

Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord

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In the developing spinal cord, signals from the roof plate are required for the development of three classes of dorsal interneuron: D1, D2, and D3, listed from dorsal to ventral. Here, we demonstrate that absence of Wnt1 and Wnt3a, normally expressed in the roof plate, leads to diminished development of D1 and D2 neurons and a compensatory increase in D3 neuron populations. This occurs without significantly altered expression of BMP and related genes in the roof plate. Moreover, Wnt3a protein induces expression of D1 and D2 markers in the isolated medial region of the chick neural plate, and Noggin does not interfere with this induction. Thus, Wnt signaling plays a critical role in the specification of cell types for dorsal interneurons.

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During development of the vertebrate central nervous system (CNS), highly proliferative cells in the ventricular zone of the neural tube serve as progenitors of the various types of neurons, such as interneurons and motor neurons. In the ventral half of the spinal cord, the secreted signaling molecule Sonic hedgehog (Shh) functions as a gradient signal for the generation of five distinct classes of neurons along the dorsoventral axis. Shh secreted from the notochord and floor plate controls the specification of ventral cell types in a dose-dependent manner (Roelink et al. 1995; Ericson et al. 1996; Tanabe and Jessell 1996; Jessell 2000).

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Three subclasses of interneuron, called D1, D2, and D3 positioned from the dorsal side, are indicated by the expression of homeodomain proteins LH2, Islet1, and Lim1/2 in the dorsal half of the spinal cord (Liem et al. 1997). These dorsal interneurons are derived from progenitors in the ventricular zone. These progenitors are also subdivided by expression of the basic helix-loop-helix (bHLH) proteins Math1, Neurogenin1 (Ngn1), and Mash1 (Lee et al. 1998, 2000). It has been established that *Math1*-expressing cells give rise to LH2⁺ neurons (Helms and Johnson 1998). Recent studies indicate that the roof plate is the major source of inductive signals controlling the generation of the D1 and D2 classes of dorsal interneuron (Lee and Jessell 1999). Experiments using cultured chick neural plate tissue have demonstrated that signals from the roof plate are sufficient to promote dorsal interneuron differentiation in vitro (Liem et al. 1997). Moreover, absence of D1/D2 class neurons in mouse embryos lacking the roof plate, either by homozygosity of the *dreher* (*dr*) allele carrying a loss-of-function mutation in the LIM-homeobox gene *Lmx1*, or by genetic roof plate ablation, has provided compelling evidence for the roof plate as the determinant source of dorsal interneurons (Lee et al. 2000; Millonig et al. 2000).

Around the time when dorsal interneurons are generated, cells at the dorsal end of the neural tube express secretory proteins belonging to BMP, FGF, and Wnt families (Lee and Jessell 1999). It has been shown in the chick that BMP family proteins mimic the roof plate in the induction of dorsal interneurons in the isolated medial region of the neural plate (Liem et al. 1997; Lee et al. 1998). Thus, BMP family proteins have been considered to be the major signaling molecules originating from the roof plate and defining the dorsal interneurons of the spinal cord.

Gene disruption studies on the function of BMP family members, expressed in the roof plate, have revealed several essential roles of this group of secreted protein. However, studies have not thus far indicated a requirement for these BMP proteins in the development of dorsal interneurons, except in the case of GDF7. In *Bmp7*-deficient mice, the development of eye and kidney is perturbed (Dudley et al. 1995; Luo et al. 1995), but defects of neurogenesis in the spinal cord have not been reported. In addition, the disruption of *Bmp6* has no effect on the developing nervous system (Solloway et al. 1998). *Bmp4*-deficient embryos show severe defects around the gastrulation stage and do not survive to the stages at which protein function in neuronal development could be analyzed (Winnier et al. 1995). In contrast, in *Gdf7*-deficient mice, the generation of D1A neurons, the dorsal subclass of D1 class interneurons, is eliminated, whereas the generation of D1B and other identified dorsal interneurons remains unaffected. The limited effect of the deficiency of BMP proteins in the dorsal spinal cord may be accounted for either by functional redundancies among coexpressed BMP family proteins (Dudley and Robertson 1997), or by the contribution of other signaling molecules expressed in the roof plate, such as Wnt proteins.

