THE JOURNAL OF COMPARATIVE NEUROLOGY 374:246–258 (1996)

Early Deletion of Neuromeres in *Wnt-1*$^{-/-}$ Mutant Mice: Evaluation by Morphological and Molecular Markers

GRANT S. MASTICK, CHEN-MING FAN, MARC TESSIER-LAVIGNE, GEORGE N. SERBEDZIJA, ANDREW P. McMAHON, AND STEPHEN S. EASTER, JR.
Department of Biology, University of Michigan, Ann Arbor, Michigan 48109 (G.S.M., S.S.E.); Howard Hughes Medical Institute, Department of Anatomy, University of California, San Francisco, San Francisco, California 94143 (C.-M.F., M.T.-L.); Harvard University, Department of Cellular and Developmental Biology, Biological Laboratories, Cambridge, Massachusetts 02138 (G.N.S., A.P.M.)

ABSTRACT

The *Wnt-1* gene is required for the development of midbrain and cerebellum; previous work showed that knockout of *Wnt-1* causes the loss of most molecular markers of these structures in early embryos and deletion of these structures by birth. However, neither the extent of early neuronal defects nor any possible alterations in structures adjacent to presumptive midbrain and cerebellum were examined. By using a neuron-specific antibody and fluorescent axon tracers, we show that central and peripheral neuronal development are altered in mutants during initial axonogenesis on embryonic day 9.5. The absence of neuronal landmarks, including oculomotor and trochlear nerves and cerebellar plate, suggests that both mesencephalon and rhombomere 1 (r1) are deleted, with the remaining neural tube fused to form a new border between the caudalmost portion of the prosencephalon (prosomere 1, or p1) and r2. Central axons accurately traverse this novel border by forming normal longitudinal tracts into the rhombencephalon, implying that the cues that direct these axons are aligned across neuromeres and are not affected by the deletion. The presence of intact p1 and r2 is further supported by the retention of markers for these two neuromeres, including a marker of p1, the *Sim-2* gene, and an r2-specific lacZ transgene in mutant embryos. In addition, alterations in the *Sim-2* expression domain in ventral prosencephalon, rostral to p1, provide novel evidence for *Wnt-1* function in this region. © 1996 Wiley-Liss, Inc.

Indexing terms: axon guidance, mesencephalon, cranial nerves, rhombencephalon, forebrain

The first regional differentiation in the brain is surely controlled genetically, and many candidate regulatory factors are expressed early in regionally restricted patterns. Of the three primary vesicles, prosencephalon, mesencephalon, and rhombencephalon, most work has focused on the latter, where nested expression patterns of homeobox transcription factors are thought to specify rhombomeric identity (Wilkinson and Krumlauf, 1990; Hunt et al., 1991). A large number of genes have also been shown to be expressed early in the more rostral neural tube (reviewed in Puelles and Rubenstein, 1993), but few of these genes have been tested for functional roles in brain development.

One of the best characterized of these candidates is the *Wnt-1* gene, a member of a family of related genes that are expressed early in vertebrate embryogenesis (McMahon, 1992; Nusse and Varmus, 1992). Like its *Drosophila* homologue, *wingless* (*wg*), the mouse gene *Wnt-1* encodes a secreted glycoprotein that may play a role in directing cell fates. On embryonic day (E) 8.0, this gene is transcribed in a restricted region of the neural plate, probably the presumptive mesencephalon excluding the ventral midline, and as the neural tube forms, the expression pattern changes dynamically (Wilkinson et al., 1987; Parr et al., 1993). By E9.0–E9.5, expression in the brain becomes restricted to a narrow circumferential ring just rostral to the border between the mesencephalon and the rhombencephalon, and two strips, one dorsal and one ventral, both of which extend rostrally through the mesencephalon into the prosencephalon. Expression is absent in rhombomere 1 (r1), but a dorsal strip of expression initiating in r2 extends caudally through the rhombencephalon and the spinal cord.

Accepted May 23, 1996.

Address reprint requests to Grant Mastick, Department of Biology, University of Michigan, Ann Arbor, MI 48109. E-mail: grants@umich.edu

© 1996 WILEY-LISS, INC.

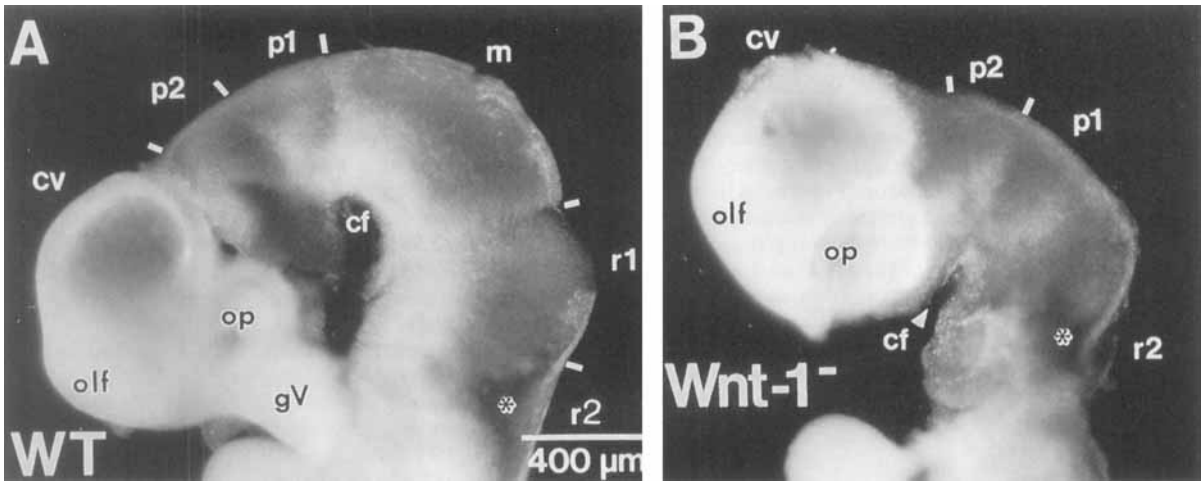

Fig. 1. Neuromeric subdivisions of the neural tube on E9.5. **A:** Wildtype embryo. The neuromeres (prosomeres 1 and 2, mesencephalon, and rhombomeres 1 and 2: p1, p2, m, r1, and r2, respectively) are separated by interneuromeric constrictions indicated by short lines at the dorsal surface. An additional boundary marker is the thin triangular roof (*) that lies just caudal to the r1–r2 border. The cephalic flexure (cf) is the ventrally directed hairpin turn of the neural tube. The front of the neural tube, marked roughly by the olfactory placode (olf) in the overlying skin, is rotated nearly 180° relative to the rhombencephalon. The cerebral vesicle (cv), optic vesicle (op), and trigeminal ganglion (gV) are shown. **B:** *Wnt-1*$^{-/-}$ embryo, littermate of A. The p2–p1 constriction is evident, but the p1–m and m–r1 constrictions are not. The rostral border of r2 is evident from the thin roof (*). The trigeminal ganglion was removed to reveal the very narrow cephalic flexure (cf). The front of the neural tube is rotated about 45° less than in the wildtype, which is consistent with a deletion of a wedge, wide side dorsal, including most or all of mesencephalon and r1.

