcription factor patterns. In ventral regions, the primary determinants of neuronal specification are combinations of HD transcription factors (Briscoe et al., 2000; Ericson et al., 1997; Pierani et al., 2001). By contrast, dorsal progenitor domains have largely been defined by bHLH transcription factors (Gowan et al., 2001). A role for bHLH factors in specification of dorsal neurons was first suggested by expression domains in the dorsoventral axis of Math1 (Atoh1 – Mouse Genome

Informatics), Ngn1 (Neurog1 – Mouse Genome Informatics), Mash1 (Ascl1 – Mouse Genome Informatics) (Gowan et al., 2001; Helms and Johnson, 1998; Lo et al., 1991; Ma et al., 1997; Sommer et al., 1996) and, more recently, Olig3 (Müller et al., 2005). Each of these factors represents a distinct subclass of neuronal bHLH (Bertrand et al., 2002). Within the dorsal neural tube, Math1 is in ventricular zone cells adjacent to the roof plate (Helms and Johnson, 1998), Ngn1 is just ventral to the Math1 domain (Gowan et al., 2001; Lee et al., 1998) and Mash1 is ventral to the Ngn1 domain extending almost to the sulcus limitans (Gowan et al., 2001; Guillemot and Joyner, 1993; Lo et al., 1991). There is little if any overlap in Math1, Ngn1 and Mash1 in individual cells (Gowan et al., 2001). The bHLH factors have been shown to have at least two functions during neural development: to induce neuronal differentiation and to specify neuronal sub-types (Cau et al., 2002; Farah et al., 2000; Nakada et al., 2004; Parras et al., 2002; Perez et al., 1999). Furthermore, cross-inhibitory regulation of expression between Math1, Ngn1 and Mash1 has been suggested as a mechanism for refining distinct progenitor domains (Gowan et al., 2001; Parras et al., 2002). By contrast, Ngn2, a bHLH factor most closely related to Ngn1, partially overlaps with Ngn1 and Mash1 (Fig. 1) (A.W.H. and J.E.J., unpublished), whereas Olig3 overlaps with Math1, Ngn1 and dorsal Mash1 (Müller et al., 2005), suggesting different rules and additional complexities for the functions of Ngn2 and Olig3.

Although inroads have been made into identifying important players in the specification of spinal cord neurons, the underlying logic behind a combinatorial code for specification of these neurons is far from complete. Indeed, in the dorsal neural tube a progenitor/neuron relationship has only been defined for Math1+ progenitors with dI1 neurons, Ngn1+ progenitors with dI2 neurons (Gowan et al., 2001) and Olig3+ progenitors with dI1-dI3 (Müller et al., 2005). The generation of dI3, dI4 and dI5 from Mash1+ progenitors has only been inferred by position in the dorsoventral axis (Gross et al., 2002; Müller et al., 2002; Qian et al., 2002). In this study, we show that cells with the highest levels of Mash1 appear to give rise to dI3 and dI5, but not dI4, neurons. Consistent with this lineage relationship, dI5 and most dI3 are lost in the *Mash1* mutant, whereas the dI4 neuronal population appears to increase. By contrast, Ngn2 overlaps with Mash1 and Ngn1 but has later temporal characteristics. Loss of Ngn2 alone, or in combination with Mash1, reveals a function for Ngn2 downstream of Mash1 in modulating the number of Mash1-dependent neurons that form. Mouse mutants where the balance and temporal characteristics of Mash1 and Ngn2 levels have been altered were used to refine a model for how these factors function in generation of the correct composition of neurons in the dorsal spinal cord.

Materials and methods

Bacterial artificial chromosome construct

The Roswell Park Bacterial Artificial Chromosome (BAC) library was screened to identify clones that contained the *Mash1* protein-coding region. RPCI-428P21 was chosen for further study. It contains a genomic insert of 305 kb with 98 kb 5′ and 206 kb 3′ of the *Mash1* translation start codon (see Fig. S1 in the supplementary material). The *Mash1*-coding region was precisely replaced by *EGFP* (Clontech) and *CRE* separated by an IRES using homologous recombination (Yang et al., 1997). The resultant modified BAC named *M1-GIC* was verified by Southern blot.

Transgenic mouse generation and mouse mutant strains

Transgenic mice were generated by standard procedures (Hogan et al., 1986) using fertilized eggs from B6D2F1 (C57B1/6×DBA) crosses. *M1-GIC* BAC was prepared using a modified Qiagen Midi Prep procedure as directed by manufacturer. The *M1-GIC* BAC was then injected into the pronucleus of fertilized mouse eggs at 0.5-1 ng/μl in 10 mM Tris (pH 7.5), 0.1 mM EDTA, 100 mM NaCl. Transgenic animals were identified by PCR analysis using tail or yolk sac DNA with primers to CRE (5′ GGACATGTTCAGGGATCGCCAGGCG 3′ and 5′ GCATAACCAGTGAAACAGCATTGCTG 3′).

The mouse mutant strains used in this study have been previously published: *Mash1* (Guillemot et al., 1993), *Ngn2* (Fode et al., 1998), $Mash1^{KINgn2}$ and $Ngn2^{KIMash1}$ (Parras et al., 2002), and *R26R-YFP* (Srinivas et al., 2001). Embryos were staged based on assumed copulation at E0, halfway through the dark cycle. All procedures on animals follow NIH Guidelines and were approved by the UT Southwestern Institutional Animal Care and Use Committee.

Immunofluorescence and mRNA in situ hybridization

Staged embryos were dissected in cold 0.1 M sodium phosphate buffer (pH 7.4), fixed in 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 hours at 4°C, sunk in 30% sucrose in 0.1 M sodium phosphate buffer pH 7.4 overnight at 4°C, embedded in OCT, cryosectioned at 30 μm and processed for immunofluorescence or mRNA in situ hybridization. All sections shown are from the level of the upper limbs.

For immunofluorescence, slides were incubated in the appropriate dilution of primary antibody in PBS/1% goat serum/0.1% Triton X-100, followed by either goat anti-rabbit, mouse or guinea pig IgG conjugated with Alexa Fluor 488, 594 or 647 (Molecular Probes). Primary antibodies used for this study include: rabbit anti-Mash1 (Horton et al., 1999), mouse anti-Ngn2 (Lo et al., 2002), mouse anti-Lhx1/5 (4F2), mouse anti-Islet1/2 (39.4D5), mouse anti-Lmx, (Developmental Studies Hybridoma Bank), rabbit anti-GFP (Molecular Probes), rabbit and guinea pig anti-Brn3a (Fedtsova and Turner, 1997), rabbit, rat and guinea pig anti-Lbx1 (Gross et al., 2002; Müller et al., 2002), guinea pig anti-Lmx1b (Müller et al., 2002), rabbit anti-Islet1/2 (Tsuchida et al., 1994), rabbit anti-Pax2 (Zymed), and mouse anti-BrdU (Becton Dickinson). For BrdU labeling, pregnant mothers were injected with 200 μg BrdU per gram body weight 1 hour before sacrifice. For double labeling experiments using the anti-BrdU antibody, either Mash1 or Ngn2 antibody staining was carried out in full, followed by treatment with 2 N HCl for 20 minutes, 0.1 M sodium borate (pH 8.5) for 20 minutes and incubation with mouse anti-BrdU antibody as described above. Cell death was detected using TUNEL analysis (Roche) on E10.5 and E11.5 sections. Fluorescence imaging was carried out on a BioRad MRC 1024 confocal microscope. For each experiment, multiple sections from at least three different embryos were analyzed and counted.

mRNA in situ hybridization was performed essentially as described using a combined protocol (Birren et al., 1993; Ma et al., 1998). A detailed protocol is available upon request. *Mash1*, *Ngn1* and *Ngn2* antisense probes were made from plasmids containing the coding region of each gene (Gowan et al., 2001).

In ovo chick electroporation

Fertilized White Leghorn eggs were obtained from the Texas A&M Poultry Department (College Station, TX) and incubated at 37°C. Solutions of supercoiled plasmid DNA (2 μg/ml) in PBS/0.02% Trypan Blue were injected into the lumen of the closed neural tube at stage HH13-14, and embryos electroporated as previously described (Funahashi et al., 1999; Muramatsu et al., 1997; Nakada et al., 2004; Suemori et al., 1990). A GFP expression vector (*CMV-eGFP*;

Clontech) was co-injected as a control to monitor efficiency and extent of electroporation. Embryos were harvested 24 hours later at HH23-24, fixed in 4% formaldehyde for 1 hour, and processed as above for cryosectioning and immunofluorescence. For each experiment, multiple sections from at least three electroporated embryos were analyzed and counted. All sections shown were taken between the upper and lower limb regions.

