Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system

Mary E. Dickinson¹*, Robb Krumlauf² and Andrew P. McMahon¹*

¹Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA
²Laboratory of Developmental Neurobiology, Medical Research Council, National Institute of Medical Research, London NW7 1AA, UK

*Present address: The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

SUMMARY

The analysis of mutant alleles at the Wnt-1 locus has demonstrated that Wnt-1-mediated cell signalling plays a critical role in development of distinct regions of the embryonic central nervous system (CNS). To determine how these signals participate in the formation of the CNS, we have ectopically expressed this factor in the spinal cord under the control of the Hoxb-4 Region A enhancer. Ectopic Wnt-1 expression causes a dramatic increase in the number of cells undergoing mitosis in the ventricular region and a concomitant ventricular expansion. Although this leads to consistent changes in the relative proportions of dorsal and ventral regions, Wnt-1 does not appear to act as a primary patterning signal. Rather, our experiments indicate that Wnt-1 can act as a mitogen in the developing CNS.

Key words: neural development, Wnt family, spinal cord, transgenic mouse, cell signalling, cell proliferation, pattern formation

INTRODUCTION

The vertebrate central nervous system (CNS) arises as a flattened plate of neuroepithelium, which rounds up to form a hollow tube (see Schoenwolf and Smith, 1990, for review). Initially the neural tube consists of a single layer of morphologically indistinguishable cells. During the course of embryogenesis, this simple epithelium is transformed, through progressive changes in cell growth and differentiation, into a highly ordered structure with a vast complexity of cell types. As early as the neural plate stage, there is a clear difference between the anterior end of the embryo, which will become the brain, and the posterior neural tube, which will form the spinal cord. Within the brain, the forebrain, midbrain and hindbrain are demarcated by the time that the neural tube closes. Moreover, transplantation studies indicate that regional cell fate is fixed soon after these domains are clearly visible (Chung and Cooke, 1978; Nakamura, 1990; Alvarado-Mallart et al., 1990; Martinez et al., 1991; Itasaki et al., 1991). A large body of evidence supports the idea that cells within the vertebrate nervous system develop according to the signals that they receive rather than a program of development inherited from their ancestors (Turner et al., 1990; Martinez et al., 1991; Storey et al., 1992; Sanes, 1993). For example, Martinez et al. (1991) have shown that Engrailed (En)-expressing regions of the metencephalon are capable of inducing prosencephalic tissue to express En, and subsequently to adopt a midbrain phenotype. Thus, extracellular signals are implicated in the establishment of cell fate within the CNS.

The Wnt gene family consists of at least fifteen vertebrate members, many of which are expressed in the early neural tube (for review see McMahon, 1992; Nusse and Varmus; 1992; Parr et al., 1993). In fact, several Wnt genes are expressed in defined patterns along the anterior-posterior axis of the nervous system, consistent with a role in establishing regional differences. These genes encode secreted glycoproteins, which probably act as intercellular signalling molecules during embryonic development (Van den Heuvel et al., 1989; Gonzalez et al., 1991; Jue et al., 1992). However, Wnt-1 and wingless, its Drosophila orthologue, do not encode freely diffusable proteins. Rather, these proteins disperse only a few cell diameters beyond expressing cells, probably due to interactions with the extracellular matrix and/or cell surface components (Papkoff and Schryver, 1990; Bradley and Brown, 1990). Thus, in the vertebrate CNS, Wnt factors are likely to be acting as short-range signalling molecules with limited, sometimes overlapping, distributions.

We are interested in how Wnt signals are interpreted by cells in the embryonic nervous system and in how these signalling events are involved in early CNS development. Recent experiments have shown that at least one member of the Wnt gene family, Wnt-1, is essential for the development of a large region of the brain. Wnt-1 is normally expressed throughout most of the midbrain region prior to neural tube closure. By the 14-somite stage (8.75 d.p.c.), expression within the midbrain becomes restricted to the dorsal midline and to a ring of cells that encircle the midbrain-hindbrain border. In addition, dorsal midline expression can be seen just anterior to the midbrain in the diencephalon, as well as in the myelencephalon and along the entire length of the spinal cord.
MATERIALS AND METHODS

Expression constructs

Two constructs were used to express Wnt-1 ectopically under the control of the Hoxb-4 Region A enhancer. The original construct was generated by simultaneously inserting the 5′ KpnI-SacI fragment of the Wnt-1 gene (including the first exon, part of the first intron and 1334 bp upstream of the start codon) and the 3′ SacI-BglII fragment (which contains the remaining exons and introns, as well as the 3′ untranslated region and the polyadenylation signal) into the KpnI-BglII sites of pSP72 (Promega); construct no. 728. The resulting construct was then linearized using an XhoI site contained within pSP72 which was converted to a blunt end cloning site using a Klenow fill-in reaction. A blunt end 3.3 kb HindIII-NcoI fragment containing the Hoxb-4 Region A enhancer was cloned into this construct upstream of the Wnt-1 gene using the modified XhoI site.

We obtained constructs in which the enhancer was in both possible orientations. For these experiments, we utilized a construct that had the Region A enhancer in the opposite orientation with respect to how it is positioned downstream of the Hoxb-4 gene (Whiting et al., 1991) (see Fig. 1; construct no. 723). This original construct was used in the first five experiments. Subsequent experiments were performed with a nearly identical construct, which contains a 245 bp Ncol-AccI fragment of the neomycin phosphotransferase gene (neo) from pMC1neo (Stratagene) that has been blunt end ligated into a filled in pMC1neo site located in the 3′ untranslated region of the Wnt-1 gene (Fig. 1; construct no. 724). The neo fragment is in the construct in the antisense orientation and we can use this sequence to detect specifically mRNA being transcribed from the transgene by using a sense strand in situ hybridization probe from this region of the neo gene. Both constructs were linearized for microinjection using SalI-BglII digests. Fragments were prepared for microinjection either by separating fragments by sucrose gradient centrifugation followed by dialysis against injection buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6) or by separating the fragments on a low melting temperature agarose gel and using the Gelase method of preparing DNA from gel slices (Epigenic Technologies, Madison WI). (We follow the Gelase ‘high activity’ protocol and add a phenol/chloroform extraction step to this procedure, after the overnight Gelase digestion but before the final precipitation.) The DNA injection solution was adjusted to 1.5 ng/µl for sucrose-purified DNA and 3 ng/µl for Gelase-isolated DNA using injection buffer.

Production of transgenic embryos

Transgenic embryos were generated following the basic procedures as outlined in Hogan et al. (1986). C57BL/6J×CBA/F1 mice were used as embryo donors, stud males and pseudopregnant recipients. CD-1 outbred males were used as vasectomized males. Embryos were cultured overnight in M-16 media following microinjection and transferred to the 2-cell stage to pseudopregnant recipient females. Noon of the day of the transfer was considered to be 0.5 days post coitum (d.p.c.). For many experiments, pregnant females were injected intraperitoneally with 50 µg/g body weight of 5-bromo-2′-deoxyuridine (BrdU) (Sigma) 2-3.5 hours prior to killing. Staged embryos were collected at 9.5 d.p.c., 10.5 d.p.c., 11.5 d.p.c., 12.5 d.p.c., 14.5 d.p.c. and 15.5 d.p.c.