Wnt-1 mutants generated by gene targeting result in embryos with large deletions in the brain. The boundaries of these deletions were estimated partly from morphology but mostly from molecular markers (immunoreactivity or in situ hybridization) on the assumption that the absence of a marker implied the loss of the subdivision in which the marker normally appeared. On E9.5, the brain of *Wnt-1*$^{-/-}$ embryos is smaller than that of the wildtype (McMahon et al., 1992), principally because the mesencephalon (recognized as the most sharply curved part of the dorsal surface of the brain) is smaller in the mutant, as a comparison of Figure 1A and 1B shows. In addition to the change in morphology, the expression patterns of other *Wnt* genes are altered on E9.5; *Wnt-7b* expression in caudal dorsal mesencephalon is lost, and a *Wnt-5a* expression domain, normally extending from the ventral forebrain into the ventral mesencephalon, is reduced in size, suggesting that part of the mesencephalon must be missing. This conclusion is supported by the loss of *engrailed* (*En*) expression in the mutant (McMahon et al., 1992). Both *En-1* and *En-2* are expressed initially in both the mesencephalon and r1, but expression later disappears at stages when it persists in wildtype. These observations led McMahon et al. (1992) to estimate that "much of the midbrain and some of the metencephalon are deleted in the *Wnt-1*$^{-/-}$ embryo" (p. 582). Many days later in development, the adult brain structure can be assessed with histological sections and a comparison with the normal neuroanatomy. By these criteria, homozygous *Wnt-1*$^{-/-}$ mice lack the cerebellum and most or all of the midbrain on E14.5 and E16.5 (McMahon and Bradley, 1990; but see Thomas and Capecchi, 1990). The midbrain is derived from the mesencephalon and the cerebellum from the mesencephalon and r1 (Martinez and Alvarado-Mallart, 1989), so the late embryonic deficits correlate well with those noted earlier. But the issue of whether entire early subdivisions (e.g., mesencephalon and/or r1) were deleted requires examination of the early brain with independent markers that can reveal the neural organization at that time.

Current understanding of the organization of the early brain is based on neuromeres and the initial patterns of neurons, which are evident at E9.5, when the *Wnt-1*$^{-/-}$ embryo shows the first defects. Soon after neural tube closure, the primary vesicles are subdivided into neuromeres, a series of annular subdivisions marked by transverse bulges, and separated from one another by constrictions in the wall of the neural tube (reviewed in Puelles et al., 1987). The neuromeres have regional properties that are thought to be important developmentally, including rate of proliferation (Bergquist and Kallen, 1954; Guthrie et al., 1991), patterns of gene expression (Bulfone et al., 1993; Puelles and Rubenstein, 1993) and of neurogenesis (Puelles et al., 1987; Lumsden and Keynes, 1989), and restriction of cellular migration (Fraser et al., 1990; Figdor and Stern, 1993). The number of neuromeres increases steadily with development, but we are concerned primarily with the rhombomeres (r1–r7), the mesencephalon (mes; which is not apparently subdivided at this time), and the two most caudal subunits in the prosencephalon, prosomeres 1 and 2 (p1 and p2; see Fig. 1A). The nerves, tracts, and the neuronal cell populations that form the sources of these axons in the early mouse brain have recently been described (Easter et al., 1993; Mastick and Easter, 1996). Briefly, there are two motor nerves: the tracts are arranged orthogonally into a pair of longitudinal pathways (one dorsal, one ventral) and several dorsoventral ones, and there are eight sources. More details will be given in the appropriate section of Results.

In summary, the loss of *Wnt-1* function results in the early disruption of brain morphogenesis and in the subsequent deletion of large regions of the mature brain. In previous reports, the extent of the early disruptions could

not be assessed very accurately from the wholemounted or sectioned embryos because neither neurons nor neuromeres were examined. This paper defines more precisely the effects of the lesion on the initial formation of nerves, tracts, and their sources. We examine early neuronal development in *Wnt-1*$^{-/-}$ embryos by using immunocytochemistry and fluorescent tracers to label early neurons and their axons. We find that neuronal markers in mes and r1 are deleted both dorsally and ventrally so completely that a juxtaposition between p1 and r2 has been created. We use molecular markers to confirm that p1 and r2 develop normally by examining the expression pattern of a newly identified gene, *Sim-2*, that marks the p1–mes border, and a lacZ transgene that marks r2. Rostral to p1, however, the *Sim-2* expression pattern is altered, providing novel evidence that *Wnt-1* is also required in ventral prosencephalon.

MATERIALS AND METHODS

Mutant alleles of *Wnt-1* were constructed by the targeted insertion of a *neo* cassette into the *Wnt-1* transcribed region, resulting in a probable complete loss of function (McMahon and Bradley, 1990). *Wnt-1*^{neo} mice were maintained by the intercrossing of heterozygotes. Embryos were collected from crosses between heterozygotes, resulting in litters containing embryos with normal appearance (both +/+ and +/− embryos) and embryos with deletions (−/− embryos), as confirmed by genotyping as previously described (McMahon et al., 1992). Mating was assumed to occur at midnight, so that noon of the next day was designated as E0.5. When the embryos were at E9.5, the females were killed by cervical dislocation (as approved by the University of Michigan University Committee on the Use and Care of Animals), the embryos dissected free from the uterus into phosphate buffered saline (PBS), and fixed in 4% paraformaldehyde overnight.

For immunocytochemistry, the TuJ1 mouse monoclonal antibody against neuron-specific class III β-tubulin was used (Moody et al., 1987; Lee et al., 1990) as previously described (Easter et al., 1993). In brief, embryos were permeabilized in a graded series of ethanols, followed by xylene, and back through the series to water. After treatment with cold acetone, the embryos were rehydrated, blocked for 1 hour, and incubated in diluted TuJ1 (1:500) overnight. A goat anti-mouse secondary coupled to horseradish peroxidase and subsequent peroxidase reaction with diaminobenzidine revealed the antibody label. The reacted embryos were bisected sagittally and mounted in DPX.

Wholemount in situ hybridization used previously described methods (Wilkinson, 1992; Parr et al., 1993), with digoxigenin-labeled RNA derived from a plasmid containing the mouse *Sim-2* cDNA (Fan et al., 1996). The reacted embryos were bisected sagittally, cleared in 80% glycerol/PBS, and mounted. The expression of a r2-specific lacZ transgene was examined by crossing the transgene into the *Wnt-1*^{neo} heterozygous line, and litters were treated with X-gal to reveal β-galactosidase activity. The treated embryos were cleared and photographed as intact wholemounts.

Measurements on labeled embryos were made by using a digitizing tablet (MOP-3, Zeiss) to trace distances between landmarks on camera lucida drawings of labeled embryos. A graticule allowed the conversion of millimeters on the drawing to micrometers in the microscope field.

For immunocytochemistry on sections, embryos were permeabilized after fixation and prepared for cryostat sectioning by equilibration for several hours each in 5% sucrose/0.1 M phosphate, 15% sucrose/0.1 M phosphate, then in 7.5% gelatin/15% sucrose/0.1 M phosphate at 37°C (Kathryn Tosney, personal communication). After setting the embryos in molds at room temperature, the embryos were frozen in block in isopentane/dry ice and stored at −30°C. Frozen sections were cut with a cryostat at 10 μm thickness and dried, and gelatin was removed by washing at 30°C in 0.1 M phosphate. TuJ1 immunocytochemistry was carried out as above, followed by toluidine blue counterstaining and mounting in DPX.