All electroporations used the expression vector pMiwIII, which drives expression through a chick β-actin promoter (Matsunaga et al., 2001; Suemori et al., 1990). PCR fragments containing the coding regions of rat *Mash1* and mouse *Ngn2* were cloned into *Nco*I and *Xba*I sites of a modified pMiwIII expression vector. Protein expression was verified by immunofluorescence with antibodies to Mash1 and Ngn2.

Results

Mash1 and Ngn2 partially overlap in the ventricular zone adjacent to dI3, dI4 and dI5 neuronal populations

Mash1 and Ngn2 are present in the ventricular zone of the neural tube at E10.5. Mash1 is in cells adjacent to where dI3, dI4 and dI5 neurons form and in a smaller ventral domain (Gowan et al., 2001; Gross et al., 2002; Müller et al., 2002). By contrast, Ngn2 is present in the ventral and dorsal neural tube, and in the dorsal domain it spans the region where dI2-dI5 are formed (Fig. 1A). As a first step in examining the roles of Mash1 and Ngn2 in dorsal neuron specification, we examined their colocalization in detail at E10.5 using previously characterized rabbit polyclonal antibodies against Mash1 (Horton et al., 1999) and mouse monoclonal antibodies against Ngn2 (Lo et al., 2002). There are many cells where Mash1 and Ngn2 are colocalized, and these are found at higher levels, but not exclusively, in the more lateral ventricular zone (Fig. 1B, arrows). Thus, contrary to the distinct cross-inhibited domains that were observed between Math1, Ngn1 and Mash1, the other bHLH factors present at this time (Gowan et al., 2001), Ngn2 overlaps with Mash1 in a subpopulation of cells in the dorsal neural tube.

Mash1 is present throughout the mediolateral extent of the ventricular zone, whereas Ngn2 is enriched more laterally (Fig. 1A,B). This spatial pattern suggests these two factors are acting at different times during neuronal development as cells move laterally out of the ventricular zone when they exit the cell cycle and initiate a program of neuronal differentiation. To characterize Mash1 and Ngn2 relative to cell proliferation, we used BrdU incorporation to detect cells in S phase. In E10.5 embryos exposed to BrdU for 1 hour before analysis, a subset of Mash1+ cells were detected that incorporate BrdU (Fig. 1C,E, arrows). By contrast, Ngn2+ cells did not score positively for BrdU incorporation (Fig. 1D,F). These results suggest that Mash1 is present at an earlier stage than Ngn2 during neuronal differentiation, and that temporal regulation of these factors may be important for their activities. Alternatively, Mash1 and Ngn2 could be revealing a code for dorsal interneuron specification such that ventricular zone cells containing each bHLH singly, or in combination, give rise to a distinct neuronal population. In this study, we used both loss-of-function and gain-of-function experiments to address the role of Mash1 and Ngn2 in specification of dorsal neuronal subtypes dI2-dI5.

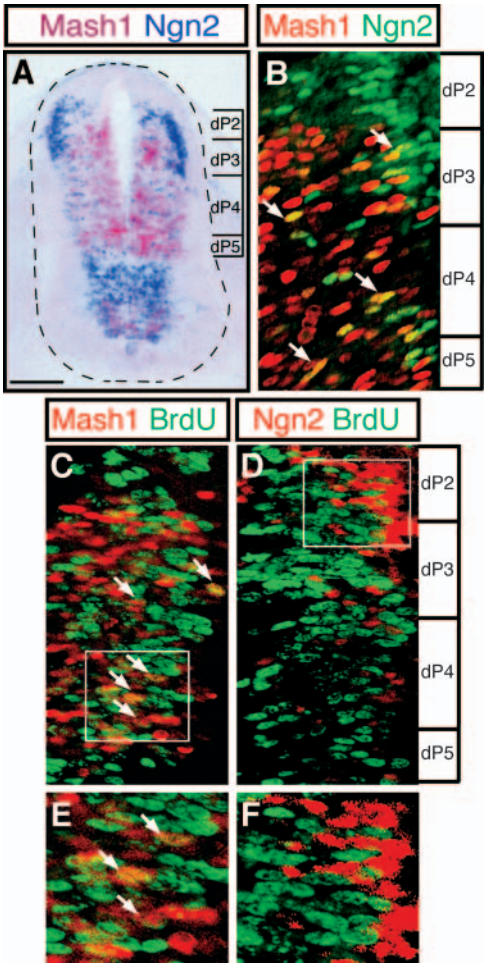


Fig. 1. Colocalization of Mash1 and Ngn2 in the ventricular zone of the dorsal neural tube. E10.5 embryo transverse sections were processed for mRNA in situ hybridization (A) or immunofluorescence (B-F). (A) Pseudocolored overlay of adjacent sections hybridized with antisense *Mash1* (pink) or *Ngn2* (purple) probes to illustrate expression in the dorsoventral axis and possible overlap. (B) Double-label immunofluorescence shows Mash1 (red) and Ngn2 (green) in ventricular zone cells in the dorsal neural tube (region shown by bracket in A; ventricular zone is at the left in each panel). Arrows indicate Mash1 and Ngn2 co-expressing cells (yellow). (C-F) Co-labeling with BrdU incorporation was performed to detect cells in S phase. (C,E) Mash1 (red) cells incorporate BrdU (green) (detected as yellow, arrows). (D,F) Ngn2 (red) cells rarely score positive for BrdU incorporation (green). (E,F) Higher magnification images of the regions boxed in C,D. Scale bars: 90 μm in A; 25 μm in B-D; 15 μm in E,F.

Mash1 is required for the formation of dI3 and dI5 neuronal populations

Mash1-null embryos were analyzed for alterations in the number of dI2-dI6 neurons using multi-label immunofluorescence at E10.5. The neurons are defined by the presence of HD transcription factors, their position in the dorsoventral axis, and a birthdate prior to E11.5 (Table 1). Although Mash1 is in ventricular zone cells adjacent to dI3, dI4 and dI5 neurons, in the *Mash1* null there is a striking loss of only two of these populations. There is a 70% loss of dI3

Table 1. Homeodomain (HD) factors used to define dI1-dI6 dorsal interneuron populations at E10.5*

Interneuron population	HD factor
dI1	Lhx2/9, Brn3a
dI2	Lhx1/5, Brn3a
dI3	Islet1, Brn3a
dI4	Lhx1/5, Pax2
dI5	Lmx1b, Brn3a
dI6	Lhx1/5, Pax2

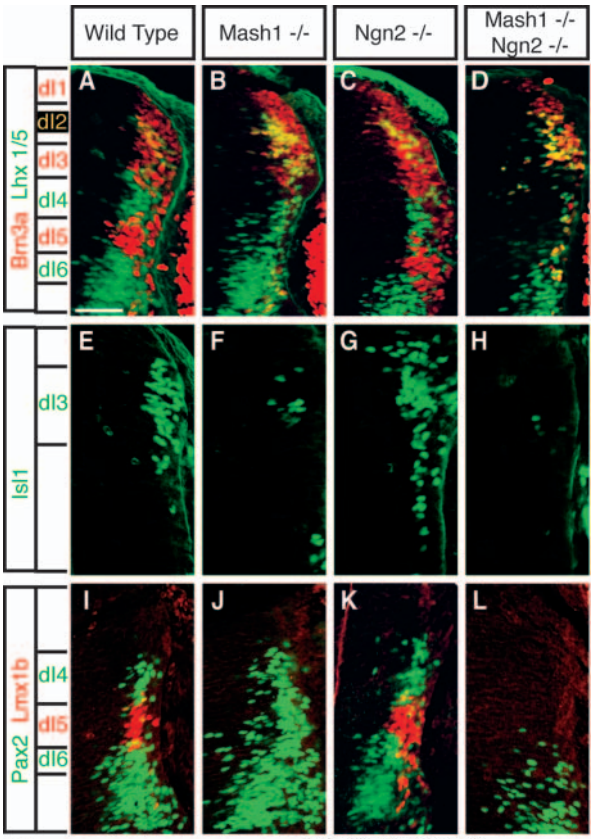
*See Helms and Johnson (2003) for references.

neurons (Isl1) and a complete loss of dI5 neurons (Lmx1b) (Fig. 2, compare E,F,I,J). By contrast, there is a dramatic increase in dI2 (Lhx1/5;Brn3a) and dI4/6 (Pax2) neurons, which appear to fill in at the location of the missing dI3 and dI5 neurons (Fig. 2, compare A,B,I,J). Although in the *Mash1* null dI4 and dI6 are not distinguishable, overexpression and lineage analysis (see below) plus the lack of expansion of the ventral *ngn1* domain, which marks dI6 progenitors (Fig. 5H), suggest the increase in Pax2 is due to an increase in dI4 neurons. These results demonstrate the requirement for Mash1 in the formation of both dI3 and dI5 neurons, and the repression of dI2 and dI4/6 neurons.