Embryos from crosses between line JL-64 [construct no. 9 in Whiting et al., 1991] and B6CBAF1 mice were obtained in order to study the regulation of the Hoxb-4 Region A enhancer during spinal cord development. Embryos were stained for β-galactosidase activity and embedded in paraffin wax as described in Whiting et al. (1991).

PCR analysis

Yolk sacs were isolated from individual embryos and used for PCR analysis in order to detect the presence of the transgene. Yolk sacs were digested overnight at 55°C in 50 mM KCl, 10 mM Tris-HCl pH 8.0, 2.5 mM MgCl2, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20 (Hanley and Merlie, 1991) in the presence of 12.5 µg/ml of Proteinase K (Boehringer Mannheim). Samples were then boiled for 10 minutes and spun at 13,000 revs/minute for 30 minutes to pellet undigested debris. The supernatant from these samples was then diluted.
Measurements and statistical analyses on either Kodak Technical Pan film or Kodak 64T film. Measurements were fixed and embedded as described above. Photomicrographs were as described in Miller and Nowakowski (1988) except that embryos et al., 1990). Immunohistochemical detection of BrdU was performed with the use of an Olympus SZH photo-dissecting microscope using Fujichrome Velvia film while the embryos were in 70% ethanol alcohol following fixation. Embryos were serially sectioned at 4-6 μm in the transverse plane. Representative sections were stained with haematoxylin and eosin and photographed on a Leitz Aristoplan-2 photomicroscope using Kodak 64T film. Remaining sections were used for in situ hybridization analysis or for the immunohistochemical detection of BrdU incorporation.

Embryos stained for β-galactosidase activity were sectioned at 8 μm in the transverse plane. Sections were then rehydrated through an ethanol series, mounted in 80% glycerol and photographed under Nomarski optics using a Leitz DMRB photomicroscope and Kodak 160T film. Individual slide images were scanned into the Adobe Photoshop software package (Adobe Systems, Inc.) using a Kodak RFS 2035 film scanner in order to make the composite, which was printed with a Mitsubishi CP210U color video processor.

Histological analysis
Embryos were fixed in 4% paraformaldehyde overnight at 4°C, subsequently dehydrated in a series of graded ethanol and embedded in paraffin wax (Fibrowax, BDH) as described in Wilkinson et al. (1987). Whole-mount photomicrographs of embryos were obtained with the use of an Olympus SZH photo-dissecting microscope using Fujichrome Velvia film while the embryos were in 70% ethanol alcohol following fixation. Embryos were serially sectioned at 4-6 μm in the transverse plane. Representative sections were stained with haematoxylin and eosin and photographed on a Leitz Aristoplan-2 photomicroscope using Kodak 64T film. Remaining sections were used for in situ hybridization analysis or for the immunohistochemical detection of BrdU incorporation.

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In situ hybridization and immunohistochemical detection of BrdU
In situ hybridization analysis was performed as described in Wilkinson et al. (1987) using single-stranded 35S-labelled probes to several genes expressed differentially in the spinal cord. The Wnt-1 probe used (referred to as Wnt-13) has been described in McMahon et al. (1992). The Wnt-7b probe is a 306 bp PstI-EcoRI fragment from the 3′ untranslated region of the Wnt-7b gene (Gavin et al., 1990). The Pax-3 probe is a 516 bp HindIII-PsrI fragment of the Pax-3 gene as described in (Goulding et al., 1991). The Pax-6 probe is a 260 bp EcoRI-Nhel fragment as described in (Walther and Gruss, 1991). The Wnt-3a probe is a 250 bp probe, which has been described elsewhere (Roelink and Nusse, 1991). Dov-6 mRNA was detected using a 266 bp PvuII-SacI fragment encoding part of the mature polypeptide (Lyons et al., 1989). The Steel factor (kit ligand) gene probe is a 396 bp NcoI-SstII fragment of the cDNA (Huang et al., 1992). The Islet-1 probe is a ~400 bp Clal-XbaI fragment of the rat cDNA (Karlsson et al., 1990). Immunohistochemical detection of BrdU was performed as described in Miller and Nowakowski (1988) except that embryos were fixed and embedded as described above. Photomicrographs were taken using a Leitz Aristoplan-2 photomicroscope and were recorded on either Kodak Technical Pan film or Kodak 64T film.

Measurements and statistical analyses
The number of BrdU-labelled cells was determined by counting the number of immunoperoxidase-stained nuclei in 4 μm transverse sections of the spinal cord. Estimates were taken from three sections through the caudal thoracic region of each of the two 12.5 d.p.c. transgenic embryos. Five sections taken from the same axial level of a non-transgenic littermate were analyzed as controls. The area of the ventricular zone was calculated by overlaying enlarged photographs of the same BrdU-stained sections with graph paper and then tracing the border of the ventricular zone onto the grid. (The same sections used to calculate the number of labelled cells were used to calculate the area of the ventricular zone.) Area was then determined by counting the number of square divisions within the traced area. Statistical analysis of BrdU-labelled cell estimates and ventricular area estimates were performed using an unpaired t-test with the aid of Statview 4.0 software (Abacus Concepts, Inc., Berkeley, CA). These results were verified using Dunn’s approximation to the Kruskal-Wallis rank sum multiple comparisons test for treatments versus controls. The P-values for these analyses were in good agreement with those obtained using the unpaired t-test.