For axon tracing, injections of 1,1′dioctadecyl-3,3,3′,3″-tetramethylindocarbocyanine (diI; Molecular Probes) were used to label groups of neuronal cell bodies and their axons in fixed embryos (Honig and Hume, 1986; Godemont et al., 1987). A 0.1% solution of diI in dimethyl formamide was injected into the wall of the neural tube by using a pulled microcapillary pipet and Picospritzer pressure injection apparatus. The diI diffused overnight (incubation at 37°C), and the embryos were bisected, mounted in 50% glycerol, and examined with a fluorescence microscope.

RESULTS

The deletion

Morphology. As Figure 1A,B shows, the mutant brain is obviously shorter along the rostral–caudal axis. The most notable differences are the loss of the interneuromeric constriction that marks the boundary between mes and r1, the more gradual dorsal convexity, and the reduced rotation of the rostral brain about the cephalic flexure. The prosencephalon (including the optic and cerebral vesicles, p2, and the constrictions on either side of it) is structurally normal, but it is closer to the rhombencephalon than it is in wildtype mice and the caudal boundary of p1 is undefined. The thin triangular roof of r2 is evident, so r2 is apparently intact, but because the rostral boundary of this dorsal tissue (presumptive choroidal tissue) may lie in caudal r1, the thin roof is an ambiguous landmark. This morphology is consistent with the conclusion that most or all of mes and r1 had been deleted (McMahon and Bradley, 1990), but the uncertainty about the caudal boundary of p1 and the rostral boundary of r1 make it impossible, from morphology alone, to locate the boundaries of the deletion. Additional landmarks are necessary to establish if all or part of p1, mes, and r1 are missing.

Neurons. To determine neuronal defects in mutant embryos, wildtype (n = 12) and *Wnt-1*$^{-/-}$ littermates (n = 7) from four litters were labeled with TuJ1 on E9.5 (25–30+ somites).

The peripheral nervous system is evident in immunolabeled wholemounts (Fig. 2A,B). In both wildtype and mutant, the peripheral ganglia (gV through gX) project axons ventrally into the branchial arches, in a pattern that reflects the underlying segmental organization of the rhombencephalon. The two placodes, olfactory and otic, are also similar in both wildtype and mutant. The major difference between the two lies in the oculomotor and trochlear nerves (nIII and nIV, respectively). nIII extends from the ventral surface of mes into the space created by the cephalic flexure toward the optic stalk. The pioneer axons of nIV exit the wildtype brain near the dorsal midline near the mes–r1 border. Both nerves are completely absent in the mutant.