Wnt proteins constitute a large family of signaling molecules that have important roles in the regulation of embryonic patterning, cell proliferation, and cell deter-

mination (Wodarz and Nusse 1998). *Wnt1* and *Wnt3a* are expressed in extensively overlapping regions within the CNS, predominantly along the dorsal midline from the diencephalon to the spinal cord (Wilkinson et al. 1987; Roelink and Nusse 1991; McMahon et al. 1992; Parr et al. 1993). Expression in the roof plate continues throughout the period of neurogenesis. Absence of both genes results in reduction of neural crest cell populations and a deficiency of dorsolateral neural precursors in the developing hindbrain (Ikeya et al. 1997).

This study demonstrates that mouse embryos lacking both *Wnt1* and *Wnt3a* are indeed defective in determination of dorsal interneurons. Generation of D1 and D2 classes of dorsal interneurons is impaired; this loss of the dorsal interneurons is compensated by a dorsal expansion of D3 interneuron populations. Most importantly, expression of BMP family proteins is not significantly affected in these mutant embryos. Moreover, the induction of D1 and D2 class interneurons by *Wnt3a* protein in the isolated medial region of chick neural plate is demonstrated. Together, these observations clearly indicate that Wnt signaling has a critical role in the generation of D1 and D2 dorsal interneurons.

Results and Discussion

A previous study indicated that in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos, expression of *Pax3* and *Pax6* in the dorsal and medioventral regions of the neural tube, respectively, was not significantly affected in the spinal cord, indicating that a basic dorsoventral division was normally established (Ikeya et al. 1997). We extended the analysis to finer dorsal subdivisions employing expression of homeodomain proteins *LH2*, *Islet1*, *Pax2*, and *Lim1/2* as markers of neural identity. D1, D2, and D3 subclasses of dorsal interneurons are identified by the combined expression of these proteins; D1 neurons express *LH2*, D2 neurons express *Islet1*, and D3 neurons express *Pax2* and *Lim1/2* (Burrill et al. 1997; Liem et al. 1997). In *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos, only trace cell populations expressing *LH2* or *Islet1* remained at the dorsal margin of the neural tube. This indicated that D1 and D2 neurons were largely absent (Fig. 1A,D–F,I,J). On the other hand, there was a twofold increase in the population of cells marked by expression of *Pax2* and *Lim1/2* in the dorsal half of *Wnt1*^{-/-}; *Wnt3a*^{-/-} spinal cord (Fig. 1K,N–R). Thus the number of D3 neurons was increased in the dorsal spinal cord, compensating for the absence of D1 and D2 neurons. This observation clearly indicates that the activity of Wnt proteins is required for proper generation of the interneuron subclasses D1 and D2. The loss of D1 and D2 interneurons occurred only in *Wnt1*^{-/-}; *Wnt3a*^{-/-} double mutant embryos but not in *Wnt1*^{-/-} or *Wnt3a*^{-/-} single mutants (Fig. 1B,C,E,G,H,I,L,M,O), indicating that expression of one of these

Wnt proteins in the roof plate is sufficient to generate normal populations of D1/D2 interneurons.

To gain insight into the mechanisms of the loss of D1/D2 interneurons and of dorsal expansion of D3 neurons, the progenitor populations in the ventricular zone were examined. Progenitors of the dorsal interneurons are characterized by the expression of one of the bHLH proteins. These proteins are *Math1*, *Ngn1*, and *Mash1*, from the dorsal toward the ventral aspect, respectively (Helms and Johnson 1998; Lee et al. 1998, 2000). In *Wnt1*^{-/-}; *Wnt3a*^{-/-} neural tube, *Math1*⁺ cells and *Ngn1*⁺ cells are present in the dorsalmost region of the ventricular zone, but are significantly reduced in population size (to ~15%; Fig. 2A–D), whereas *Mash1*⁺ cells expanded their territory dorsally (Fig. 2E,F). Thus it can be seen that a reduction in the potential population of dorsal interneurons proximal to the roof plate has already occurred at the progenitor stage, and this situation is exacerbated as the neurons differentiate (Fig. 2G,H).