To corroborate the conclusions from the Mash1 loss-of-function analysis, over-expression of Mash1 in the chick neural tube was used to test whether Mash1 is sufficient to promote dI3 and dI5 neuronal fates. A *Mash1* expression plasmid (*pMiWIII-Mash1*) was electroporated into chicken embryos at HH13-14, and its effect on dorsal neuronal markers was analyzed 24 hours later. Excess levels of Mash1 increased the number of dI3 (Isl1) and dI5 (Lmx1b) cells when compared with the control side (Fig. 3C,G, right panel of each pair is the injected side). Furthermore, excess Mash1 decreased the number of dI2 (Lhx1/5;Brn3a) and dI4 (Pax2) neurons (Fig. 3A,E). These results are the opposite of those seen in the *Mash1* null, and demonstrate that Mash1 is sufficient for the generation of dI3 and dI5 neurons. Interestingly, the increase observed for each marker does not generally extend over the whole dorsoventral length of the neural tube, but rather it is localized around the area of its normal expression domain. This suggests that regional differences along the dorsoventral axis modulate the activity of Mash1, and demonstrate the context-dependent nature of its specification function.

Ngn2 acts to limit Mash1 activity in dI3 and dI5 neuron formation

The role of Ngn2 in specification of dorsal neuronal populations was also examined to test the hypothesis that Mash1 and Ngn2 provide a combinatorial code for dorsal neuron identity. In E10.5 embryos null for Ngn2, all neuronal populations (dI1-dI6) were generated (Fig. 2), demonstrating that Ngn2 is not required for any specific dorsal cell type. However, the composition of neurons that formed was altered with a subtle increase in the number of dI3 (Isl1) and dI5 (Lmx1b) neurons relative to wild-type embryos (Fig. 2E,G,I,K), and no significant changes in dI2 (Lhx1/5;Brn3a) and dI4/6 (Pax2) populations were detected (Fig. 2A,C,I,K). Excess levels of Ngn2 in the chick neural tube resulted in a complementary phenotype to the Mash1 experiments in that dI3 (Isl1) and dI5 (Lmx1b) were dramatically decreased, but



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Interneuron Population	# of cells per quadrant: Wild Type	Mash1 -/-	Ngn2 -/-	Mash1-/- Ngn2 -/-
dI2 (Lhx1/5; Brn3a)	18+/-2	43+/-10**	16+/-6	29+/-7**
dI3 (Isl1)	29+/-10	7+/-6**	50+/-22*	6+/-8**
dI4/6 (Pax2)	48+/-11	91+/-14**	62+/-14	18+/-5**
dI5 (Lmx1b)	27+/-8	0.2+/-0.4**	42+/-12*	1+/-1**

* p<0.01 ** p<0.001

Fig. 2. Mash1 is required for dI3 and dI5 neuron formation, while Ngn2 represses formation of these populations. Immunofluorescence on transverse sections of neural tubes in wild-type (A,E,I), *Mash1*$^{-/-}$ (B,F,J), *Ngn2*$^{-/-}$ (C,G,K) and *Mash1*$^{-/-}$;*Ngn2*$^{-/-}$ (D,H,L) E10.5 mouse embryos. (A-D) Co-labeling with Brn3a and Lhx1/5 (yellow) marks dI2 neurons. There is a significant increase in dI2 neurons in the *Mash1*$^{-/-}$ and *Mash1*$^{-/-}$;*Ngn2*$^{-/-}$ embryos, but not *Ngn2*$^{-/-}$ relative to wild type. (E-H) Isl1 identifies dI3 neurons (green). In the *Mash1*$^{-/-}$ and *Mash1*$^{-/-}$;*Ngn2*$^{-/-}$ mutant embryos, there is a dramatic decrease in dI3 neurons relative to wild type. By contrast, an increase in dI3 neurons in *Ngn2*$^{-/-}$ is detected. (I-L) Pax2 marks dI4 and dI6 neurons (green), and Lmx1b labels dI5 neurons (red). In the *Mash1*$^{-/-}$ and *Mash1*$^{-/-}$;*Ngn2*$^{-/-}$ embryos, there is a complete loss of dI5. This is the opposite in *Ngn2*$^{-/-}$ embryos, where there is an increase in dI5 relative to wild type. dI4/6 neurons increase in the *Mash1*$^{-/-}$, are unchanged in the *Ngn2*$^{-/-}$, and are dramatically reduced in the *Mash1*$^{-/-}$;*Ngn2*$^{-/-}$ relative to wild type embryos. (M) Individual populations in each mutant were counted on at least three sections from at least three embryos and cell counts are shown in the table. Because dI4 and dI6 neuronal populations cannot be distinguished in the *Mash1* null, all Pax2 cells dorsal to the boundary where the first ventral Pax2 cell was found within the ventricular zone were counted, and as such they are labeled as dI4/6. Scale bar: 50 μm.

dI4 (Pax2) was slightly increased relative to the non-injected side (Fig. 3B,D,F,H, see graph for cell counts). Together, these results demonstrate that Ngn2, although not required for any individual neuronal subtype, limits the generation of dI3 and dI5 neurons, two populations that require Mash1 activity.

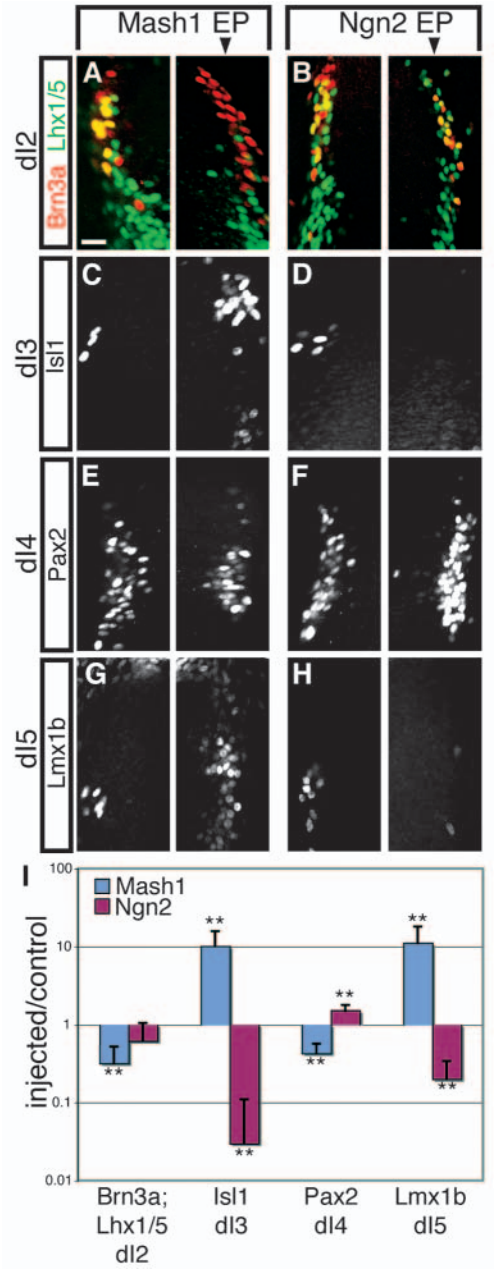


Fig. 3. Excess Mash1 promotes dI3 and dI5 populations, while excess Ngn2 represses them in the chick neural tube. Immunofluorescence on transverse sections of E4 (HH23-24) chick neural tubes electroporated with *pMiWIII-Mash1* (A,C,E,G) or *pMiWIII-Ngn2* (B,D,F,H) at E3 (HH13-14). The arrow above each row of panels indicates the electroporated side of the neural tube. Neuronal population dI2 was labeled by Brn3a;Lhx1/5 (yellow) (A,B), dI3 by Isl1 (C,D), dI4 by Pax2 (E,F) and dI5 by Lmx1b (G,H). Each panel is representative of the phenotype seen in at least three sections from at least three electroporated embryos. (I) Relative induction or repression is expressed as the number of cells on the injected side divided by the number of cells on the control side and plotted in the graph on a logarithmic scale. ** $P<0.001$. Scale bar: 25 μm.