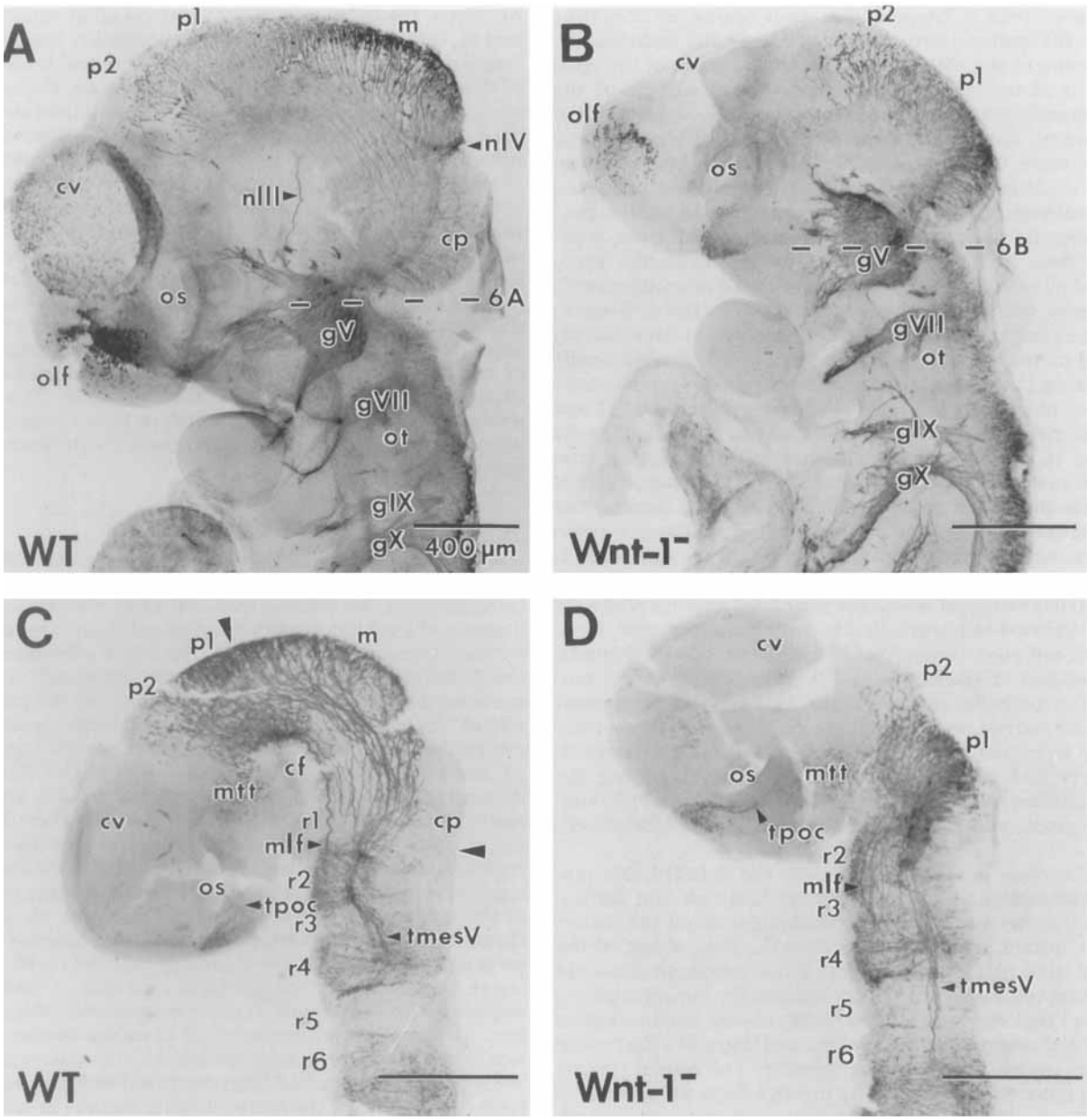

Fig. 2. Neuronal organization on E9.5. Littermates were labeled with an antibody against neuron-specific β-tubulin to reveal central and peripheral neurons and their axons. **A,B:** Wholemounts with skin and underlying structures intact. Peripheral ganglia (gV, trigeminal; gVII, facial; gIX, glossopharyngeal; gX, vagal), motor nerves (nIII, oculomotor; nIV, trochlear), placodes (olf, olfactory; ot, otic), and the cerebellar plate (cp) are indicated. The ganglia and the placodes are normal in the mutant, but the cerebellar plate is reduced, and no oculomotor or trochlear axons were visible in the mutants at any plane of focus. Dashed lines indicate transverse planes of sections in rhombomere 2 shown in Figure 6A,B. **C,D:** Wholemounted brains from which the skin, peripheral ganglia, and cranial nerves have been removed. The anterior prosencephalon in D was inadvertently damaged during dissection and is fragmented. The optic stalk (os), which is elongated rostrocaudally, provides a convenient indication of the different rotation of the prosencephalon in wildtype and mutant brains. The first four tracts, the medial longitudinal fasciculus (mlf), the tract of the mesencephalic nucleus of the trigeminal nerve (tmesV), the tract of the postoptic commissure (tpoc), and the mammillotegmental tract (mtt) are indicated. In C, the large arrowheads mark the rostral boundary of mesencephalon and the r1–r2 boundary. The number of labeled cells on the ventral side of the neural tube differs regionally. In the wildtype, r5, r3, r1, and the caudal part of mesencephalon have relatively few labeled cells, whereas r6, r4, r2, the rostral part of the mesencephalon, and p1 are rich in labeled cells. In the mutant, the cell-poor gap that includes r1 and caudal mesencephalon is missing, and r2 is contiguous rostrally with a cell-rich region.

This result is consistent with the recent observation that neither nIII nor nIV could be retrogradely labeled by axon tracers from the periphery on E10.5 (Fritzsch et al., 1995).

The central tracts and their sources are more evident in preparations from which the skin and ganglia have been removed (Fig. 2C,D). The groups of labeled neurons are

similar in both wildtype and mutant brains, as is evident when the various structures are considered individually, beginning at the rostral end. The cross section of the optic stalk is elongated parallel to the ventral surface of the prosencephalon, and at the base of the stalk (at the front of the brain), the initial axons of the tract of the postoptic commissure (tpoc) project from their source, the anterobasal nucleus. The dorsal part of the prosencephalon, the cerebral vesicle, contains few neurons, but the ventral part contains the source of the mammillotegmental tract, separated from the ventral midline by a neuron-free zone. Dorsal p2 is characterized by sparse dorsal neurons extending axons ventrally, but between p2 and r2 the differences between mutant and wildtype become obvious. In wildtype, dorsal p1 and mes contain a large number of labeled cells and axons that contribute to the tract of the mesencephalic nucleus of the trigeminal nerve (tmesV), and ventral p1 and rostral mes also contain many labeled neurons whose axons course in the medial longitudinal fasciculus (mlf). The caudal mes contains few labeled cells. The mutant differs because the dorsal group is much shorter and because the labeled-cell-poor ventral region is missing. Despite these reductions, the two tracts, tmesV and mlf, are still evident, although somewhat reduced in size. Wildtype r1 is characterized by the dorsal cerebellar plate that appears relatively clear (labeled-cell-poor) in the wholemounts and by a labeled-cell-poor ventral half that abuts the labeled-cell-rich ventral r2 (Lumsden and Keynes, 1989). In the mutant, the cerebellar plate is greatly reduced, and the labeled-cell-poor ventral region (normally continuous with a similar region in caudal mes) is not evident. Caudal to this point, wildtype and mutant brains are essentially identical; the even-numbered rhombomeres have labeled-cell-rich ventral regions, and the odd numbered ones are labeled-cell-poor.

These observations coupled with the detailed information about tracts and their sources (Mastick and Easter, 1996) lead to reasonably firm conclusions about the nature of the mutant brain. Consider first the ventral half of the neural tube. nIII originates from a mesencephalic cluster of neuronal cell bodies that abuts against the border with p1. The fact that this nerve is completely absent implies that its somata of origin must be missing, and therefore the rostral part of ventral mes must be missing. The caudal ventral mes is apparently also missing to judge from the absence of the extended cell-poor region that normally extends through caudal mes and r1. nIV also originates from a single neuromere, r1, with its source abutting on the border with mes, and the absence of this nerve implies that rostral ventral r1 must also be missing in the mutant. What about caudal ventral r1? If part of r1 remains, then a lightly labeled ventral region should be evident rostral to r2, but the skinned embryos revealed no such gap (compare Fig. 2C with 2D). Therefore, r1 has been deleted ventrally, and we conclude that the caudal border of the deletion is probably at the rostral border of r2. What about the rostral border of the deletion? The mlf arises from neuronal cell bodies on both sides of the p1–mes border. The presence of the mlf, albeit reduced in size, implies that part of its source must have been spared by the lesion. The mesencephalic portion is at the same rostrocaudal level as that of nIII neurons and is therefore probably missing. For this reason, some and perhaps all of ventral p1 must be spared, so we place the rostral boundary of the deletion at the caudal boundary of p1. Thus, the deletion seems to include all of ventral mes and r1, but we cannot rule out the possibility that a small remnant of either or both remains. The dorsal boundaries of the deletion are more difficult to estimate. Dorsal neurons in mes are morphologically indistinguishable from their neighbors in p1, and both contribute axons to the tmesV. The presence of a tmesV in the mutant brains (Fig. 2D) implies that some and perhaps all of dorsal p1 must be present, and this suggests that the rostral boundary of the deletion is probably at the caudal boundary of p1. The assignment of the dorsal caudal boundary of the deletion is based on the altered cerebellar plate, which is reduced and may be totally absent. But the difficulty of distinguishing dorsal r1 neurons from those of dorsal r2 makes the border more tentative here than elsewhere. We assume that all of r1 has been deleted and tentatively set the dorsal caudal limit at the rostral boundary of r2 ventrally. Thus, our analysis of neuronal landmarks leads us to conclude that all of mes and r1 are missing, but adjacent neuromeres are normal in the mutant.

"Graphical" deletion

Our interpretation suggests that p1 and r2 must be juxtaposed in the mutant. As a way of evaluating this interpretation, we deleted mes and r1 in a camera lucida drawing of a wildtype embryo and joined the two remaining parts of the neural tube (Fig. 3A). The length of immunopositive dorsal tissue is smaller in both the "graphical" and real mutants than the length in the wild type, and the trajectories of the axons exiting p1 in the graphical mutant are compatible with those entering r2. However, the joining of p1 and r2 is clearly an imperfect model of the *Wnt-1*$^{-/-}$ phenotype because the cut edges do not match, and the rostral end of the brain is rotated by 90° less than that in the mutant. The reduced rotation suggests that the dorsal region of the mutant is larger than predicted. This idea was confirmed by measuring the dorsal extent of three regions in the wildtype (n = 4): p1 plus p2, mes plus r1, and r2 through r5 and the corresponding regions believed to be present in the mutant (n = 3), and p1, p2, and r2–r5. If the tissue that would ordinarily form mes and r1 had been respecified and developed as other neuromeres, the overall lengths (from rostral border of p2 to caudal border of r5) would be equal in both wildtype and mutant. Alternatively, if the deleted regions had been destroyed and not replaced, then the lengths of the corresponding regions in wildtype (p2, p1, and r2–r5) should be similar. Figure 3B shows that neither prediction was fulfilled precisely. The wildtype overall length substantially exceeded that of the mutant (2,061 ± 86 μm vs. 1,221 ± 68 μm). However, the lengths of the corresponding regions were about 20% greater in the mutant than in the wildtype (1,221 ± 68 μm vs. 991 ± 65 μm). These results suggest either that some tissue from mes or r1 is retained dorsally or that expansion in the remaining p1 or r2 partially compensates for the deletion, or both. We favor the second of these alternatives for reasons that will be given in the Discussion.