RESULTS
Production of transgenic embryos
In order to express Wnt-1 specifically within the embryonic spinal cord, we made expression constructs that link the Wnt-1 gene (van Ooyen and Nusse, 1984) and the Hoxb-4 (formerly Hox-2.6) Region A enhancer (Whiting et al., 1991) (Fig. 1A,B). Like other genes in the Hoxb cluster, Hoxb-4 is expressed in a dynamic pattern within the spinal cord (see Fig. 1C), as well as in other regions of the embryo (Graham et al., 1988, 1991; Whiting et al., 1991). During early stages of development (8.5-10.5 d.p.c.), Hoxb-4 is expressed within the ventricular zone of the hindbrain and spinal cord from the rhombomere 6/7 boundary to the caudal extent of the axis. Thus, Hoxb-4 is expressed by proliferating precursor cells capable of giving rise to both neuronal and glial cell types. By 12.5 d.p.c., Hoxb-4 expression is localized to the dorsal intermediate zone and, at 14.5 d.p.c., transcripts are abundant in the dorsal horns. The Hoxb-4 Region A enhancer mimics the expression of the endogenous Hoxb-4 gene within the spinal cord (Whiting et al., 1991). In order to examine the regulation of the Hoxb-4 Region A enhancer at a cellular level, we have studied transverse sections of stained embryos that carry a Hoxb-4 Region A-lacZ marker gene construct (see Materials and Methods; Whiting et al., 1991). Our results confirm previous studies as β-galactosidase activity is detected within the neural tube and its derivatives (Fig. 1C; Whiting et al., 1991). However, throughout development, enhancer driven expression is weaker in the hindbrain and rostral spinal cord (anterior to the forelimb) than in the region of the spinal cord posterior to the forelimb. For instance, at 9.5 d.p.c. expression in the hindbrain and rostral spinal cord is not only much weaker, but also does not include cells in the most dorsal and ventral regions of the neural tube, in contrast to the strong expression all along the dorsoventral axis of the caudal spinal cord (Fig. 1C left panel). Rostrocaudal differences in expression are also evident at 10.5 d.p.c. (Fig. 1C, middle panels), 12.5 d.p.c. (Fig. 1C, right panels) and 15.5 d.p.c. (data not shown). Although the levels of expression vary along the axis, the enhancer regulates expression along the dorsoventral axis in a manner that is consistent with what is known about the regulation of the Hoxb-4 locus. At 10.5 d.p.c. β-galactosidase-positive cells are found in the ventricular zone as well as in the intermediate zone (Fig. 1C top, middle panel) and, by 12.5 d.p.c., nearly all of the expressing cells are located in the dorsal intermediate zone (Fig. 1C bottom, right panel). Migrating neural crest cells and structures composed of neural crest cells, such as dorsal root and sympathetic ganglia, also show β-galactosidase activity (Fig. 1C left and middle panels), indicating that the Hoxb-4 enhancer is active in these cells or their progenitors. Enhancer
driven expression persists in the spinal cord at least until 15.5
d.p.c., although expression appears weak (data not shown).

Our analysis confirms that the Region A enhancer contains
only the regulatory elements necessary for correct expression
in the hindbrain, spinal cord and its derivatives, such as neural
crest cells, but not other regions of the developing embryo
where the Hoxb-4 gene is normally expressed (Fig. 1C; Whiting et al., 1991). This has enabled us to enlarge the
domain of Wnt-1 expression within the neural tube without
affecting other regions of the embryo. Because we expected
the ectopic expression of Wnt-1 to cause postnatal lethality,
founder (generation 0 [G0]) embryos were examined for
aberrant phenotypes (Table 1). Subsequently, all embryos were
genotyped by Polymerase Chain Reaction (PCR) using primers
specific for the transgene (Fig. 1).

The spinal cords of transgenic embryos consistently
appeared enlarged and ‘wavy’ at all stages examined. Only 3
of 42 embryos had defects outside the spinal cord in keeping
with the specificity of the Region A enhancer and limited
diffusion of Wnt-1 protein. These three embryos were necrotic
when harvested and displayed multiple developmental defects,
perhaps caused by widespread activation of the transgene
construct by insertion near another strong enhancer or because
the transgene itself has disrupted a gene necessary for proper
early development. These embryos were not analyzed beyond
the level of visual inspection. At 10.5 d.p.c. and 12.5 d.p.c.,
the phenotype in the spinal cord can easily be seen when trans-
genetic embryos are compared to wild-type controls (Figs
2A, D, G, J, 3). Spinal cords of transgenic embryos contain a
much deeper, wider lumen than controls and the neural tube

**Fig. 1.** Wnt-1 ectopic expression constructs and expression pattern of the Hoxb-4 gene. For both constructs, the Hoxb-4 Region A enhancer has
been placed upstream of the entire Wnt-1 gene, including 650 bp of Wnt-1 promoter sequence (see Materials and methods). This 650 bp
minimal promoter cannot promote functional transcription without an appropriate enhancer (Y. Echelard, G. Vassileva and A. P. M.,
unpublished data). #1 and #2 refer to the position of PCR primers used to detect the transgene in yolk sac DNA samples from injected embryos.
The construct shown in A was used in the initial stages of the project and the construct shown in B was substituted in later experiments. These
constructs produce identical results and they differ only in their 3′ untranslated regions where the construct shown in B was engineered to carry
a 245 bp fragment of the neomycin transferase gene to allow for specific detection of transgenic mRNA. (C) Regulation of β-galactosidase
expression by the Hoxb-4 region A enhancer. Transverse sections through embryos at 9.5, 10.5 and 12.5 d.p.c. stained for β-galactosidase
activity. The left panel shows a section from a 9.5 dpc embryo, through the hindbrain and the spinal cord, caudal to the forelimb. Middle panels
show transverse sections from a 10.5 d.p.c. embryo. The section shown in the top panel (ant) is at the level of the forelimb and the bottom panel
(post) shows a section just rostral to the hindlimb. The panels on the right show sections from a 12.5 d.p.c. embryo. The top panel (ant) is a more rostral section
taken from the caudal thoracic region and the bottom panel (post) shows a section from the lumbar region. Embryos were obtained from stable transgenic lines
(IL-64) which carry construct #9 as shown previously in Whiting et al. (1991; see Materials and methods). drg, dorsal root ganglia; fp, floor plate; hb, hindbrain;
iz, intermediate zone; nc, neural crest; sc, spinal cord; vz, ventricular zone.
Wnt-1 can act as a mitogen in the CNS has an abnormal, asymmetric appearance. Embryos examined at all stages had a similar phenotype, but exhibited variable degrees of severity. This is best seen in the largest population of embryos that were recovered at 12.5 d.p.c. These embryos were grouped into four severity classes based on superficial inspection of spinal cord irregularity [+/− (weakest effect), +, ++ and +++ (strongest effect)] (Fig. 3). The phenotypic class was determined by comparing the size and morphology of the spinal cord to normal littermates. In +/− embryos, only slight irregularities in spinal cord structure were noted and the overall size of the spinal cord appeared quite normal. Progressively greater disruptions in the linear morphology of the neural tube

Fig. 2. Dysmorphology of the spinal cord in transgenic embryos at 10.5 d.p.c. A,D,G and J are whole-mount photomicrographs. Embryos are all oriented dorsal up, anterior to the left (see forelimb (fl) and hindlimb (hl) for orientation). B,C,F,H,I,K,L are photomicrographs of hematoxylin and eosin (H&E)-stained transverse sections from two levels through each embryo. The level of each section is depicted by arrows on each of the corresponding whole-mount photomicrographs. B,E,H,K are more rostral sections relative to those shown in C,F,I,L (A–C) A control embryo; (D–F) embryo Tg 2815; (G–I) embryo Tg 8815, and (J–L) embryo Tg 9815. The notochord in E,F,H,I,K and L is arrowed. The ventricular zone is demarcated by a dashed line. A large haemangioma in D,F is indicated by the asterisk (*) in F. Transgenic embryos at several different stages showed disruptions in the normal vasculature da, dorsal aorta; drg, dorsal root ganglia; no, notochord; rp, roof plate; vh, ventral horn. The scale bar in panel B is equivalent to 100 µm and B,C,E,F,H,I,K,L were all photographed at the same magnification.
and small differences in the size could be detected in + and ++ embryos. In the most extreme cases (+++), the spinal cord appeared very convoluted and the expansion of spinal cord tissue formed a very pronounced bulge along the dorsal midline. Differences in the degree of abnormality are predictable within a G0 population. Variability in the copy number of the transgene, position effects caused by the site of integration or mosaicism caused by integration of the transgene after the first cleavage are likely to account for the observed differences. Despite this, over 50% of the transgenic embryos recovered at this stage have a common phenotype (Table 1) and 22% of these embryos exhibit severe defects (+++, Fig. 3). In addition to differences in severity amongst embryos, we also noticed that the hindbrain and rostral spinal cord (roughly to the level of the forelimb bud) showed little disruption in normal morphology, yet caudal regions of the same embryos were severely affected. This result is consistent with the weaker and less widespread expression seen with the Region A element in these anterior regions.