Mash1 is epistatic to Ngn2 in the formation of dI3 and dI5 neurons

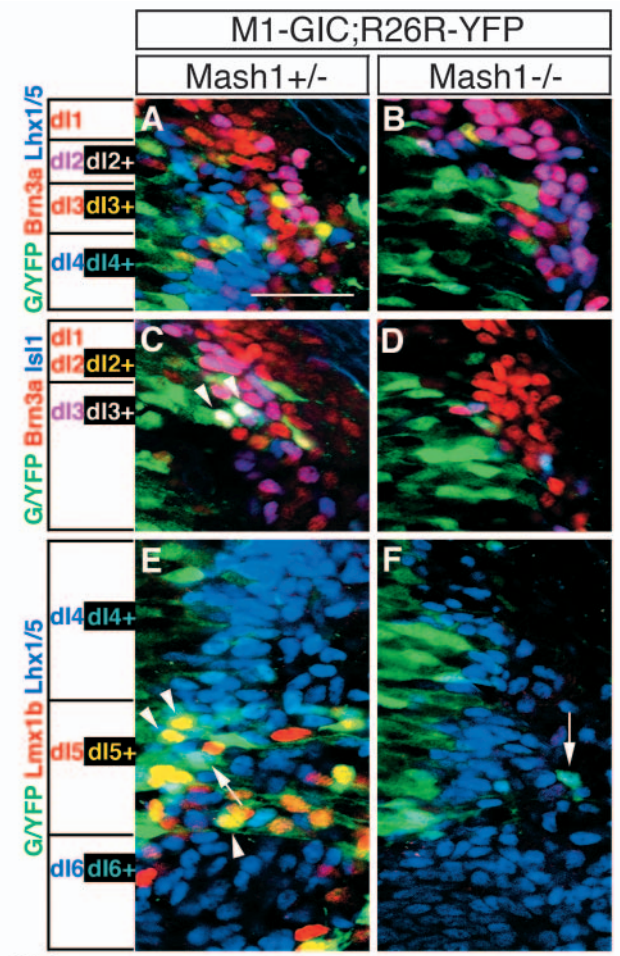
The single mutants described above demonstrate that Mash1 and Ngn2 have opposite effects on the number of dI3 and dI5 neurons that form. Furthermore, the expression pattern suggests that Ngn2 may function downstream of Mash1 rather than setting up a combinatorial code for neuronal subtype specification. If this interpretation of temporal expression is correct, the prediction is that the loss of both Mash1 and Ngn2 should phenocopy the Mash1 null. In support of this hypothesis, embryos null for both Mash1 and Ngn2 have a 70% loss of dI3 and complete loss of dI5 neurons, just as seen in the single *Mash1* null (Fig. 2H,L). Furthermore, the increase in dI2 neurons detected in the *Mash1* null is also seen in the double mutant (Fig. 2D). These results are consistent with Mash1 functioning upstream of Ngn2 in these populations.

Surprisingly, embryos null for both *Mash1* and *Ngn2* have an apparent loss of dI4 neurons (Fig. 2L), a phenotype not predicted from the single mutants. Although dI4 and dI6 cannot be distinguished in the absence of dI5, the position of the Pax2-positive cells in the *Mash1/Ngn2* double knockout strongly suggests a complete loss of dI4 neurons, and possibly also dI6 neurons (Fig. 2L). This loss of dI4 in the double knockout is in contrast to the single knockouts, where there was no indication that either Mash1 or Ngn2 is required for dI4 generation. In fact, dI4/6 cells are significantly increased in the Mash1 null (Fig. 2J). Thus, there is an apparent redundant function for Mash1 and Ngn2 in the generation of dI4 neurons.

Mash1-positive cells give rise primarily to dI3 and dI5, but not dI4 neurons

Given the presence of Mash1 in the ventricular zone throughout the dorsoventral domain adjacent to dI3, dI4 and dI5 neurons, it was surprising that dI4 neurons increased in the *Mash1* knockout, while dI3 and dI5 were lost. This finding suggests either Mash1+ cells become dI4 neurons but do not require Mash1, or that there are distinct low- or non-Mash1+ cells in the ventricular zone that give rise to the dI4 neurons. We used recombination-based lineage tracing in vivo to distinguish between these two possibilities. A transgenic mouse was used (*M1-GIC*) that expresses both GFP and Cre in the *Mash1* expression pattern from a bacterial artificial chromosome containing 300 kb of genomic sequence surrounding the *Mash1* protein-coding region (see Fig. S1 in the supplementary material). By crossing the *M1-GIC* mouse line with a Cre reporter line *R26R-YFP*, any cell that has expressed the transgene will be permanently labeled with YFP (Srinivas et al., 2001).

Embryos at E11.5 were examined by triple-label immunofluorescence to determine the fate of Mash1+ cells in the dorsal neural tube. An antibody to GFP detects GFP and YFP simultaneously, and thus, these cells are referred to as GFP/YFP-positive cells. GFP/YFP-positive cells in the marginal zone were counted and scored for co-labeling with markers of dI2-dI6 neurons (Fig. 4, Table 2). The majority of GFP/YFP+ cells become dI5 (Lmx1b, 73%) and dI3 (Isl1, 17%), rarely, dI2 (Lhx1/5;Brn3a, 2%) and no dI6 (Lhx1/5, 0%), consistent with the requirement for Mash1 specifically in



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Interneuron Population	Wild Type		Mash1 -/-	
	Total	GFP/YFP+	Total	GFP/YFP+
dI2 (Lhx1/5; Brn3a)	20+/-5	1+/-1	46+/-2**	4+/-2
dI3 (Isl1)	35+/-4	10+/-3	13+/-4**	3+/-1**
dI4 (Lhx1/5)	144+/-14	5+/-2	125+/-22	8+/-2
dI5 (Lmx1b)	85+/-8	44+/-5	3+/-0.4**	2+/-1**
dI6-V2 (Lhx1/5)	NC	0+/-0.0	NC	0+/-0.0

*p<0.01 **p<0.001

Fig. 4. Mash1 cells primarily become dI3 and dI5 neurons. Immunofluorescence on transverse sections of *Mash1*$^{+/-}$ (A,C,E) or *Mash1*$^{-/-}$ (B,D,F) mouse embryos at E11.5 containing the transgene *M1-GIC* (see Fig. S1 in the supplementary material) and the *Cre* reporter allele *R26R-YFP*. (A-F) Anti-GFP antibodies detect both GFP from *M1-GIC* and YFP when *R26R-YFP* reports the Cre activity from *M1-GIC*. These green labeled cells reflect cells with Mash1 or progeny of Mash1+ cells. (A,B) Brn3a;Lhx1/5 (pink) label dI2. These cells would appear white if they co-labeled with antibodies to GFP/YFP. The absence of white cells indicate dI2 do not derive from Mash1+ precursors (A). (C,D) Brn3a;Isl1 (pink) label dI3. White cells indicate co-labeling with antibodies to GFP/YFP and demonstrate Mash1+ cells give rise to dI3 neurons (C, arrowheads) and these cells are absent in the null (D). (E,F) Lmx1b (red) labels dI5. The co-labeling with GFP/YFP (yellow) indicates dI5 are derived from Mash1+ cells (E, arrowheads) and these cells are absent in the null (F). Lhx1/5 (blue) labels dI4. Co-labeling with GFP/YFP (turquoise) indicates rare dI4 cells derived from Mash1+ cells (E, arrow), and these cells do not increase in *Mash1*$^{-/-}$ (F). The increase in dI2 and dI4 seen at E10.5 cannot be attributed to fate switching of dI3 and dI5 precursor cells. The color of each neuronal population is shown on the left without and with (black background) co-labeling with the GFP/YFP. The total number of cells for each neuronal subtype and the number of each that co-labels with GFP/YFP are shown in the table. (G) Individual populations in each mutant were counted on at least three sections from at least three embryos each. Scale bar: 25 μm.

dI3 and dI5. A notable percentage of GFP/YFP-expressing cells co-label with dI4 (Lhx1/5, 8%) suggesting a Mash1+ cell can become a dI4 neuron. However, this represents only a small percentage of the dI4 cells generated (~3%) and when identified, these co-labeled cells border the dI3 and dI5 domains (Fig. 4E, arrow). We estimate that Cre induced recombination of the reporter gene in the *M1-GIC* embryos is ~50% because essentially all dI5 neurons require Mash1, but we detect only 52% of the dI5 neurons co-labeled with GFP/YFP (44 of 85 total Lmx1b cells). Furthermore, as we detect 25% of the dI3 neurons co-labeled with GFP/YFP (nine out of 35 total Isl1 cells), using the efficiency factor we predict ~50% of dI3 neurons are derived from Mash1+ cells, consistent with the partial loss of dI3 detected in the *Mash1* null (Fig. 2). Taken together, these results suggest that the function of Mash1 in the generation of dI3 and dI5 neurons is cell-autonomous, and demonstrate that dI2, the majority of dI4, and dI6 neurons develop from cells that contain Mash1 at levels not detected using this transgenic reporter mouse line. Indeed, heterogeneity in endogenous Mash1 levels is detected by immunofluorescence where ventricular zone cells adjacent to dI3 and dI5 have Mash1 at higher levels than cells adjacent to dI4 (Fig. 5D).