Outside the deletion

Our results with neuronal landmarks have found defects only in mes and r1, suggesting that in other domains of *Wnt-1* expression (dorsal and ventral prosencephalon, posterior hindbrain, and spinal cord) development was normal. We have evaluated this possibility further with two novel

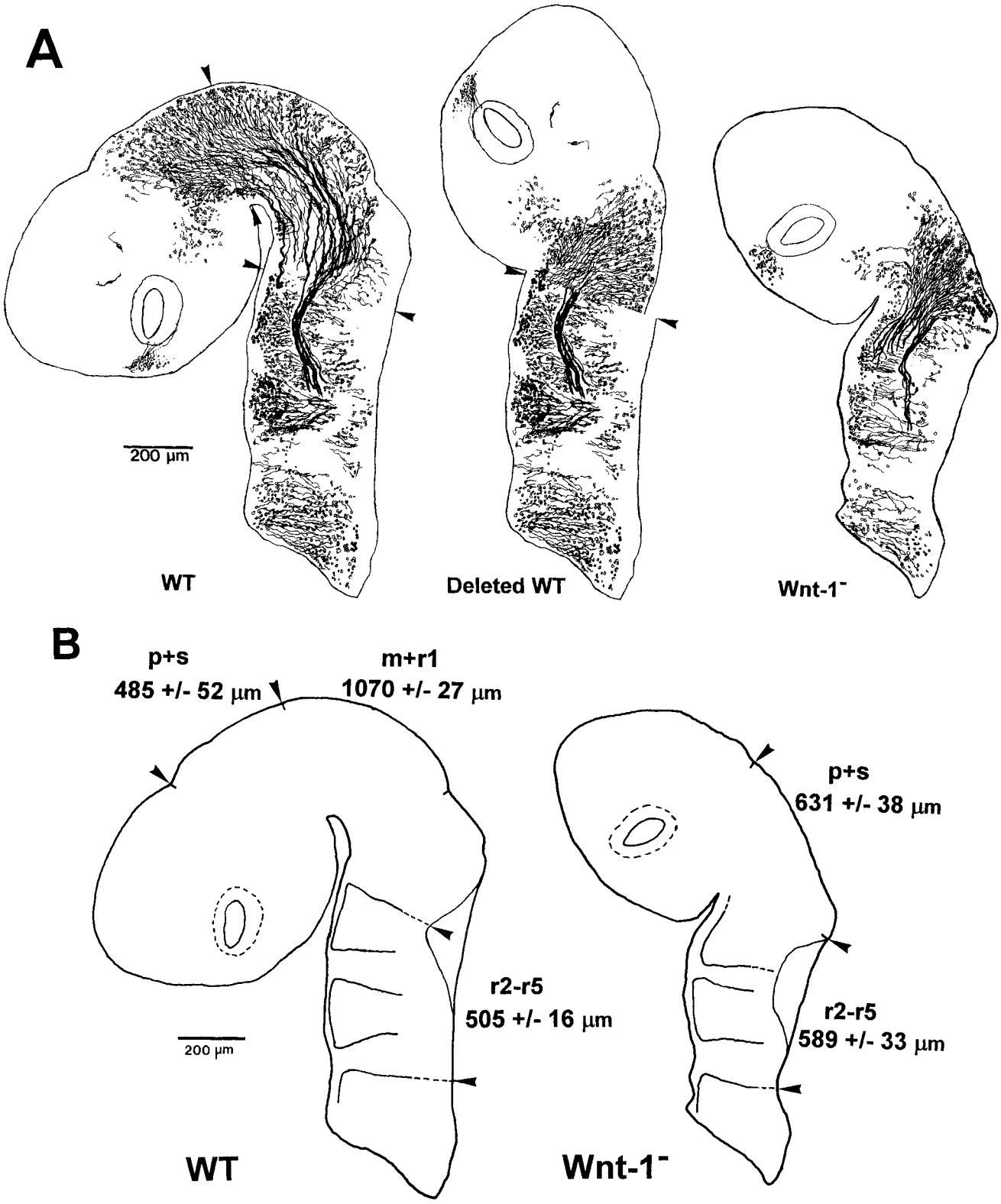

Fig. 3. Graphical evaluations of the hypothesis that the mesencephalon and r1 are precisely deleted. **A:** A comparison of graphic versus genetic deletions. Camera lucida drawings of immunolabeled wildtype (WT) and mutant (*Wnt-1*$^{-/-}$) embryos are shown. The central "embryo" (Deleted WT) was created graphically by cutting out the portions between the arrowheads in WT and joining the remaining fragments along the line given by the arrowheads in the Deleted WT. The distribution of labeled cells and axons in the region of the junction in the graphic deletion matches the *Wnt-1*$^{-/-}$ drawing quite well, but the dorsal border is discontinuous and the rotation of the front of the brain is more pronounced. The mutant embryo depicted is a different embryo than that shown in Figure 2. **B:** A comparison of the lengths along the dorsal surface of hypothetically corresponding regions (marked by arrowheads). On WT, the distances from the rostral border of p2 to the caudal border of p1 (p1 + p2), from the rostral border of the mesencephalon to the caudal border of r1 (m + r1), and from the rostral border of r2 to the caudal border of r5 (r2 – r5) are shown. On *Wnt-1*$^{-/-}$, the estimated boundaries of p1 + p2 and r2 – r5 are shown. In each case, distances are given as mean ± S.E.M.