We chose a representative subset of embryos recovered at various stages for further characterization. Three 10.5 d.p.c. transgenic embryos (the three most extreme of the six recovered), six 12.5 d.p.c. transgenic embryos (1+, 1++ and 4+++) and two 15.5 d.p.c. transgenic embryos together with two non-transgenic controls were subjected to a detailed analysis. These embryos were embedded in paraffin wax, serially sectioned and analyzed using several different techniques.
Histological analysis of the spinal cord phenotype

Transverse sections through transgenic and control embryos were examined histologically to identify disruptions in normal anatomy. The spinal cord is functionally subdivided into three major regions: the ventricular zone, the intermediate zone and the marginal zone (Boulder Committee, 1970). The ventricular zone consists of actively dividing precursors for both neuronal and glial cells (Nornes and Carry, 1978). Neurons are generated in a specific sequence both with respect to time and their position along the dorsoventral axis (Nornes and Carry, 1978). As each cell begins to differentiate, it ceases mitosis and migrates away from the ventricular zone into the intermediate zone, where postmitotic neuronal cells complete their maturation. Generally, cells within ventral regions cease dividing and begin to differentiate at earlier times than cells that constitute more dorsal parts of the spinal cord. As cells differentiate,
extending axons and processes, the marginal zone, which contains only fiber tracts, becomes prominent.

At 10.5 d.p.c., control spinal cords have a large ventricular zone composed of darkly stained cells, densely arranged along the luminal surface (outlined by dotted lines in Fig. 2B,C). The ventral horns are prominent regions of lightly stained, large motor neurons adjacent to the floor plate (Fig. 2B,C marked vh; Nornes and Carry, 1978). No distinguishable ventral horns have formed in transgenic embryos by 10.5 d.p.c. and the ventricular region is greatly expanded (compare Fig. 2B,C with remaining panels). In addition, in many sections taken from transgenic embryos the notochord appears to have been flattened (Fig. 2E,F,I,K,L, small arrowhead), perhaps due to mechanical stress imposed by the expanding neural tube.

In normal embryos at 12.5 d.p.c., cells in the dorsal half of the spinal cord are undergoing differentiation (Nornes and Carry, 1978) and the dorsal ventricular region is very pronounced (Fig. 4A,B large arrowhead). In contrast, as the majority of ventral cells have been born by this time (Nornes and Carry, 1978), the ventral ventricular region contains only a small group of darkly stained cells, which lie adjacent to the lumen (see Fig. 4A,B small arrowhead). The intermediate zone, where nuclei are less densely arranged, surrounds the ventricular zone (iz, Fig. 4A,B) and the marginal zone can be best detected in the most ventral regions (mz, Fig. 4A,B). In embryos with a less severe phenotype, constrictions in the ventricular zone can be seen (+, Fig. 4D), which indicate that normal spinal cord morphology has been perturbed. In more severe embryos (+++) the ventricular region is greatly enlarged (compare Fig. 4A,B with E,F). Moreover, the area of the dorsal ventricular zone (Fig. 4E,F large arrowhead) appears to be enlarged to a greater extent than ventral regions within the same embryo (Fig. 4E,F small arrowhead). Overall, considerably more spinal cord tissue is present in transgenic embryos than in control embryos. In extreme cases, the dorsal part of the spinal cord is very tightly opposed to the surrounding meningeal tissue and the overlying skin. In addition, the roof plates of transgenic spinal cords are often thinner than normal (Fig. 4E,F, asterisk and Fig. 2H,I,L), perhaps as a result of stretching caused by forces exerted by the expanding dorsal ventricular layer.

By 15.5 days of development, the lumen of the spinal cord in control embryos has narrowed and is barely detectable. Virtually all neuronal cells have migrated into the intermediate zone and the ventricular layer consists of relatively few, darkly stained cells, which line the central canal. Dorsal and ventral horns are clearly visible and the marginal zone can easily be recognized (Nornes and Carry, 1978; Altman and Bayer, 1984; Fig. 5A,D). Sections through transgenic embryos indicate that the ventricular zone is constricted (Fig. 5C,F small arrowhead), and cells around the lumen have a disorganized appearance (Fig. 5B,C,E,F). Extra luminal regions which are continuous with the central canal can be detected in some of the sections (Fig. 5F). In contrast to the highly organized and symmetric configuration of cells that can be seen in the control sections, the cells in transgenic spinal cords have an irregular organization. However, both dorsal and ventral cell types can be readily detected based on morphological criteria. For instance, cells of the dorsal horn have small, dense, darkly stained nuclei and little cytoplasm, whereas motor neurons are large, lightly stained cells with a clearly visible cytoplasm. Although ventral horns could not be distinguished in 10.5 d.p.c. transgenic spinal cords, discrete groups of large motor neurons are present in transgenic spinal cords at 15.5 d.p.c. (Fig. 5, arrows). A large number of dorsal horn cells can be detected within transgenic spinal cords at this stage and the dorsal half of the spinal cord appears to be expanded to a greater extent than ventral regions (Fig. 5B,C,E,F), but individual classes of differentiated cells have not been quantified. Although it is not clear exactly which cells are overrepresented in spinal cords in which Wnt-1 is ectopically expressed, transgenic spinal cords are clearly much larger. Moreover, the expansion of the spinal cord appears to have caused pressure on the surrounding meninges, forcing the meningeal layer into the spinal cord (Fig. 5E,F, asterisk).

**Characterization of Wnt-1 ectopic expression**

In order to correlate ectopic expression of Wnt-1 with the resulting phenotype, we have used 35S-labelled RNA probes to characterize the distribution of Wnt-1 transcripts. Throughout normal embryogenesis, Wnt-1 is expressed in the dorsalmost part of the spinal cord, beginning at early somite stages (Wilkinson et al., 1987; McMahon et al., 1992; Fig. 6B and E, arrow). In contrast to the localized expression of Wnt-1 in control embryos (Fig. 6B, arrow), strong ectopic expression was detected uniformly along the dorsoventral axis of the caudal thoracic spinal cord in all three of the 10.5 d.p.c. transgenic embryos assayed (Fig. 6D and data not shown).