Mash1 null cells are stalled in the ventricular zone at E11.5 and do not appear to trans-fate to dI4 neurons

Analysis of the Mash1 knockout revealed the loss of dI3 and dI5 with an increase in dI2 and dI4/6 that appeared to complement the loss (Fig. 2). To determine if the cells that would have become dI3 and dI5 switch their fate to dI2/dI4 in the absence of Mash1, we examined these neuronal populations in *M1-GIC;R26R-YFP;Mash1*$^{-/-}$ embryos at E11.5. The first phenotype noted was that the GFP/YFP level is much higher in the mutant when compared with wild type (see Fig. S1 in the supplementary material). This difference in level reflects the negative autoregulation at the *Mash1* locus that has previously been reported (Casarosa et al., 1999; Horton et al., 1999; Meredith and Johnson, 2000). The *Mash1* null sections showing GFP/YFP (Fig. 4B,D,F) were imaged at much lower GAIN than similar sections from embryos wild-type for Mash1 (Fig. 4A,C,E) (see Fig. S1 in the supplementary material for images with matched GAIN).

Other than negative autoregulation, the most dramatic phenotype detected using these mouse strains is that the cells that should have expressed Mash1 (GFP/YFP) appear stalled in the ventricular zone with only a few cells detected in the marginal zone (Fig. 4B,D,F). These stalled cells aberrantly located in the ventricular zone have at least partially initiated a differentiation program; they express Lbx1, a marker that is normally restricted to the marginal zone, and they rarely

Table 2. Distribution of total GFP/YFP expressing marginal zone cells in M1-GIC E11.5 dorsal neural tube

	dI1	dI2	dI3	dI4	dI5	dI6	Total GFP/YFP
Number of cells	0	1±1	10±3	5±2	44±5	0	60±2
%	0	2%	17%	8%	73%	0	

incorporate BrdU, demonstrating that many have exited the cell cycle (see Fig. S2 in the supplementary material). TUNEL labeling shows no detectable increase in cell death in the neural tube at E10.5 and E11.5 (data not shown). Taken together, the precursors to dI3 and dI5 are not leaving the ventricular zone in the *Mash1* null at E10.5/E11.5, and do not appear to significantly contribute to the increase in dI2 and dI4 as there was no increase in the proportion of GFP/YFP cells co-labeled with dI2 and dI4 markers (Fig. 4B,D,F). Thus, the ectopic dI2 and dI4/6 neurons in the *Mash1* null at E10.5 cannot be accounted for by a switch in fate of dI3 and dI5 precursor cells. Furthermore, cells with undetectable levels of Mash1 must give rise to dI2 and dI4/6 neurons, and the number of these cells increase in the *Mash1* knockout.

Mash1 and Ngn2 levels are independent of each other

Previously, it was demonstrated that cross-inhibition between the three bHLH family members, Mash1, Math1 and Ngn1, was used to control cell number and cell-type formed (Gowan et al., 2001; Scardigli et al., 2001). To determine whether this type of regulation is occurring between Mash1 and Ngn2, we examined the levels of Ngn2 in the *Mash1* null at E10.5 and vice versa. We observed no significant change in the number of Ngn2+ cells in the dorsal neural tube of *Mash1* nulls (Fig. 5A-C) and no significant change in the number of Mash1+ cells in *Ngn2* nulls (Fig. 5D-F). These results suggest that Mash1 and Ngn2 do not use cross-inhibition as a mechanism to control the number of dorsal neurons formed, consistent with the co-expression seen with these two bHLH factors. Although difficult to quantify, there may be increased protein levels of Mash1 in individual cells in the most dorsal region in the *Ngn2* null and vice versa in the *Mash1* null (Fig. 5, compare A with B and D with E) and this may account for the increase in dI3 neurons in the *Ngn2*-null embryos.

We have previously shown that an increase in Ngn1 in the dorsal neural tube leads to an increase in dI2 neurons (Gowan et al., 2001). To test whether an increase in Ngn1 could explain the increase in dI2 neurons in the *Mash1* and the *Mash1/Ngn2* double knockouts, we examined *Ngn1* expression. Indeed, in the *Mash1* and the *Mash1/Ngn2* double knockouts, we detected an increase in *Ngn1* expression in the dorsal neural tube at E10.5 relative to wild type (Fig. 5, compare G with H and J). Consistent with the lack of change of the dI2 population in the *Ngn2* null, there was no change in Ngn1 detected (Fig. 5I). Thus, the excess dI2 cells in the *Mash1* and *Mash1/Ngn2* knockouts is probably due to loss of cross-inhibition of *Ngn1* by Mash1 in its dorsal domain of expression (Gowan et al., 2001).

Ngn2 does not directly block Mash1 function in specifying dI3 and dI5 neurons

The preceding data strongly suggest that Ngn2 opposes Mash1 function in generation of dI3 and dI5 neurons, and thus, the levels and timing of Mash1 and Ngn2 determine the number of dI3 and dI5 neurons that form. A possible mechanistic model to explain the phenotypes involves Ngn2 directly opposing Mash1 function by forming a non-functional heterodimer or by competing with Mash1 on target genes, analogous to interactions between Olig2 and Ngn2 recently reported (Lee et al., 2005). The ability of Ngn2 and Mash1 to form non-functional heterodimers, or to bind similar DNA recognition sites, has been shown in vitro (Gradwohl et al., 1996). To test these models in vivo, we used mouse mutant lines that contain replacement mutations where either the *Ngn2* protein-coding region was swapped into the *Mash1* locus

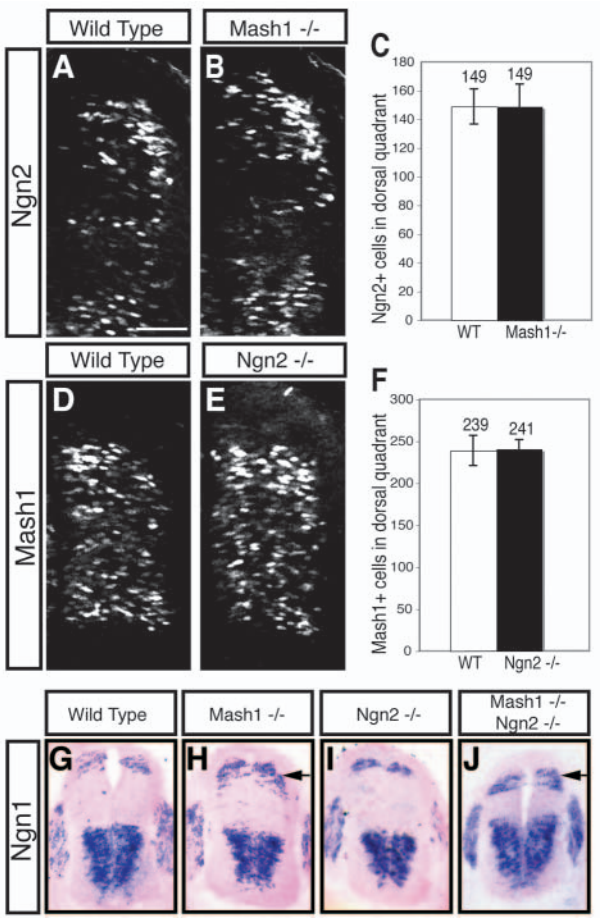


Fig. 5. Mash1 inhibits *Ngn1* but not *Ngn2* expression in the dorsal neural tube. Immunofluorescence (A,B,D,E) and in situ hybridization (G-J) on transverse sections of E10.5 mouse neural tubes. (A-C) There is no change in levels of Ngn2 between wild-type (A) and *Mash1*$^{-/-}$ (B) embryos. (D-F) There is no change in expression of Mash1 between wild-type (D) and *Ngn2*$^{-/-}$ (E) embryos. (G-J) The dorsal domain of Ngn1 is expanded in *Mash1*$^{-/-}$ (H, arrow) and *Mash1*$^{-/-}$;*Ngn2*$^{-/-}$ (J, arrow) relative to wild-type (G) embryos. No change was detected in Ngn1 in *Ngn2*$^{-/-}$ (I) embryos. Each panel is representative of the phenotype seen in at least three sections from at least three embryos. Scale bar: 50 μm in A,B,D,E; 225 μm in G,H.