Given the indication that the ability of the roof plate to induce dorsal characteristics in the spinal cord involves BMP proteins (Liem et al. 1997; Lee et al. 1998), it is important to determine whether the loss of D1 and D2

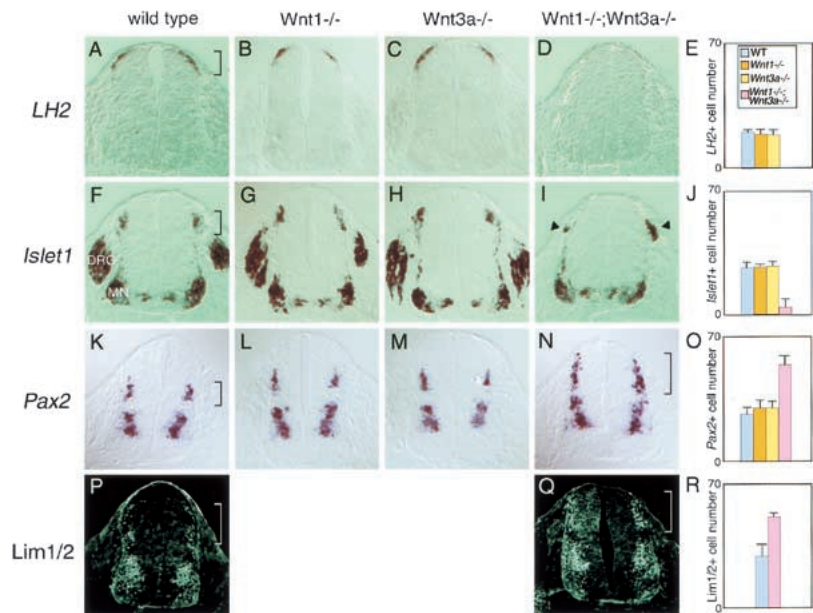


Figure 1. Defects of dorsal interneuron classes in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos. Expression of *LH2* (A–D), *Islet1* (F–I), *Pax2* (K–N) and *Lim1/2* (P, Q), in the cervical spinal cord of E10.5 wild-type (A, F, K, P), *Wnt1*^{-/-} (B, G, L), *Wnt3a*^{-/-} (C, H, M), and *Wnt1*^{-/-}; *Wnt3a*^{-/-} (D, I, N, Q) embryos. D1 neurons expressing *LH2* (A–D) and D2 neurons expressing *Islet1* (F–I) were significantly reduced (to ~5%) in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos. D3 neurons expressing *Pax2* (K–N) and stained with anti-*Lim1/2* antibody (P, Q), normally generated from a medial position, were found in the dorsal third of the spinal cord in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos. Domains expressing *LH2* (A), *Islet1* (F), *Pax2* (K, N) and *Lim1/2* (P, Q) in the dorsal neural tube are indicated by brackets. These results were confirmed by analysis of four embryos of each genotype. *Islet1* is also expressed by motor neurons (MN) and dorsal root ganglia (DRG). *Pax2* and *Lim1/2* are also expressed by ventral interneurons. In *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos, development of the dorsal root ganglion was impaired as already reported (I, arrowheads; Ikeya et al. 1997). (E, J, O, R) Cell count analyses for individual neural markers in the dorsal spinal cord. Averages were taken for four serial sections per embryo over two embryos of a genotype. There was a twofold increase in the population of *Pax2*- and *Lim1/2*-expressing cells in *Wnt1*^{-/-}; *Wnt3a*^{-/-} dorsal spinal cord compared with normal neural tube (O, R). The population sizes of the interneurons were comparable to wild-type embryos in *Wnt1*^{-/-} or *Wnt3a*^{-/-} single mutants.