By 12.5 d.p.c., ectopic expression can only be detected in embryos that have the most extreme phenotypes (in three of four +++ embryos examined). At this and all other stages, ectopic expression is restricted to the ventricular layer of the spinal cord (Fig. 6G,I,K), despite the fact that Hoxb-4 is normally expressed only in the dorsal intermediate zone at these later stages in development. Strong, uniform expression can be detected in a number of transverse sections taken from Tg 6720, which has the most severe phenotype, whereas only patchy expression is detected in Tg 11720 and Tg 36916, which have weaker phenotypes.

Only one of the two embryos examined at 15.5 d.p.c. showed ectopic expression. This was restricted to the ventricular layer surrounding the lumen and was very weak (data not shown). These experiments demonstrate that the transgene establishes strong ectopic expression by 10.5 d.p.c., and most likely earlier but, by 12.5 d.p.c., the majority of embryos no longer ectopically express Wnt-1. Those embryos maintaining ectopic expression have the most severe phenotypes and in regions of the embryo that routinely show a less severe phenotype, such as the hindbrain and rostral spinal cord,
Wnt-1 can act as a mitogen in the CNS.

Fig. 5. Histological analysis of the spinal cord morphology of transgenic embryos at 15.5 d.p.c. (A-C) Sections taken from the mid-thoracic region and (D-F) sections from the caudal thoracic region. (A,D) Photomicrographs of sections from control embryos. B,E and C,F are sections from transgenic embryos Tg 17727 and Tg 17618, respectively. The arrows in all panels indicate the position of motor neurons. Arrowheads in C and E represent constrictions of the ventricular zone. Asterisks in E and F indicate meningeal tissue that has been forced into the spinal cord. cc, central canal; dh, dorsal horn; mz, marginal zone; vh, ventricular zone. The scale bar in panel A is equivalent to 100 µm. All photomicrographs were taken at the same magnification.
Fig. 6. Ectopic Wnt-1 expression in 10.5 d.p.c. and 12.5 d.p.c. transgenic embryos. All sections were hybridized with a 35S-labelled Wnt-1 riboprobe. 10.5 d.p.c. control and transgenic sections are shown in A,B and C,D (Tg 8815), respectively. Sections from 12.5 d.p.c. control and transgenic embryos are shown in D,E and F,G (Tg 11720), H,I (Tg 36916), J,K (Tg 6720), respectively. Wild-type Wnt-1 expression in the roof plate is arrowed in B and E. A,C,D,F,HJ are bright-field photomicrographs and B,D,E,G,I and K have been photographed using dark-ground illumination. Scale bars are equivalent to 100 µm.
Fig. 7. Examination of Wnt-7b expression in the ventricular zone of Wnt-1 transgenics at 12.5 and 15.5 d.p.c. All sections have been hybridized with an 35S-labelled riboprobe which specifically detects Wnt-7b mRNA. A,E and C,G represent control sections hybridized with the Wnt-7b probe, whereas B,F and D,H are sections from transgenic embryos (Tg 36916 and Tg 17727, respectively). A,B and E,F are from 12.5 d.p.c. embryos and C,G and D,H are from 15.5 d.p.c. embryos. A-D represent bright-field images and E-H were photographed using dark-ground illumination. Arrows in G and H indicate Wnt-7b hybridization to ventricular cells surrounding the central canal. Scale bars are equivalent to 100 µm.
expression is either absent or significantly reduced as compared to more caudal regions of the same embryo (in the two +++ embryos examined; data not shown). Therefore, the severity of the phenotype correlates with ectopic Wnt-1 expression.

**Wnt-7b expression in transgenic embryos**

Histological analysis of 10.5, 12.5 and 15.5 d.p.c. transgenic embryos indicates that ectopic expression of Wnt-1 results in ventricular expansion. To analyze this in greater detail, we examined Wnt-7b expression in transgenic embryos. Wnt-7b is expressed in the ventricular layer of the normal spinal cord at 12.5 d.p.c. (Fig. 7E). In transgenic embryos examined at this stage, Wnt-7b-expressing cells extend further ventrally than normal, indicating that the inner ventricular zone has expanded along the dorsoventral axis (Fig. 7F). Thus, either Wnt-7b transcription is activated by Wnt-1 ectopic expression or Wnt-1 enhances the proliferation of Wnt-7b-expressing cells.

By 15.5 d.p.c., proliferation in the ventricular zone has greatly declined, as neurogenesis is complete (Nornes and Carry, 1978). A few dividing cells remain, which are probably glia. These cells divide throughout the life of the animal (see Sturrock, 1982 for discussion). At this time, Wnt-7b can be detected in the darkly stained ventricular cells which surround the shrunken lumen (Fig. 7G). In transgenic embryos, Wnt-7b is expressed in ventricular cells surrounding the central canal as well as within groups of darkly stained cells in more lateral regions of the spinal cord (Fig. 7H arrow). By analyzing serial sections, we have concluded that the lateral groups of Wnt-7b-expressing cells are adjacent to lumens which are continuous with the central canal (see also Fig. 5C). The presence of ventricular cells in these lateral regions suggests that the expanded ventricular zone collapsed and folded during development.

**Wnt-1 expression enhances the proliferation of spinal cord precursor cells**

The expansion of the ventricular zone within transgenic embryos suggests that Wnt-1 signalling is leading to enhanced or prolonged mitotic activity in this region. To investigate this issue further, we directly assayed proliferative activity by monitoring the incorporation of bromodeoxyuridine (BrdU) at 12.5 d.p.c. (Fig. 8). On average, approximately 3.3 and 7.5 times the number of proliferating cells could be detected in embryos Tg 11720 and Tg 6720, respectively (Fig. 9A) as compared to the number of dividing cells in control sections. These increases are statistically significant (wt versus Tg 11720, \( P=0.0075 \); wt versus Tg 6720, \( P<0.0001 \), unpaired t-test).

In addition to quantifying the number of labelled cells per section, we also measured the area of the ventricular zone. The mean areas observed for Tg 11720 and Tg 6720 were approximately 3.0-fold and 7.4-fold greater than control embryos, respectively (Fig. 9B). These increases are also statistically significant (wt versus Tg 11720 \( P=0.0153 \), wt versus Tg 6720 \( P<0.0001 \), unpaired t-test). The increases in area correlate well with the observed increases in the number of labelled cells, indicating that there were no differences in the density of labelled cells within the ventricular zone. Thus, the increase in proliferating cells accounts for the increase in the area of the ventricular zone.

No significant increase in the number of BrdU-labelled cells could be detected in 15.5 d.p.c. transgenic embryos (data not shown). Therefore, ectopic Wnt-1 expression causes a pronounced enhancement of ventricular proliferation in the spinal cord, which ceases by 15.5 d.p.c. These results correlate with Wnt-1 ectopic expression. There is an increase in proliferation in embryos where Wnt-1 expression has been maintained at 12.5 d.p.c. and no increase in mitotic activity at later stages when ectopic expression has declined.