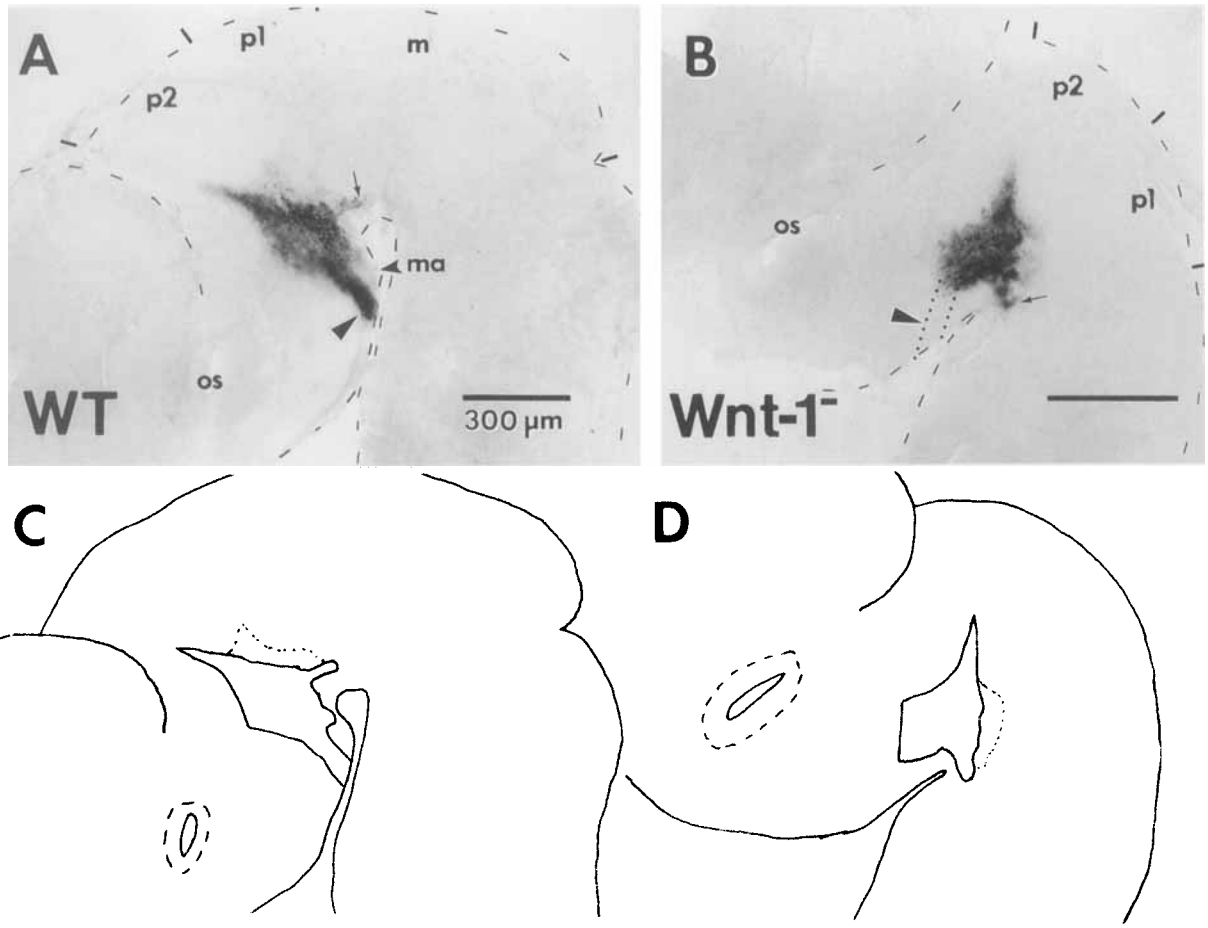

Fig. 4. *Sim-2* expression is altered in the mutant. E9.5 wildtype and mutant embryos labeled by wholemount in situ hybridization with a *Sim-2* probe, shown here as bisected, flattened wholemounts. **A,B:** Photomicrographs of the wildtype and mutant, respectively. The large arrowhead indicates a stripe of *Sim-2* expression that extends to the ventral midline rostral to the mammillary bulge (ma; small arrowhead); label is absent at the same location in the mutant (dotted lines), with no apparent ma. In ventral p1, light labeling (small arrow) extends to the caudal border of p1 in both wildtype and mutant embryos. **C,D:** Line drawings of A and B, respectively, showing corresponding regions.

molecular markers and a detailed examination of axon guidance in the formation of initial longitudinal tracts.

Sim-2 expression in the ventral prosencephalon is altered in the mutant

The mouse gene *Sim-2* (Fan et al., 1996) is related to the *Drosophila* gene *single-minded* (Crews et al., 1988; Thomas et al., 1988). In the wildtype, *Sim-2* expression is limited to a single domain of cells in the ventral region of the prosencephalon (Fig. 4A,C; n = 7), part of which overlaps with *Wnt-1* expression at E9.5 (see below). The core of expression is a trapezoidal region at approximately the same location as the source of the mammillotegmental tract (compare Fig. 4A with Fig. 2C,D). Three prominent strips extend from the core. One stretches dorsally along the constriction that marks the rostral boundary of p2. A second stretches caudally through ventral p1 to the p1–mes border. The third, most heavily labeled, extends ventrally to cross the ventral midline just rostral to the mammillary process, a ventral bulge that is a landmark for p4 (Puelles and Rubenstein, 1993). This region corresponds to the rostralmost extent of the ventral *Wnt-1* stripe on E9.5 (see Fig. 6F in Parr et al., 1993); therefore, *Wnt-1* is in a position to influence *Sim-2* expression.

Sim-2 expression in the mutant supports our earlier conclusions about the extent of the lesion and provides novel evidence for changes outside of the deletion. The patch of *Sim-2* expression in the mutant is rotated with the rest of the prosencephalon, which is consistent with the earlier interpretation based on neuronal markers (Fig. 4B,D; n = 8). The central core is evident. The dorsal strip is present, thus supporting the earlier conclusion that the rostral border of p2 forms normally. The caudal strip is present and is roughly the same length in the mutant. Because of this and because it extends to the p1/mes border in the wildtype, p1 is apparently intact. This finding is independent support for our earlier conclusion that ventral p1 is intact in the mutant. In contrast, the ventral strip is completely missing; where it normally forms a stirrup-like domain across the ventral midline, a clear unlabeled gap appears in the mutant. It is in this ventral midline region that both *Sim-2* and *Wnt-1* are expressed in the wildtype. The mammillary process is less prominent in mutant embryos (Fig. 4B), suggesting that the development of this

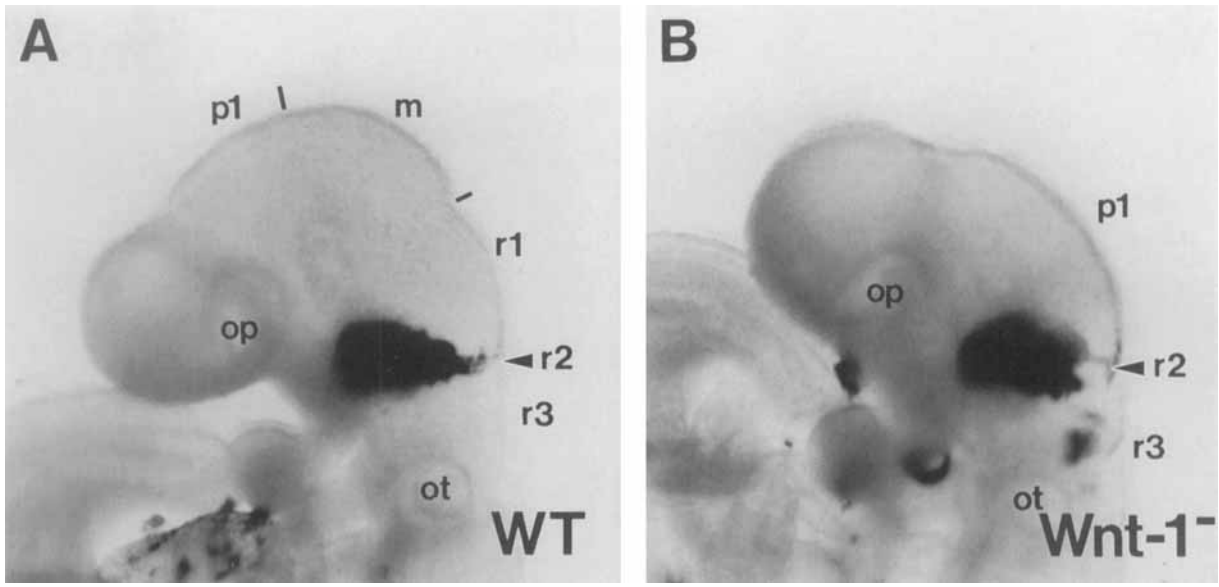

Fig. 5. The expression of an r2-specific transgene is unaltered in the mutant. E9.5 wildtype (**A**) and mutant (**B**) embryos from a line carrying an additional lacZ transgene and stained for β-galactosidase activity. The small patch of apparent label in dorsal r3 is artifactual.

region is altered or delayed. These changes provide the first evidence that *Wnt-1* has a function in this region.