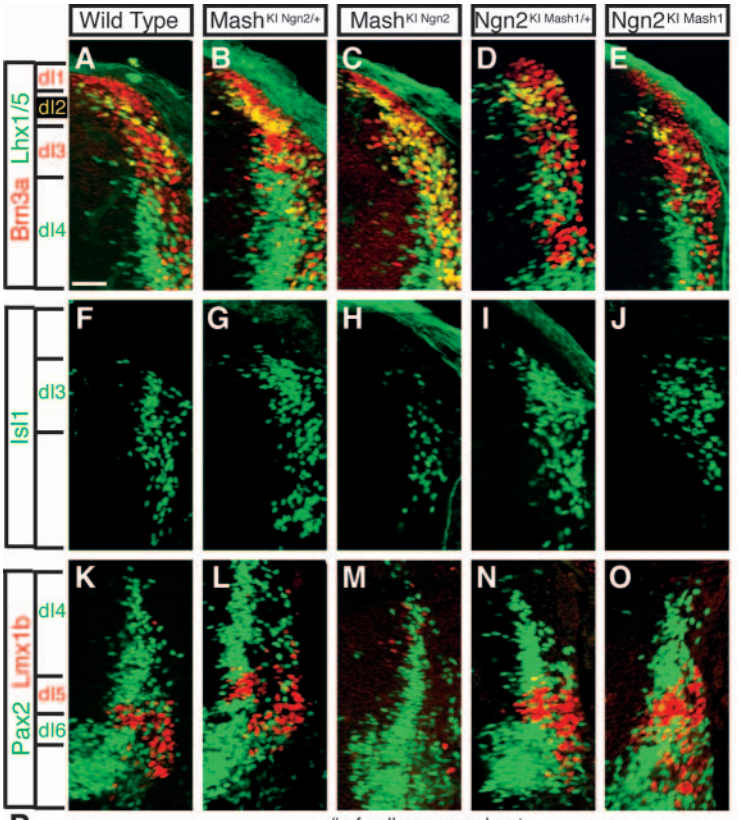
(*Mash1*$^{KI\ Ngn2}$) or the *Mash1* protein-coding region was swapped into the *Ngn2* locus (*Ngn2*$^{KI\ Mash1}$) (Parras et al., 2002). The heterozygous embryos in each strain shift the balance and temporal relationship of Mash1 and Ngn2. If Ngn2 directly opposes Mash1 function, as predicted above, then the *Mash1*$^{KI\ Ngn2/+}$ would approximate the Mash1 knockout, and the *Ngn2*$^{KI\ Mash1/+}$ would approximate a Mash1 gain-of-function phenotype. In *Ngn2*$^{KI\ Mash1/+}$ embryos, we see an increase in dI3 and dI5, reflecting the Mash1 gain-of-function phenotype, as predicted from the specification function of Mash1 (Fig. 6F,I,K,N). However, rather than losing dI3 and dI5 in *Mash1*$^{KI\ Ngn2/+}$ embryos, dI3 and dI5 are increased (Fig. 6F,G,K,L). Owing to variability between these mutant embryos, only the dI3 increase was statistically significant. There is also a significant increase in dI2 neurons in *Mash1*$^{KI\ Ngn2/+}$ embryos, possibly reflecting the role of Ngn2 in generation of these neurons (Gowan et al., 2001) (Fig. 6A,B). Although the results confirm the importance of Mash1 in specifying dI3 and dI5 neurons, they contradict the model that Ngn2 directly opposes this activity of Mash1. Rather, these results fit a model that highlights distinct functions for Mash1 and Ngn2, where Mash1 has a major role in neuronal specification and Ngn2 has a role in temporal control of neuronal differentiation.

The Mash1 and Ngn2 knock-in strains have also been used to address whether these related bHLH factors are able to compensate for each other's function. It has been reported that Ngn2 and Mash1 share the ability to induce neuronal differentiation, but are distinct in specifying neuronal identity (Parras et al., 2002). The functions of Mash1 and Ngn2 in the dorsal neural tube are similar to those attributed to them in other regions of the nervous system. Ngn2 is unable to compensate for Mash1 function in specification as shown by its inability to rescue the generation of dI3 and dI5 neurons (Fig. 6F,H,K,M), and its inability to suppress the ectopic formation of dI2 and dI4/6 neurons (Fig. 6A,C,K,M). Similarly, Mash1 does not compensate for Ngn2 as in both *Ngn2*$^{-/-}$ and *Ngn2*$^{KI\ Mash1/+}$ embryos the number of dI3 and dI5 neurons is elevated (Fig. 6F,J,K,O), reflecting the specification function of Mash1 distinct from Ngn2. The number of dI2 neurons was decreased in this mutant line (Fig. 6A,E), suggesting that ectopic Mash1 in dI2 precursors is inhibitory for dI2 generation as is predicted from Mash1 repression of *Ngn1* expression (Gowan et al., 2001). Together, these results confirm that Mash1 function is crucial for the specification of dorsal neuronal subtypes dI3 and dI5, and Ngn2 cannot replace this function. In addition, although no specification function can be ascribed to Ngn2, it functions in controlling the number of different neuronal subtypes that form.

Discussion

A transcription factor code for specification of dorsal interneurons

Formation of the neuronal network in the dorsal spinal cord requires the generation of correct numbers of neurons with specified identities. Regulation of this process starts early in nervous system development, as cells in the ventricular zone of the neural tube have distinct identities that are determined by transcription factors of the HD and bHLH families. We demonstrate that Mash1 is in precursors to, and is required for, dI3 and dI5 neurons. Furthermore, the number of dI3 and dI5 neurons that form depends on the activity of Ngn2. Thus, the balance of Mash1 and Ngn2 activity plays a central role in



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Interneuron Population	# of cells per quadrant: Wild Type	Mash$^{KI\ Ngn2/+}$	Mash$^{KI\ Ngn2}$	Ngn2$^{KI\ Mash1/+}$	Ngn2$^{KI\ Mash1}$
dI2 (Lhx1/5; Brn3a)	29+/-5	39+/-5**	63+/-13**	26+/-6	21+/-5**
dI3 (Isl1)	109+/-15	139+/-19**	41+/-8**	160+/-29**	135+/-19*
dI4/6 (Pax2)	169+/-27	216+/-37	225+/-32*	162+/-33	168+/-21
dI5 (Lmx1b)	92+/-13	107+/-19	1+/-1**	132+/-25**	126+/-19**

* p<0.01 ** p<0.001

Fig. 6. Distinct functions for Mash1 and Ngn2 in specification of dorsal neurons. Immunofluorescence on cross-sections of E11.5 mouse neural tubes where *Mash1* and *Ngn2* have been swapped into the *Ngn2* and *Mash1* locus, respectively: wild type (A,F,K), *Mash1*$^{KI\ Ngn2/+}$ (B,G,L), *Mash1*$^{KI\ Ngn2}$ (C,H,M), *Ngn2*$^{KI\ Mash1/+}$ (D,I,N) and *Ngn2*$^{KI\ Mash1}$(E,J,O). (A-E) Co-localization of Brn3a (red) and Lhx1/5 (green) detects dI2 neurons (yellow). (F-J) Isl1 (green) detects dI3 neurons. (K-O) Pax2 (green) and Lmx1b (red) detect dI4 and dI5 neurons, respectively. Cell counts for each marker on at least three sections from at least three embryos of each genotype are shown in the table. (P) Because dI4 and dI6 populations cannot be distinguished in the *Mash1* null, all Pax2 counts were completed by drawing a boundary line at the first Pax2-expressing cell found nearest the ventricular zone, and as such, they are labeled as dI4/6. **P<0.001 and *P<0.01. Scale bar: 50 μm.

setting up the correct composition of neurons found in the dorsal spinal cord.