**Analysis of dorsoventral patterning in transgenic embryos**

Interestingly, substantial ventral proliferation is observed in the most severe transgenic embryo at 12.5 d.p.c. in contrast to control embryos where few mitotically active cells are present in ventral regions of the spinal cord (Fig. 8 compare A,D to C,E, arrowhead). Several hypotheses could account for the observed distribution of BrdU-labelled cells. (1) Cells in the ventral ventricular zone could actually be dorsal cells that have been forced into ventral territories. If this were the case, dorsal and ventral cells would be intermingled in ventral regions. (2) The fate of ventral cells could have been altered by the ectopic expression of Wnt-1 so that these ventrally located cells now have a dorsal identity and continue to divide according to the dorsal schedule. According to this scenario, dorsal cell types would exist at the expense of ventral cell types and ventral cell types would no longer be present. (3) Wnt-1 could, either directly or indirectly, be providing a proliferative stimulus which causes cells to remain dividing, without primarily affecting the dorsoventral distribution or patterning of cells in this region. We have used several in situ hybridization markers in order to distinguish between these hypotheses.

Two members of the vertebrate Paired-box (Pax) gene family, Pax-3 and Pax-6, are useful markers for distinguishing dorsal and ventral cell types in the ventricular region of the spinal cord. Pax-3 is expressed in a broad, dorsal area of the ventricular zone, whereas Pax-6 is expressed in a domain that overlaps the ventral border of Pax-3 expression and extends ventrally to include a large region of the ventral ventricular zone, excluding the floor plate (Goulding et al., 1991, 1993; Walther and Gruss, 1991; see Fig. 10B,C and H,I). In situ hybridizations were performed with \(^{35}\)S-labelled Pax-3 and Pax-6 probes to adjacent sections from 10.5 d.p.c. and 12.5 d.p.c. embryos. The normal expression pattern of Pax-3 and Pax-6 at 10.5 d.p.c. is shown in Fig. 10B,C. In transgenic embryos at 10.5 d.p.c., the domain of Pax-3 expression is expanded ventrally (Fig. 10E) and the region of the dorsal ventricular zone that expresses this marker has increased. In contrast, the size of the ventral domain of Pax-6 expression does not appear to be affected and sharp borders delimit the Pax-3 and Pax-6 domains. Thus, the expression of this ventral marker is unaffected despite the ectopic expression of Wnt-1 in ventral ventricular cells (see Fig. 6D). Multiple sections from all three transgenic embryos gave similar results, differing only in the extent to which the dorsal Pax-3 domain is expanded (data not shown).

Both Pax genes maintain similar distributions at 12.5 d.p.c. (Fig. 10H,I). Expression was examined in five transgenic embryos at 12.5 d.p.c. (1 ++ and 4 +++). In embryos with the most extreme phenotypes (+++, Tg 36916 and Tg 6720), it is clear that the Pax-3-expressing dorsal ventricular zone is greatly expanded (Fig. 10K,N). Despite this increase, a sharp ventral border of expression is still apparent. Interestingly, the
majority of Pax-6-expressing cells are concentrated around the ventral border of Pax-3 expression and in regions just ventral to the Pax-3 expression domain (Fig. 10L,O large arrow), as is the case in control sections (Fig. 10I), although the dorsal border of Pax-6-expressing cells does not always appear to be sharply delineated, as some of these cells are intermingled with more dorsal cells (Fig. 10L,O small arrow). These experiments indicate that the dorsoventral polarity of the spinal cord is unchanged by Wnt-1 expression. Even in the most extreme case, where a greater number of cells are proliferating in ventral regions, Pax-6, but not Pax-3, is expressed by these ventral proliferating cells (Fig. 8E and Fig. 10N,O represent adjacent sections). Therefore, ventral cells have not been transformed to a dorsal cell fate, nor have dorsal cells been pushed into the ventral regions where enhanced cell proliferation is seen. These markers also illustrate that there is a disproportionate increase in dorsal ventricular cells compared to ventral ventricular cells. Given these results, we conclude that the ectopic expression of Wnt-1 can enhance the proliferation of spinal cord cells, but the expression of Wnt-1 does not appear primarily to affect the dorsoventral pattern of the spinal cord. This conclusion is further supported by examining the expression of markers that identify the extreme dorsal (roof plate) and ventral (floor plate) regions of the spinal cord. Wnt-3a and Dvr-6 (Vgr-1) transcripts are localized to cells in the roof plate in transgenic embryos (data not shown and Fig. 11A-D) as they are in normal embryos (Roelink and Nusse, 1991; McMahon et al., 1992; Jones et al., 1991). Moreover, Dvr-6 (Vgr-1) and Steel factor (kit ligand) transcripts are localized to cells in and adjacent to the floor plate as expected (Jones et al., 1991; Matsui et al., 1990; Keshet et al., 1991), even in transgenics with severe (+++) phenotypes (Fig. 11E-H). In addition, we have also examined the expression of the Islet-1 gene, which is restricted to the ventral horns of the spinal cord at 10.5 d.p.c. (Ericson et al., 1992; Fig. 12B). Islet-1 transcripts can be detected on either side of the floor plate in 10.5 d.p.c. transgenic embryos (Fig. 12D), despite the apparent lack of distinguishable ventral horns (see also Fig. 2). Therefore, we have not detected any changes in the dorsoventral pattern of cells within the spinal cord based on the expression of these six markers.
DISCUSSION

Ectopic expression of Wnt-1 causes an increase in the number of proliferating cells

Our studies clearly demonstrate that expanding the domain of Wnt-1 expression disrupts the normal morphology of the spinal cord in over 50% of the transgenic embryos examined. The ‘wavy’ appearance results from an expansion of the ventricular zone within a confined space which apparently causes the spinal cord to buckle and fold. Analysis of BrdU incorporation indicates that by 12.5 d.p.c. there are three to seven fold more proliferating cells in the spinal cords of transgenic embryos than in non-transgenic littermates. Thus, a substantial increase occurs in a relatively short period of time after the onset (approximately 8.5-9.5 d.p.c.) of ectopic Wnt-1 expression. The increase in mitotic ventricular cells can be readily visualized in sections probed with another Wnt family member, Wnt-7b. At 12.5 d.p.c., Wnt-7b transcripts are restricted to proliferating ventricular cells which largely occupy the dorsal half of the spinal cord. In transgenic embryos with the most severe phenotypes, there is a dramatic increase in Wnt-7b expression.

The degree to which the ventricular region is expanded and the overall number of mitotic cells can be correlated with the extent of Wnt-1 expression. At 10.5 d.p.c. and in some 12.5 d.p.c. embryos, Wnt-1 transcripts are abundant and in these embryos the numbers of proliferating cells are clearly increased. At later stages (15.5 d.p.c.), when little to no ectopic expression can be detected, there is no increase in the number of proliferating cells. Unfortunately, we are unable to analyze the distribution of Wnt-1 protein, as there are no available antibodies that will detect this protein in vivo. Nonetheless, based on the distribution of ectopic mRNA, it appears that cells in the spinal cord proliferate in response to ectopic Wnt-1 expression. This conclusion is consistent with previous studies of the oncogenic action of Wnt-1 and the normal role of its Drosophila orthologue wingless.