Expression of an r2-specific transgene is unaltered in the mutant

We confirmed that r2 remains intact by examining the pattern of expression of an r2-specific transgene in *Wnt-1*$^{-/-}$ mutant stocks. In this transgene, a lacZ insertion (likely under the regulation of a *Hox-1.6* enhancer; T. Gridley, personal communication) results in β-galactosidase activity that labels a wedge of cells, corresponding precisely to r2 on E9.5 (Fig. 5A). In *Wnt-1*$^{-/-}$ embryos, the labeled wedge is present, indicating that r2 is present (Fig. 5B). In fact, dorsal r2 may be slightly expanded in the mutant but otherwise develops normally, despite the deletion of r1 and mes, which agrees with our observations of morphology and neuronal labeling.

Initial tract formation in *Wnt-1*$^{-/-}$ embryos

Our analysis indicates that the mutant brain has a novel border between p1 and r2; it seemed possible that this structural change might alter the tracts (mlf and tmesV) that originate in p1 and normally traverse mes and r1. In r2, both tracts were readily identified and occupied their normal positions in both wholemounts (Fig. 2C,D) and sections (Fig. 6A,B). They were smaller in the mutant brain, as would be expected from the reduced numbers of somata caused by the deletion. The caudalmost point of both tmesV and mlf was roughly the same in both mutant and wildtype; there was no consistent retardation or acceleration in the mutant embryos versus their littermates that we examined. There was no sign of axons accumulating at the p1–r2 boundary, as would be expected if they had difficulty crossing it. Most of the axons appeared to course in an orderly caudal trajectory, although the heavy immunolabeling made it difficult to resolve individual axons.

To determine more precisely the accuracy of axon navigation through the new border, small groups of neurons and their axons were selectively labeled by microinjections of dil in p1. In both wildtype and mutant, small groups of axons from p1 projected with smooth caudal trajectories into mes in wildtype embryos and into the rhombencephalon in mutant embryos (Fig. 7A,B). One example of an apparent error, a growth cone-tipped axon oriented rostrally, was observed in a wildtype embryo (Fig. 7A), but no similar errors were noted in any of the mutant embryos. There were no apparent discontinuities associated with the novel border in mutant embryos. The lack of any obvious pathfinding errors confirms the general impression from TuJ1-labeled wholemounts that axons navigate correctly through the novel border.

DISCUSSION

Defining the *Wnt-1*$^{-/-}$ deletion

In this paper, we have used an extensive battery of landmarks, both morphological and molecular, in both dorsal and ventral halves of the neural tube to define the deletion. We have shown that the missing ventral structures are the most clearly defined and indicate a precise deletion of mes and r1. This finding is in accord with the earlier data showing a reduction in the ventral expression of *Wnt-5a* (McMahon et al., 1992). Dorsal defects were also present, although the boundaries were less clear. The possibility remains that some very small remnants of mes and r1 may be present, although perhaps changed in fate. Our conclusion that mes and r1 are deleted is consistent with previous observations of the complete loss of *En* expression in mes and r1 by E9.5 (McMahon et al., 1992) and the failure of the cerebellum to develop (McMahon and Bradley, 1990; Thomas and Capecchi, 1990).

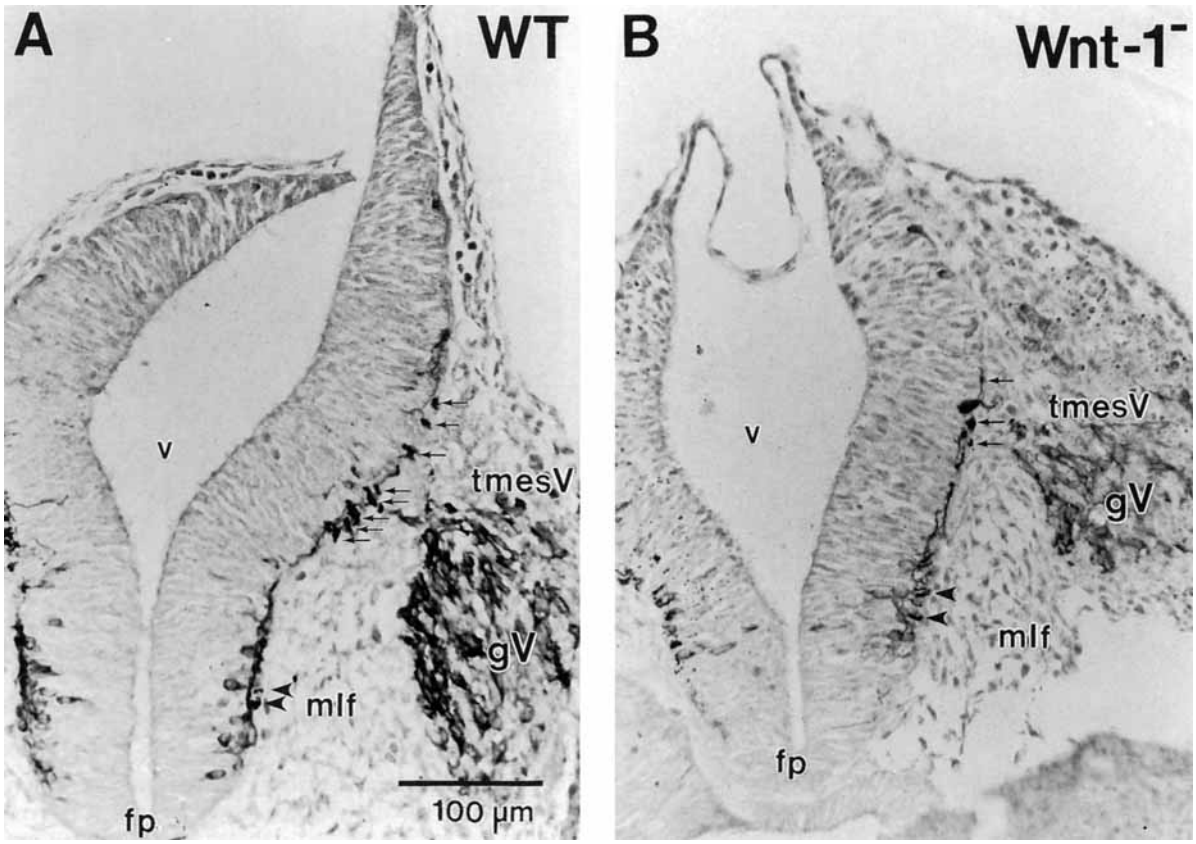

Fig. 6. The initial longitudinal tracts occupy the same locations in the rhombencephalon in both wildtype and mutant. Cryostat sections of E9.5 littermates (wildtype n = 2, *Wnt-1*$^{-/-}$ n = 2) were cut transversely in the plane indicated in Figure 2A,B and immunolabeled to show the locations of the longitudinal bundles of mlf and tmesV axons relative to neuronal landmarks in r2. Dorsal is up. Floor plate (fp) and the ventricle (v) are indicated. **A:** Wildtype embryo (WT). Intensely labeled axon bundles form the mlf (arrowheads) that passes superficially through the midst of rhombomeric neurons. Axon bundles of the tmesV (small arrows) are located more dorsally, both slightly dorsal and ventral to the dorsal root of gV. Rhombomeric neurons are located superficially (near the pial surface) in the ventral half of the neural tube and extend axons dorsally. **B:** *Wnt-1*$^{-/-}$ embryo. The mlf and tmesV axon bundles are found in the same locations in mutant embryos.