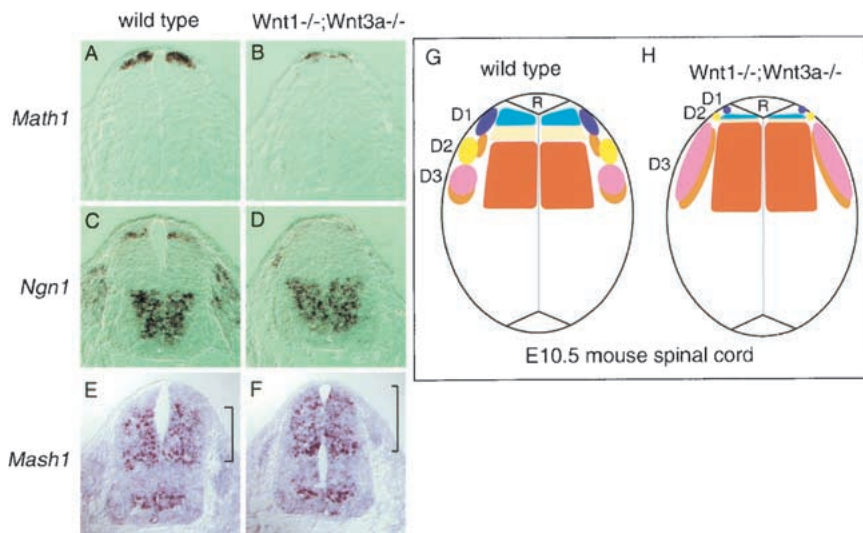


Figure 2. Defects of dorsal neural progenitors in *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos. Expression of *Math1* (A,B), *Ngn1* (C,D) and *Mash1* (E,F) in the cervical spinal cord of E10.5 wild-type (A,C,E) and *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos (B,D,F). In *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos, dorsal neural progenitors expressing *Math1* or *Ngn1* were greatly reduced in number (to ~15%). *Ngn1*-expressing cells were located more dorsally than normal in *Wnt1*^{-/-};*Wnt3a*^{-/-} neural tube (D). *Mash1*⁺ progenitors, normally excluded from the dorsal neural tube, occupied the dorsal area of the spinal cord in *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos (F). These results were confirmed by analysis of four embryos of each genotype. (G,H) Summary of the phenotype of dorsal interneuron development in *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos. D1, D2, and D3 subclasses of the dorsal interneuron are indicated by the expression of homeodomain proteins LH2 (blue), *Islet1* (yellow), *Pax2* (pink), and *Lim1/2* (orange), respectively. The progenitors in the ventricular zone are also subdivided by the expression of bHLH proteins, *Math1* (pale blue), *Ngn1* (light yellow), and *Mash1* (deep orange). Absence of *Wnt1* and *Wnt3a* led to diminished development of D1 and D2 neurons and to a compensatory increase of D3 neurons. *Math1*⁺ progenitors and *Ngn1*⁺ progenitors are present in the dorsalmost region of the ventricular zone, but are significantly reduced in population sizes, whereas *Mash1*⁺ progenitors have increased and expanded their territory dorsally (H).

interneurons in *Wnt1*^{-/-};*Wnt3a*^{-/-} neural tube is a function of altered BMP protein expression, or a more direct consequence of Wnt protein deficiency. Therefore, the expression of *Bmp4*, *Bmp6*, *Bmp7*, *Gdf7*, and *Noggin* in the roof plate was examined. Expression of these BMP genes and the BMP-antagonist gene in the roof plate was indistinguishable between wild-type and *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos (Fig. 3A–D; data not shown). Moreover, the expression of *Msx1* and *Msx2*, which respond to BMP activity, was not altered in *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos (Fig. 3E,F; data not shown). Thus, the reduction in D1/D2 neurons and compensatory expansion of D3 neurons in *Wnt1*^{-/-};*Wnt3a*^{-/-} mutants occurred without alteration of the BMP signaling system. This result implies that Wnt proteins are direct regulators in the determination of D1 and D2 interneurons.