The Wnt-1 gene was originally identified as a proto-oncogene that could, when activated by the Mouse Mammary Tumor Virus (MMTV) promoter, cause hyperplasia leading to tumor formation in the mammary gland (Nusse and Varmus, 1982; Tsukamoto et al., 1988). Cultured mammary epithelial cells transfected with Wnt-1 display a characteristic transformed phenotype (Brown et al., 1986), that is confluent cells continue to divide when contacted by other cells while normal cells exhibit contact inhibition and cease dividing under these conditions. Thus, in these heterologous assays, Wnt-1 expression causes cells to ignore the normal cues which control cell division and responding cells continue to proliferate.

Compelling evidence that the product of the Drosophila wingless gene, controls cell proliferation has come from elegant studies which have explored the role of wg during Malpighian tubule development in the Drosophila embryo (Skaer and Martinez Arias, 1992). If functional wg protein is absent during morphogenesis, the Malpighian tubules fail to elongate due to the lack of cell proliferation. If wg is ectopically expressed in these cells at a time when wild type wg expression has ceased, the Malpighian tubules continue to divide producing supernumerary cells. Thus, a mitogenic function for Wnt-1 signalling may be evolutionarily conserved.

In our experiments, the increased number of mitotic cells which result from the ectopic expression of Wnt-1 in the spinal cord could accumulate because Wnt-1 is acting as it has been shown to act on other cell types, as a mitogen. Spinal cord precursor cells (particularly those cells which will become neurons) divide for a finite period of time before they exit the cell cycle, migrate away from the ventricular region and begin to differentiate (Nornes and Carry, 1978; Altman and Bayer, 1984). Therefore, ectopic Wnt-1 expression is either causing cells to divide at a faster rate or to remain mitotic in the presence of signals which normally instruct cells to cease dividing. In either case, more proliferating cells would accumulate in the ventricular zone causing this region to expand.

Interestingly, ectopic expression of Wnt-1 is consistently confined to the ventricular zone throughout several stages of development. Expression from the transgene construct could be detected in all of the embryos examined at 10.5 d.p.c., half of the embryos assayed at 12.5 d.p.c., and little to no expression could be detected in those analyzed at 15.5 d.p.c. Although Hoxb-4 is expressed by cells in the ventricular layer early in development, by 10.5 d.p.c. this gene begins to be expressed outside the ventricular layer and is subsequently confined to the dorsal intermediate zone (Graham et al., 1991). As we have never observed ectopic expression of Wnt-1 outside of the ventricular zone, Wnt-1 may be stimulating Hoxb-4 expressing cells to remain mitotic, preventing their normal maturation and migration into postmitotic regions of the spinal cord.

At present, it is unclear whether mitogenic effects of Wnt-1 are a direct or indirect consequence of Wnt-1 signalling. For instance, Wnt-1 could act as a signal which triggers mitosis in an indirect way, presumably by causing other mitogenic factors to be expressed or by overcoming or blocking negative regulators of proliferation. It is also possible that Wnt-1 may contribute to cell proliferation by altering the adhesive properties of precursor cells. Although our experiments cannot rule out such indirect possibilities, it is clear that the ectopic expression of this factor causes a significant increase in the number of proliferating cells, however this is achieved mechanistically.

Fig. 10. Pax-3 and Pax-6 expression indicates the presence of dorsal and ventral ventricular cells in 10.5 and 12.5 d.p.c. transgenic spinal cords. Photomicrographs represent adjacent transverse sections through a 10.5 d.p.c. control embryo (A-C), a 10.5 d.p.c. transgenic embryo (Tg 8815) (D-F), a 12.5 d.p.c. control embryo (G-I), and two sets of sections through 12.5 d.p.c. transgenic embryos (J-K, Tg 36916 +++ and M-O, Tg 6720 +++). B,E,H,K,N show sections that have been hybridized with a 35S-labelled RNA probe specific for Pax-3 and C,F,I,L,O have been hybridized with a probe specific for Pax-6. A,D,G,J,M are bright-field photomicrographs and B,C,E,F,H,I,K,L,N,O were photographed using dark-ground illumination. Pax-3 is expressed in the dorsal ventricular zone as well as within the dermamyotome of the somite (so) and the dorsal root ganglia (drg) at 10.5 d.p.c. and within the dorsal root ganglia (drg) at 12.5 d.p.c. In contrast, Pax-6 is expressed in the ventral half of the spinal cord in these sections. (Some background hybridization to a large clump of blood cells (bc) is seen in F. This is an artifact and does not represent a true site of Pax-6 expression.) The sections shown in N,O are adjacent to the section shown in Fig. 8E. Large arrows in L and O point out major areas of Pax-6 expression, whereas small arrows indicate Pax-6-expressing cells that are located dorsal to the normal border of expression. Scale bars are equivalent to 100 μm. A-F were photographed at the same magnification, as were G-O.
Fig. 11. *Dvr-6* and *Steel factor (kit ligand)* expression marks extreme dorsal and ventral regions in 12.5 d.p.c. transgenic embryos. A, B and C,D were hybridized with a $^{35}$S-labelled *Dvr-6* riboprobe and E-H were hybridized with a $^{35}$S-labelled probe specific for *Steel factor (kit ligand)* mRNA. A,B and E,F represent control sections and C,D and G,H are sections taken from Tg 36916 and Tg 11720, respectively. *Dvr-6* is expressed in both the roof plate and the floor plate (arrows), whereas the *Steel factor* gene is expressed in the floor plate (open arrows) as well as in a population of ventrolateral cells. The scale bar in A is equivalent to 100 µm. All sections were photographed at the same magnification. (note: the section in panel E is slightly larger because it was taken from a 12.5 d.p.c. CD-1 embryo.)
Wnt-1 and spinal cord patterning

To rule out the possibility that Wnt-1 expression affects cell division by instructing ventral cells to assume dorsal identities, and consequently extends the amount of time that cells remain dividing, we analyzed the dorsoventral pattern of cell types within the spinal cord using regionally expressed gene markers. Our analysis of Pax-3 and Pax-6 expression in the ventricular region convincingly shows that Wnt-1 causes cells to proliferate regardless of a cell’s dorsoventral identity. While dorsal, ventricular regions of the spinal cord are expanded to a greater extent than ventral regions in transgenic embryos, ventral, ventricular specific gene expression is retained. Thus, extra dorsal cells, which might proliferate for a longer period of time, are not accumulating at the expense of ventral cells. Moreover, in the most extreme embryo we examined, dividing cells could be found in regions only expressing Pax-6 at 12.5 d.p.c., indicating that ventral cells can also respond to Wnt-1 expression and contribute to the overall increase in mitotic precursors. In addition, we saw no change in the expression of markers for extreme dorsal and ventral cell types, such as the roof plate and floor plate cells. Thus, there is no compelling evidence that Wnt-1 expression primarily influences cell identity along the dorsoventral axis of the spinal cord. However, as signals which are thought to pattern the ventral spinal cord appear to act very early in neural development (van Stratten et al., 1988; Yamada et al., 1991; Goulding et al., 1993), a different result might be obtained if high levels of ectopic expression were initiated at earlier stages.