Surprisingly, dI4 neurons, which appear to arise from the domain containing Mash1, do not require Mash1 or Ngn2 alone. Rather, dI4 neurons appear to arise from cells with low levels of Mash1 or none at all. Thus, the control of dI4/6 cell number implies a non-autonomous mechanism with respect to Mash1. Indeed, the heterogeneous Mash1 levels may reflect function of the Notch-signaling pathway in the neural tube at this time (Lindsell et al., 1996; Ma et al., 1997). In addition, we recently identified another bHLH factor, PTF1a (Krapp et al., 1996), which is required for dI4, and is present in the dI4 precursor domain where Mash1 levels are lower (S. Glasgow and J.E.J., unpublished). We have shown that Mash1 levels are heterogeneous in the ventricular zone; thus, the cells with the highest levels of Mash1 preferentially go on to become dI3 and dI5 neurons, and the cells with distinctly lower levels of Mash1 either remain as progenitor cells or become dI4 neurons. These data, combined with previous reports demonstrating the requirement of Math1 for dI1 neurons (Bermingham et al., 2001; Helms and Johnson, 1998) and Ngn1/2 for dI2 neurons (Gowan et al., 2001), demonstrate an emerging bHLH transcription factor code for specification of the early-born dorsal neuronal populations.

Additional refinements of the code are required to explain what determines whether a Mash1 precursor will develop into a dI3 or a dI5 neuron. Overexpression of Mash1 in the chick neural tube resulted in an increase in both neuronal cell types; however, the normal position of the ectopic neurons in the dorsoventral axis was essentially maintained. This result suggests that Mash1 interacts with other factors to specify dI3 versus dI5, and that these factors are likely restricted to defined domains in the dorsoventral axis of the neural tube (Fig. 3C,G). The identity of these interacting factors is suggested in recent studies. Olig3, a bHLH factor present in precursors to dI1-dI3, when combined with Mash1, induces the dI3 phenotype but not dI5 (Müller et al., 2005). By contrast, Lbx1, a HD class transcription factor expressed just as the cells become postmitotic, is required for specification of dI4-dI6 (Gross et al., 2002; Müller et al., 2002). When Lbx1 is co-electroporated with Mash1 into the chick neural tube, there is an increase in dI5 and decrease in dI3 (A.W.H., Y.N. and J.E.J., unpublished). Furthermore, in PTF1a-deficient embryos, the loss of PTF1a leaves cells with Mash1 and Lbx1, and this combination results in a fate switch from dI4 to dI5 (S. Glasgow and J.E.J., unpublished). Thus, additional refinements to the transcription factor code suggest that combinations of factors such as Mash1 plus Olig3 specifies dI3 (Müller et al., 2005), Mash1 followed by Lbx1 specifies dI5, and Mash1^{low} plus PTF1a followed by Lbx1 specifies dI4 (S. Glasgow and J.E.J., unpublished). In addition, upstream factors controlling expression of the bHLH genes, e.g. suppression of Ngn1 and induction Mash1 expression by the HD factor Gsh2 (Kriks et al., 2005), are also crucial for generating the correct composition of neuronal subtypes. Identification of transcriptional targets for the bHLH and HD factors, as has been reported in ventral spinal cord development (Lee et al., 2005; Lee and Pfaff, 2003), will be required to determine the mechanisms controlling the specification of these dorsal neurons.

Sequential actions of Mash1 and Ngn2 in specifying dorsal horn neurons

In the dorsal telencephalon, loss of Ngn2 results in an increase in Mash1+ cells and a subsequent increase in GABAergic neurons, presumably derived from these ectopic Mash1-expressing cells (Fode et al., 2000). Thus, one function of Ngn2 in forebrain development is to suppress levels of Mash1. This interpretation is similar to the cross-repression seen between Math1, Ngn1 and Mash1 in the dorsal spinal neural tube (Gowan et al., 2001). However, in the dorsal neural tube, Ngn2 overlaps with both Mash1 (Fig. 1) and Ngn1 (A.W.H. and J.E.J., unpublished). This colocalization is found in cells that do not incorporate BrdU, suggesting that Ngn2 is somewhat temporally delayed relative to Mash1 and Ngn1. Regardless, the overlap suggests there is little if any transcriptional cross-repression mechanism regulating the expression of *Mash1* and *Ngn2* in the dorsal neural tube. Indeed, in this domain, the number of Mash1+ cells is not increased in the *Ngn2* null, nor is the number of Ngn2+ cells increased in the *Mash1* null (Fig. 5). Thus, rather than cross-inhibition, these results support a model where Mash1 appears to be temporally upstream of Ngn2.

Although Mash1 and Ngn2 do not appear to cross-repress each other's expression, it is clear that Ngn2 limits the apparent activity of Mash1 in inducing dI3 and dI5 neurons. This is evident in the *Ngn2* null, where there is an increase in the Mash1-dependent dI3 and dI5 populations, and in overexpression of Ngn2 in chick, where there is a decrease in the number of dI3 and dI5 neurons. However, the hypothesis that Ngn2 directly blocks Mash1 activity, either by forming inactive complexes or by blocking shared DNA-binding sites, as has been reported for Olig2 and Ngn2 (Lee et al., 2005), appears incorrect as increasing Ngn2 and decreasing Mash1 in a cell, as occurs in *Mash1*$^{KINgn2/+}$, does not decrease dI3 and dI5 as would be predicted.

An alternative model is that Ngn2 increases the probability that a cell will permanently exit the cell cycle. Even subtle perturbations in the probability of cell-cycle exit could modulate the number of cells of a specific cell type that are formed. This function for Ngn2 in controlling the timing of differentiation is similar to that attributed to Ngn2 in motoneuron formation, where the balance of Olig2 and Ngn2 control the number of cells that will undergo neuronal differentiation (Lee et al., 2005). In *Mash1*$^{KI\ Ngn2/+}$ embryos, Ngn2 is present earlier than in wild type, and Mash1 levels are decreased, but an increase in dI3 and dI5 is still detected. This increase in dI3 and dI5 could reflect Ngn2 inducing premature differentiation. By contrast, in the *Ngn2* null, the increase in dI3 and dI5 populations could result from extra divisions of the dI3/dI5 precursor cells owing to a subtle shift in timing of cell-cycle exit. What is clear is that there is a fundamental difference in how Ngn2 functions relative to the other bHLH factors, such as Mash1, Math1 and Ngn1. These latter factors function in specifying neuronal identity in the dorsal spinal cord. By contrast, Ngn2 is not required for any specific cell type but is required to get a normal composition of neurons formed. Further support for this difference in how Ngn2 functions relative to Mash1 is seen in the ventral neural tube where Ngn2 but not Mash1 can synergize with the HD factors

Lhx3 and Isl1 in post-mitotic cells to generate motoneurons (Lee and Pfaff, 2003).

Mash1 is required for lateral movement of differentiating cells from the ventricular zone

The generation of transgenic mice expressing *GFP* and *Cre* from a *Mash1* locus containing BAC allows a unique look at the behavior of the cells in the absence of Mash1 function. The *GFP* reporter in *M1-GIC;Mash1*$^{-/-}$ mice indicates that in the absence of Mash1, the cells do not switch fate and contribute to the increased dI4 population, but rather these cells primarily remain in the ventricular zone (see Fig. S1 in the supplementary material, compare E with F; see Fig. S2 in the supplementary material, compare A with B). Although stalled in the ventricular zone, the cells continue some aspects of the neuronal differentiation process as illustrated by ectopic Lbx1 and Tuj1 within the ventricular zone of *Mash1* nulls (Fig. S2). Furthermore, these aberrant Lbx1+ cells rarely incorporate BrdU suggesting many have left the cell-cycle (Fig. S2). Thus, in the absence of Mash1, the cells apparently undergo multiple aspects of differentiation but they do not have the characteristic lateral movement to the marginal zone.

Conclusions

The data reported here, combined with previous studies (Bermingham et al., 2001; Gowan et al., 2001; Helms and Johnson, 1998; Müller et al., 2005), demonstrate that bHLH factors are required for generating the correct number and types of neurons in the dorsal neural tube. The precise mechanisms for how these factors interact to result in this neuronal diversity is still not clear and will require identification of downstream targets to define where these pathways intersect. Furthermore, identification of additional bHLH family members and co-factors such as HD proteins, and a more detailed understanding of non-cell autonomous mechanisms involving Notch/delta signaling, will be required to fully understand the formation of the neuronal network in the dorsal spinal cord.

These studies could not be performed without the generosity of multiple laboratories in providing cell type-specific antibodies: S. Morton and T. Jessell for guinea pig anti-Lmx1b and rabbit anti-Isl1/2; E. Turner for rabbit and guinea pig anti-Brn3a; T. Müller and C. Birchmeier for guinea pig and rabbit anti-Lbx1; M. Goulding for rabbit and rat anti-Lbx1; L.-C. Lo and D. Anderson for mouse anti-Ngn2; and the Developmental Studies Hybridoma Bank for mouse anti-Lhx1/5, mouse anti-Isl1 and mouse anti-Lmx. We thank M. Goulding, T. Müller and C. Birchmeier for sharing data prior to publication. These studies also benefited from technical assistance provided by Ms P. Parab and Ms C. Weldon. This work was supported by NIH RO1 HD37932 and RO1 NS32817 to J.E.J.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/12/2709/DC1

References

Bermingham, N. A., Hassan, B. A., Wang, V. Y., Fernandez, M., Banfi, S., Bellen, H. J., Fritzsch, B. and Zoghbi, H. Y. (2001). Proprioceptor pathway development is dependent on MATH1. *Neuron* **30**, 411-422.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

Birren, S. J., Lo, L. and Anderson, D. J. (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. *Development* **119**, 597-610.