In a previous study, the potential of the roof plate to induce dorsal interneurons of the D1/D2 subclasses was demonstrated by coculturing of roof plate fragments with explants from the medial region of avian neural plate (Liem et al. 1997). If Wnt proteins account for the major component of D1/D2-inducing activity, Wnt protein added to the medial neural plate will exert an effect mimicking the cocultured roof plate. Therefore, *Wnt3a* protein was added to cultures of medial neural plate explant, in the form of a conditioned medium of *Wnt3a*-expressing L cells (Fig. 4A). Expression of *LH2A* (D1 marker), *Islet1* (D2 marker), and *Pax2* (D3 marker) in the explants was compared with that in control L cell-conditioned medium. Analysis by RT-PCR indicates that

expression of *LH2A* and *Islet1* was clearly activated by *Wnt3a* in the explants, but was not detectable in the control explants. *Pax2* expression was significantly reduced by *Wnt3a* protein (Fig. 4B). Furthermore, activation of *LH2A* and *Islet1* expression by *Wnt3a*-conditioned medium was not affected by BMP antagonist *Noggin* at 20 nM (Fig. 4C), which caused an eightfold reduction of the *Msx1* expression when applied to the dorsal neural plate (data not shown).

To examine how the effect of exogenous *Wnt3a* is spatially organized, an explant of medial neural plate was cultured in contact with a bead soaked with *Wnt3a* protein, and expression of *Islet1* and *Lim1/2* was examined by immunohistochemistry. Expression of *Islet1* was induced in cells surrounding the bead with the most frequent occurrence in the region 25–50 μm away from the edge of the neural plate tissue in contact with the *Wnt3a* beads, whereas the number of *Lim1/2*-positive cells decreased in areas within 50 μm of the *Wnt3a* beads (Fig. 4D,E). These results indicate that *Wnt3a* proteins can organize a pattern of interneurons in the medial neural plate resembling that formed in the dorsal spinal cord. Taken together, the in vitro experiments with explants of the avian neural plate indicate that *Wnt3a* protein has the activity to induce D1/D2 neurons without dependence on BMP signaling, and that D1/D2 neurons are generated at the expense of D3 neurons by the action of *Wnt3a* protein. Thus, Wnt signals appear to be directly involved in

expression of *LH2A* and *Islet1* was clearly activated by *Wnt3a* in the explants, but was not detectable in the control explants. *Pax2* expression was significantly reduced by *Wnt3a* protein (Fig. 4B). Furthermore, activation of *LH2A* and *Islet1* expression by *Wnt3a*-conditioned medium was not affected by BMP antagonist *Noggin* at 20 nM (Fig. 4C), which caused an eightfold reduction of the *Msx1* expression when applied to the dorsal neural plate (data not shown).

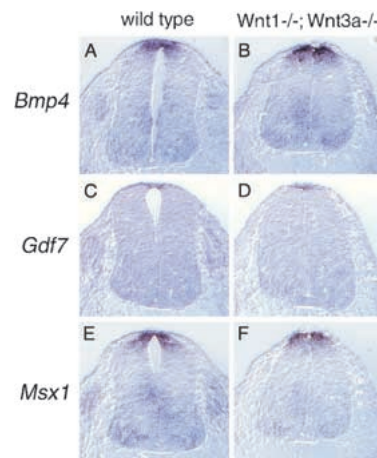


Figure 3. The expression of BMP signal-related molecules in *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos. Expression of *Bmp4* (A,B), *Gdf7* (C,D) and *Msx1* (E,F) in the cervical spinal cord of E10.5 wild-type (A,C,E) and *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos (B,D,F). All of these genes were expressed normally by roof plate cells in wild-type and *Wnt1*^{-/-};*Wnt3a*^{-/-} embryos. These results were confirmed by analysis of at least two embryos of each genotype.