It is not clear why the uniform expression of Wnt-1 along the dorsoventral axis results in a preferential expansion of dorsal cells. It is possible that dorsal regions are expanded to a greater extent because dorsal cells are more sensitive to the signal or that other Wnt family members are acting synergistically with ectopically produced Wnt-1 (see below for discussion). It is also possible that Wnt-1 expression may simply be more abundant in dorsal regions, although we have not detected any dorsal bias of expression caused by the Hoxb-4 enhancer. Another potential explanation could relate to the fact that negative regulators of proliferation, which are normally involved in controlling the ventral to dorsal cascade of proliferation may be competing against the positive proliferative stimulus of Wnt-1. If this were the case, one might expect to see a greater expansion of dorsal regions because putative negative regulators may not act in dorsal regions until later in development. This might explain why substantial ventral proliferation is seen only when high levels of expression are detected at 12.5 d.p.c., and not in other embryos with less severe phenotypes and lower levels of ectopic expression. At present little is known about the mechanism of growth regulation in the nervous system and to date, no negative regulators of CNS proliferation have been identified.

We have not yet determined whether ectopic expression of Wnt-1 can affect the distribution of differentiating cells emerging from the ventricular zone. It is possible that Wnt-1 expression can bias cells to assume a particular fate once they become postmitotic. In light of the fact that there is a more dramatic expansion of the dorsal ventricular region in our transgenic embryos, one might expect that more mature dorsal cell types will be born. In fact, small, darkly stained cells typical of dorsal interneurons may be overrepresented in 15.5 d.p.c. transgenic embryos. Further analysis using markers for distinct cell types should provide more detailed information about whether Wnt-1 expression can influence the identity of specific neuronal or glial cells in addition to causing changes in cell number.

One other hypothesis could potentially explain the accumulation of dividing cells. If Wnt-1 were to act as a survival factor, then the ectopic expression of this factor could be rescuing cells that normally die. If these cells continued to divide, this would also lead to an increase in the number proliferating cells as we have observed. Recent studies indicate that apoptotic cells can be detected in the early neural tube of the chick embryo during active proliferation (S. Homma and R. Oppenheim, personal communication). A detailed quantitative study which compares estimates of apoptotic cells from transgenic and non-transgenic embryos would be needed in order to determine whether cell survival is enhancing the number of mitotic precursors in our experiments.

The normal role of Wnt-1 and Wnt family members during CNS development

The primary defect in Wnt-1 null mutants is an absence of cells in the midbrain and a subsequent loss of cells in the dorsal metencephalon (McMahon et al., 1992). One explanation, that midbrain cells fail to proliferate, is consistent with our present findings suggesting that Wnt-1 may act as a mitogen during the normal development of the midbrain and anterior hindbrain.

Further, as Wnt-1 is expressed in the dorsal most part of the spinal cord along with Wnt-3a, the function of these factors may be to control cell division within this region. Functional redundancy between these related signals could account for the absence of an overt phenotype in Wnt-1 null mutants, a hypothesis supported by various biological assays which compare the activities of Wnt factors (Moon, 1993; for review; G. Wong,
B. Gavin and A. P. M., unpublished data). Our data indicate that there is a preferential expansion of dorsal cells in transgenic embryos, suggesting that in some way these cells are more responsive to Wnt-1 signalling. Perhaps this is because these cells respond to Wnt-1 and/or Wnt-3a during normal development. The analysis of Wnt-1/Wnt-3a double mutants should provide further insight into the function of these molecules in the spinal cord.

It is also possible that these dorsal cells respond to another Wnt family member during normal development. Wnt-1 and Wnt-7b have similar activities when expressed in other cell types (G. Wong, B. Gavin and A. P. M., unpublished data) and the domain of Wnt-7b-expressing cells is clearly expanded in embryos that ectopically express Wnt-1. Wnt-1 and Wnt-7b are expressed in non-overlapping domains during normal spinal cord development, but by ectopically expressing Wnt-1 in more ventral regions, we have created a situation where these two domains overlap in transgenic embryos. Therefore, it is possible that the combined expression of these factors produces a synergistic effect and causes a preferential dorsal expansion.

Interestingly, in addition to Wnt-1, Wnt-3a and Wnt-7b, several other Wnt genes are expressed in the embryonic spinal cord. Wnt-3 is expressed very weakly along the dorsal midline (Roelink and Nusse, 1991; Salinas and Nusse, 1992; McGrew et al., 1992; Parr et al., 1993). Wnt-4 transcripts can be detected in the dorsal ventricular zone, as well as in the floor plate, beginning at 9.5 d.p.c. and expression continues into midgestational stages (Parr et al., 1993). Wnt-7a and Wnt-7b are expressed in overlapping domains in the ventromedial part of the spinal cord at early stages (9.5 d.p.c.) (Parr et al., 1993) and like Wnt-7b, Wnt-7a expression is restricted to the inner ventricular zone by 12.5 d.p.c.. Thus, all six of these Wnt family members are expressed in the ventricular region. Like Wnt-1, other members of the Wnt family have been shown to have mitogenic properties. In particular, Wnt-3, Wnt-3a, Wnt-7a and Wnt-7b all have the ability to transform mammary epithelial cells, but Wnt-4 lacks this activity (G. Wong, B. Gavin and A. P. M., unpublished data). Therefore, several Wnt genes may be involved in the regulation of cell division along the entire dorsoventral axis of the spinal cord. We are currently investigating the properties of other Wnt factors by ectopically expressing them in the spinal cord to determine if their expression can phenocopy the effects of Wnt-1.

The localized expression of different Wnt genes may play a role in regulating the ventral to dorsal cascade of cell division which occurs during spinal cord maturation (Normes and Carry, 1978; Altman and Bayer, 1984). Also, the localized regions of Wnt gene expression, and the restricted patterns of expression of other gene families, such as the Pax genes (see Gruss and Walthcr, 1992 for review), within precursor populations indicate that these cells have received patterning instructions long before they cease dividing and begin to migrate. Analyses of cell fate decisions in other areas of the CNS have shown that cells receive instructional cues as to their fate while they are still dividing (McConnell and Kaznowski, 1991; Altschuler and Cepko, 1992), indicating that cell fate choice is taking place while cells are still located within the ventricular zone. How a dividing precursor cell is instructed to stop dividing and follow a particular fate is a process that is poorly understood. Our data suggest that Wnt factors are involved in the first step in this process by regulating the proliferation of precursor cells, a role that may also influence the identity of a cell after it ceases to divide.

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