Briscoe, J., Alessandra, P., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-445.

Casarosa, S., Fode, C. and Guillemot, F. (1999). *Mash1* regulates neurogenesis in the ventral telencephalon. *Development* **126**, 525-534.

Caspary, T. and Anderson, K. V. (2003). Patterning cell types in the dorsal spinal cord: what the mouse mutants say. *Nat. Rev. Neurosci* **4**, 289-297.

Cau, E., Casarosa, S. and Guillemot, F. (2002). *Mash1* and *Ngn1* control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. *Development* **129**, 1871-1880.

Cheng, L., Arata, A., Mizuguchi, R., Qian, Y., Karunaratne, A., Gray, P. A., Arata, S., Shirasawa, S., Bouchard, M., Luo, P. et al., (2004). Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. *Nat. Neurosci.* **7**, 510-517.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Farah, M. H., Olson, J. M., Sucic, H. B., Hume, R. I., Tapscott, S. J. and Turner, D. L. (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development* **127**, 693-702.

Fedtsova, N. and Turner, E. E. (1997). Inhibitory effects of ventral signals on the development of Brn-3.0-expressing neurons in the dorsal spinal cord. *Dev. Biol.* **190**, 18-31.

Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C. and Guillemot, F. (1998). The bHLH protein NEUROGENIN2 is a detemination factor for epibranchial placode-derived sensory neurons. *Neuron* **120**, 483-494.

Fode, C., Ma, Q., Casarosa, S., Ang, S.-L., Anderson, D. J. and Guillemot, F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. *Genes Dev.* **14**, 67-80.

Funahashi, J., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H. and Nakamura, H. (1999). Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity. *Dev. Growth Differ.* **41**, 59-72.

Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R. and Johnson, J. E. (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31**, 219-232.

Gradwohl, G., Fode, C. and Guillemot, F. (1996). Restricted expression of a novel murine atonal-related bHLH protein in undifferentiated neural precursors. *Dev. Biol.* **180**, 227-241.

Gross, M. K., Dottori, M. and Goulding, M. (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. *Neuron* **34**, 535-549.

Guillemot, F. and Joyner, A. (1993). Expression of murine Achaete-Scute and Notch homologues in the developing central nervous system. *Mech. Dev.* **42**, 171-185.

Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A. L. (1993). Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* **75**, 463-476.

Helms, A. W. and Johnson, J. E. (1998). Progenitors of dorsal commissural interneurons are defined by MATH1 expression. *Development* **125**, 919-925.

Helms, A. W. and Johnson, J. E. (2003). Specification of dorsal spinal cord interneurons. *Curr. Opin. Neurobiol.* **13**, 42-49.

Hogan, B., Costantini, F. and Lacy, E. (1986). *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Horton, S., Meredith, A., Richardson, J. A. and Johnson, J. E. (1999). Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. *Mol. Cell. Neurosci.* **14**, 355-369.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genetics* **1**, 20-29.

Krapp, A., Knofler, M., Frutiger, S., Hughes, G. J., Hagen-buchle, O. and Wellauer, P. K. (1996). The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix-loop-helix protein. *EMBO J.* **15**, 4317-4329.

Kriks, S., Lanuza, G. M., Mizuguchi, R., Nakafuku, M. and Goulding, M. (2005). Gsh2 is required for the repression of Ngn1 and specification of dorsal interneuron fate in the spinal cord. *Development* (in press).

Lanuza, G. M., Gosgnach, S., Pierani, A., Jessell, T. M. and Goulding, M.

(2004). Genetic identification of spinal interneurons that coordinate left-right locomotor activity necessary for walking movements. *Neuron* **42**, 375-386.

Lee, K. J., Mendelsohn, M. and Jessell, T. M. (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev.* **12**, 3394-3407.

Lee, K. J., Dietrich, P. and Jessell, T. M. (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. *Nature* **403**, 734-740.

Lee, S.-K. and Pfaff, S. L. (2003). Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transription factors. *Neuron* **38**, 731-745.

Lee, S.-K., Lee, B., Ruiz, E. C. and Pfaff, S. L. (2005). Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. *Genes Dev.* **19**, 282-294.

Lindsell, C. E., Boulter, J., diSibio, G., Gossler, A. and Weinmaster, G. (1996). Expression patterns of *Jagged, Delta1, Notch1, Notch2,* and *Notch3* genes identify ligand-receptor pairs that may function in neural development. *Mol. Cell. Neurosci.* **8**, 14-27.

Lo, L.-C., Johnson, J. E., Wuenschell, C. W., Saito, T. and Anderson, D. J. (1991). Mammalian *achaete-scute* homolog 1 is transiently expressed by spatially-restricted subsets of early neuroepithelial and neural crest cells. *Genes Dev.* **5**, 1524-1537.

Lo, L. C., Dormand, E., Greenwood, A. and Anderson, D. J. (2002). Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. *Development* **129**, 1553-1567.

Ma, Q., Sommer, L., Cserjesi, P. and Anderson, D. J. (1997). Mash1 and neurogenin1 expression patterns define complementary domains of neuroepithelium in the developing CNS and are correlated with regions expressing notch ligands. *J. Neurosci.* **17**, 3644-3652.

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L. and Anderson, D. J. (1998). *neurogenin1* is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **120**, 469-482.

Matsunaga, E., Araki, I. and Nakamura, H. (2001). Role of Pax3/7 in the tectum regionalization. *Development* **128**, 4069-4077.

Meredith, A. and Johnson, J. E. (2000). Negative regulation of *Mash1* expression in CNS development. *Dev. Biol.* **222**, 336-346.

Müller, T., Brohmann, H., Pierani, A., Heppenstall, P. A., Lewin, G. R., Jessell, T. M. and Birchmeier, C. (2002). The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. *Neuron* **34**, 551-562.

Müller, T., Anlag, K., Wildner, H., Britsch, S., Treier, M. and Birchmeier, C. (2005). The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord. *Genes Dev.* **19**, 733-743.

Muramatsu, T., Mizutani, Y., Ohmori, Y. and Okumura, J. (1997). Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. *Biochem. Biophys. Res. Commun.* **230**, 376-380.

Nakada, Y., Hunsaker, T. L., Henke, R. M. and Johnson, J. E. (2004). Distinct domains within Mash1 and Math1 are required for function in neuronal differentiation versus cell-type specification. *Development* **131**, 1319-1330.

Parras, C. M., Schuurmans, C., Scardigli, R., Kim, J., Anderson, D. J. and Guillemot, F. (2002). Divergent functions of the proneural genes *Mash1* and *Ngn2* in the specification of neuronal subtype identity. *Genes Dev.* **16**, 324-338.

Perez, S. E., Rebelo, S. and Anderson, D. J. (1999). Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. *Development* **126**, 1715-1728.

Pierani, A., Moran-Rivard, L., Sunshine, M. J., Littman, D. R., Goulding, M. and Jessell, T. M. (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. *Neuron* **29**, 367-384.

Qian, Y., Shirasawa, S., Chen, C., Cheng, L. and Ma, Q. (2002). Proper development of relay somatic sensory neurons and D2/D4 interneurons requires homeobox genes *Rnx/Tlx3* and *Tlx1*. *Genes Dev.* **16**, 1220-1233.

Scardigli, R., Schuurmans, C., Gradwohl, G. and Guillemot, F. (2001). Crossregulation between *neurogenin2* and pathways specifying neuronal identity in the spinal cord. *Neuron* **31**, 203-217.

Sommer, L., Ma, Q. and Anderson, D. J. (1996). Neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogenity in the developing CNS and PNS. *Mol. Cell. Neurosci.* **8**, 221-241.

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* **1**, 4.

Suemori, H., Kadodawa, Y., Goto, K., Araki, I., Kondoh, H. and Nakatsuji, N. (1990). A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous beta-galactosidase expression. *Cell Differ. Dev.* **29**, 181-186.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.

Yang, X. W., Model, P. and Heintz, N. (1997). Homologous recombination based modification in Escherichia coli and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat. Biotechnol.* **15**, 859-865.