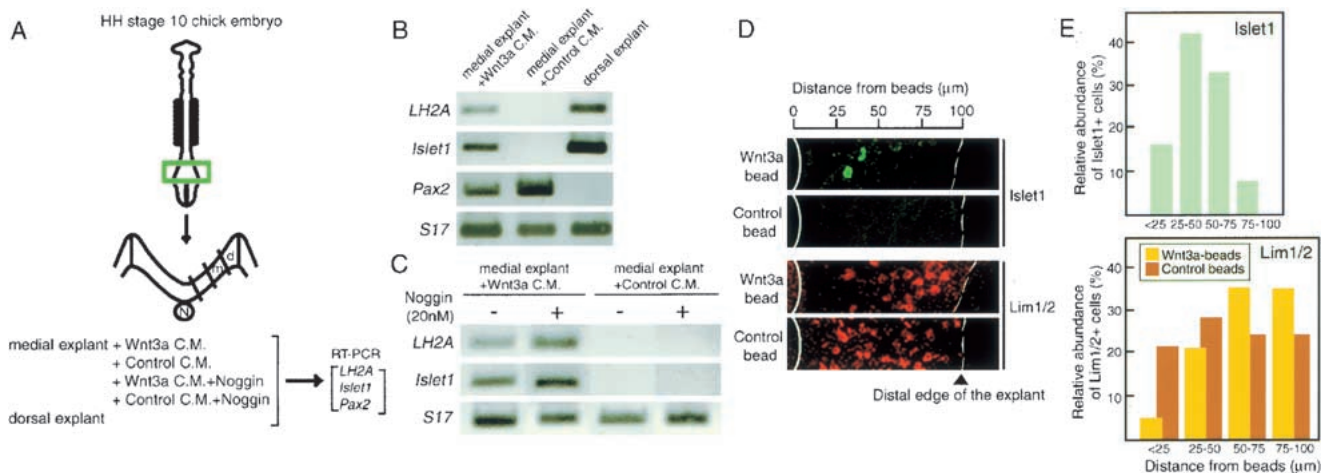


Figure 4. Wnt3a protein induces D1 and D2 interneurons in the medial neural plates of chick embryos. (A) Experimental procedure. Neural plate tissue at the caudal region of HH stage 10 chick embryo was isolated, dissected into medial (m) and dorsal (d) regions, and cultured as explants under various conditions: with Wnt3a-conditioned medium (Wnt3a C.M.), with control conditioned medium (Control C.M.), with Wnt3a-conditioned medium plus Noggin (20 nM), or with control conditioned medium plus Noggin. RNA was extracted from the explants and analyzed by RT-PCR. (N) Notochord. (B) RT-PCR analysis of Wnt3a-induced gene expression in chick neural plate explants. Analysis of medial region explants cultured for 48 h with Wnt3a-conditioned medium and of those cultured with control conditioned medium in comparison with dorsal region explants. Wnt3a treatment induced expression of both *LH2A* and *Islet1* in medial region explants ($n = 4$). In contrast, expression of *Pax2* in the medial region explant with Wnt3a was weaker than in control explants. Chick S17 ribosomal protein RNA was used to control the amount of RNA used for the RT-PCR analysis. S17 PCR products were in proportion to the amount of template RNA as confirmed by serial dilutions of the input RNA (data not shown). (C) Effect of Noggin on Wnt3a-induced gene expression in chick neural plate explants. Induction of *LH2A* and *Islet1* was not affected by addition of 20 nM Noggin protein ($n = 4$). The activity of Noggin protein was confirmed by an eightfold decrease of *Msx1* expression in dorsal region explants ($n = 3$). (D) Culture of medial region explants with Wnt3a-beads or control beads. Wnt3a-beads activated *Islet1* expression and repressed *Lim1/2* expression in cells located in regions surrounding the Wnt3a-beads. In contrast, no *Islet1*⁺ cells were observed in explants in contact with control beads. Edges of beads and distal ends of explants are indicated by solid and broken white lines, respectively. (E) Distribution of *Islet1*⁺ or *Lim1/2*⁺ cells in relation to the distance from the beads. The cell populations expressing *Islet1* or *Lim1/2* and located in specific areas (indicated by the distance from the beads) are shown as the fraction of cells among those expressing the marker proteins.

the specification of dorsal interneurons. This role defines a newly described function of Wnt proteins in neurogenesis.

The specification of the dorsal subclasses of interneurons appears to involve at least two steps of signaling cascades. BMP proteins secreted from the surface ectoderm induce the roof plate in the neural tube, and the roof plate then secretes signaling molecules required for the generation of D1 and D2 interneurons (Liem et al. 1995, 1997; Lee et al. 2000). As shown in this study, concomitant loss of Wnt1 and Wnt3a activities results in deficiency of D1 and D2 dorsal interneurons. This defect resembles that observed in mouse embryos missing the roof plate (Lee et al. 2000; Millonig et al. 2000), providing evidence for the hypothesis that Wnt proteins are the major component of the signal from the roof plate leading to dorsal interneuron specification.

On the other hand, previous *in vitro* studies have indicated that members of the BMP family, for example, BMP4, induce the expression of D1 and D2 markers in chick neural plate explants. In addition, *in vitro* assays using chick neural plate explants have shown that the inductive activity of the roof plate is inhibited by Noggin and Follistatin, antagonists of BMP-related molecules (Liem et al. 1997). Thus, BMP family proteins are considered to be another group of signaling molecules emanating from the roof plate and regulating development of dorsal interneurons. However, by use of mouse studies with targeted mutations in BMP family members expressed by roof plate cells have not yet clarified the role of BMP proteins in the development of dorsal interneurons, except for the case of *Gdf7*-deficient mice. Al-

though ocular and renal development is disrupted in the *Bmp7*-deficient mouse (Dudley et al. 1995; Luo et al. 1995), and ossification is delayed in the *Bmp6* mutant mouse (Solloway et al. 1998), no neuronal defects in the spinal cord have been reported. The *Bmp4*-deficient mouse dies around the gastrulation stage, and therefore, it is uncertain whether this gene is required for spinal cord development (Winnier et al. 1995). The only evidence for involvement of BMP proteins in the generation of dorsal interneurons is the case of *Gdf7*-deficient embryos lacking the most dorsal subclass of the interneurons, D1A (Lee et al. 1998).

Evidence indicates that Wnt signals directly control specification of dorsal interneurons without modulating BMP signaling. However, it is still possible that Wnt and BMP signals from the roof plate act in coordination on specification of dorsal interneurons. Although the cells expressing *Math1* and *Ngn1*, representing progenitors of dorsal spinal cord neural cells, were severely decreased in *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos, small cell populations located in the most dorsal regions of the neural tube still expressed these markers of dorsal neural tube (Fig. 2B,D). This is in contrast with embryos lacking the roof plate, where expression of *Math1* and *Ngn1* is completely missing (Lee et al. 2000). Thus, some signals remaining in the roof plate of *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryos, including BMP-related proteins, seem to have a subsidiary role in the development of the dorsal subclasses of interneurons. In fact, expression of *Gdf7* appeared normal in the *Wnt1*^{-/-}; *Wnt3a*^{-/-} embryo (Fig. 3H) and *Wnt1* is expressed normally in the *Gdf7* mutant embryo, whereas

loss of either GDF7 or of Wnt1/3a affects D1A neurons. This observation may suggest that Wnt1/3a and GDF7 signals in normal circumstances act coordinately to specify dorsal interneurons.

Another possible mechanism is that BMP signals are involved in regulation of the dorsal interneurons by inducing expression of *Wnt1/Wnt3a* in the roof plate. It has been shown that a constitutively active form of BMP receptor I induces ectopic expression of *Wnt1* in the neural tube (Panchision et al. 2001). Thus, BMP proteins secreted from the surface ectoderm and/or from the roof plate probably induce *Wnt1/Wnt3a* expression in the roof plate. Then, the induced Wnt proteins act to generate D1 and D2 interneurons. Induction of the D1/D2 subtypes of the dorsal interneurons by exogenous BMPs in previous reports may partly be explained by this activity of inducing Wnt protein expression.

Previous studies have shown that Wnt1 and Wnt3a have activities that promote cell proliferation in the neural tube (Dickinson et al. 1994; Ikeya et al. 1997). A slight decrease in the size of the dorsal spinal cord observed in *Wnt1^{-/-};Wnt3a^{-/-}* embryos (e.g., Fig. 1D) may be accounted for by the loss of Wnt proteins as proliferative agents. However, this work has clearly shown another important activity of the Wnt proteins, namely, specification of neuron types. The dual functions of Wnt signals thus underscore the importance of Wnt proteins in neurogenesis.

Materials and methods

Wnt1^{-/-};Wnt3a^{-/-} doubly homozygous mutant embryos

Compound heterozygotes of *Wnt1^{+/-}* and *Wnt3a^{+/-}* were produced by crosses between heterozygous mice carrying a null allele of *Wnt1* or *Wnt3a*, and maintained by backcrossing to C57/Bl6 (McMahon and Bradley 1990; Takada et al. 1994). Doubly homozygous mutants were identified among embryos derived from matings between compound heterozygotes.

In situ hybridization of embryos

In situ hybridization on frozen sections (16 μ m thickness) was carried out as described previously (Tomita et al. 2000) by use of digoxigenin-labeled probes.

Immunohistochemistry

Immunohistochemistry for neural plate explants and frozen sections was performed as described (Yamada et al. 1993; Tomita et al. 2000). Islet1 was detected with mouse monoclonal antibody 39.4D5 (from Developmental Studies Hybridoma Bank) and Lim1/2 with monoclonal antibody 4F2 (from Developmental Studies Hybridoma Bank).

Neural plate explants

The medial one third of the neural plate between dorsal and ventral thirds was isolated in the form of a strip from stage 10 chick embryos (Hamburger and Hamilton 1951) as described (Yamada et al. 1993; Liu and Jessell. 1998). A group of four isolated strips were explanted in collagen matrix (Vitrogen) with F12 medium containing penicillin/streptomycin and Mito+ Serum Extender (Collaborative Biomedical Products).

Wnt3a protein and conditioned media

Mouse Wnt3a cDNA was transfected into mouse L-cells and expressed under the PGK promoter. Wnt3a-conditioned medium was prepared as described previously (Shibamoto et al. 1998). Transfected cells were cultured in DMEM/F12 with 0.5% fetal bovine serum for 3 d and in serum-free DMEM/F12 for a further 24 h. Wnt3a-containing conditioned medium was then harvested. The concentration of Wnt3a protein in the conditioned medium was estimated as 400 ng/mL. Heparin acrylic beads (H5263, Sigma) of 0.1 mm in diameter, were soaked in Wnt3a-conditioned medium or control medium for 2 h at 4°C. Then, Wnt3a-soaked beads were placed into the medial region of a stage 10 chick neural plate

in a collagen matrix. Binding of Wnt3a proteins to heparin beads was confirmed by Western blotting with an anti-Wnt3a monoclonal antibody. Noggin proteins were purchased from R&D systems.

RT-PCR

A group of four explants was collected in 400 μ L Trizol reagent (GIBCO BRL) and RNA was prepared according to the manufacturer's protocol. Total RNA was reverse-transcribed (GIBCO BRL) by use of random hexamer primers (Takara) in a reaction volume of 20 μ L. The reverse transcripts were amplified by PCR for 35 cycles, each consisting of reactions at 94°C for 30 sec, at 60°C–65°C for 30 sec, and 72°C for 2 min. PCR products were analyzed on a 1.5% agarose gel.

Primer pairs used to amplify chick *LH2A*, *Islet1*, *Pax2*, and *Msx1* sequences were as follows: *LH2A* 5', 5'-GCATTTCACCTCGGATG TAGCTGC-3'; *LH2A* 3', 5'-GCTTGGAGTGTGAATCTGAAGTC-3'; *Islet1* 5', 5'-GAGCAACTGGTAGAAATGACTGGCCTCAGT-3'; *Islet1* 3', 5'-TCGATCCTCCTCAAGATCATTGAGTAGC-3'; *Pax2* 5', 5'-GACAAAGGATAGTGGAGCTGGC-3'; *Pax2* 3', 5'-ACGCTCAAAA CTCGATCTAAAGC-3'; *Msx1* 5', 5'-GAGATATTCGCCTCCTCCAA GACAC-3'; *Msx1* 3', 5'-ACACCCAGCCTGTTGCATACAGGATG-3'. Sequences for S17 primers were adopted from a previous publication (Trueb et al. 1988).

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