The overall length of the brain was reduced in the mutant, which is consistent with a deletion, but the mutant brain was about 20% longer than predicted by a precise deletion of mes and r1 (Fig. 3B). There are two possible explanations for this discrepancy: either the lesion was incomplete (remnants of mes and r1 remain) or the mutant brain (lacking both mes and r1) had expanded. We favor the second explanation based on the deletions that we have noted, but we acknowledge that the issue can only be decided with more specific markers of p1 and r2. Compensatory expansion would lead to larger expression domains of genes specific to remaining neuromeres. We have one marker specific for r2, the lacZ transgene, and we have shown that this expression may be expanded in dorsal r2. We have one marker specific to ventral p1, the caudally directed tail of the *Sim-2* expression domain, and we have shown that it did not expand. This result suggests that any possible expansion of p1 must be restricted to the dorsal side. Compensatory expansion could result from at least two quite different mechanisms. If some of the cells that would normally have become mes or r1 had survived and were found in p1 in the mutant, then this neuromere would be enlarged by the addition of these cells that had switched fate. If all the cells in mes and r1 had died, then p1 may have expanded passively as a result of the morphogenetic forces that shape the head. The neural tube is linked mechanically through the mesenchyme to the rest of the embryo, and those motive forces responsible for head development, particularly the formation of the cephalic flexure, would be expected to drag the shorter neural tube along with the rest of the embryo. We suggest that the regions of the neural tube outside the missing neuromeres have been passively stretched in the mutant brain.

Neuromeres as independent developmental units

Despite the disappearance of their neighboring neuromeres, the spared regions adjacent to the deletion, r2 and p1, continue much of their program of neuronal development essentially without delay or alteration. For example, p1 neurons in appropriate locations are produced and project axons caudally with the normal timing to pioneer the tmesV and mlf. Likewise, r2 neurons and a lacZ reporter gene appear normal in the mutant. In contrast, it seems likely that the ventral midline region of the forebrain that expresses *Wnt-1* (approximately p1–p4; Parr et al., 1993) is perturbed in the mutant. *Wnt-1* expression in p4

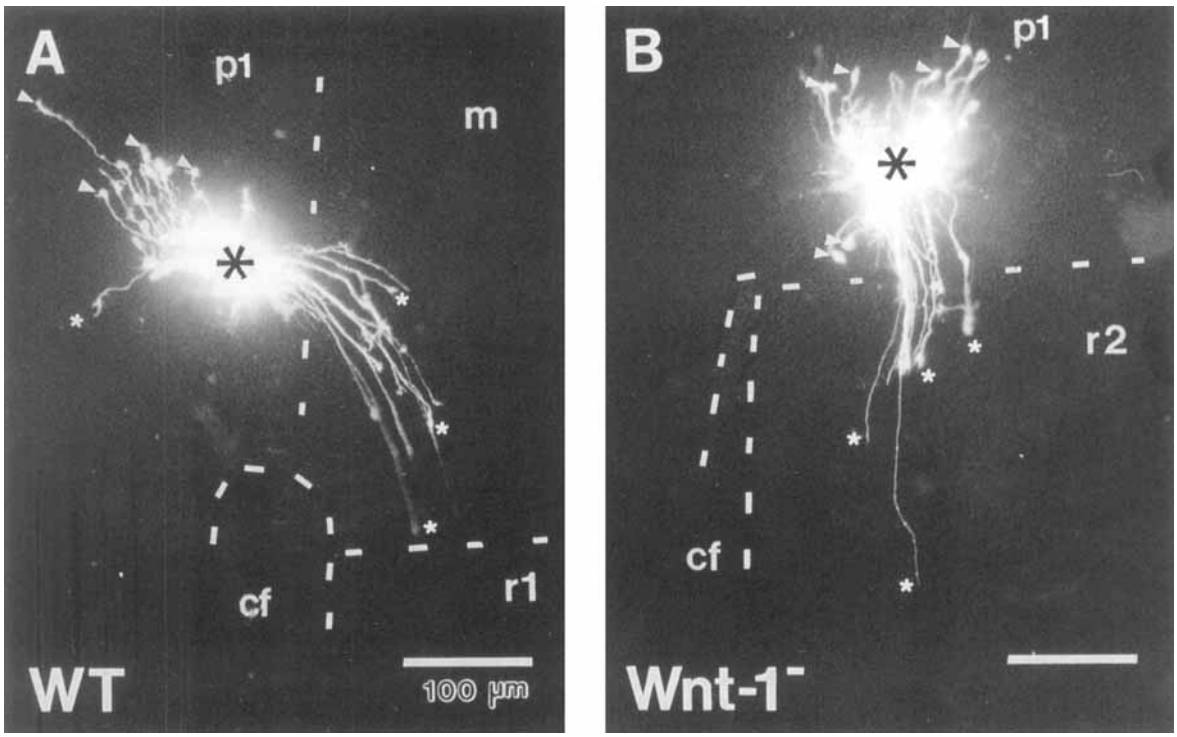

Fig. 7. Neurons in p1 accurately project axons across the novel border directly into the rhombencephalon. Microinjections of 1,1′dioctadecyl-3,3,3′,3″-tetramethylindocarbocy anine (dil; large asterisks) were made in fixed wildtype and *Wnt-1*$^{-/-}$ embryo wholemounts. Both dorsal (wildtype n = 3, *Wnt-1*$^{-/-}$ n = 2) and ventral (wildtype n = 2, *Wnt-1*$^{-/-}$ n = 2) injections were made in p1, labeling tmesV and mlf neurons, respectively. Embryos are mounted in the same orientation as those shown in Figure 1, and the outline of the ventral midline forming the cephalic flexure (cf) is shown. Boundaries (dashed lines) were estimated from examination in brightfield conditions in the dissecting microscope. Retrogradely labeled somata are indicated by arrowheads and anterogradely labeled growth cones by small asterisks. **A:** Wildtype embryo (WT). Neuronal cell bodies in p1 extend axons caudally into mes. A few axons almost reach r1 in this embryo; one exceptional axon is rostrally directed. **B:** *Wnt-1*$^{-/-}$ embryo. Neurons from p1 extend axons caudally into the rhombencephalon, with none in abnormal directions.

overlaps with the most ventral strip of *Sim-2* expression, and we have found that this *Sim-2* expression is absent in the mutant, although there does not seem to be a major loss of the cells of this region (see below). Therefore, gene expression in the *Wnt-1*-expressing ventral midline regions of p1–p3 (for which we do not have independent markers) may also be altered in the mutant. In summary, our results suggest that there is little discernable effect on those portions of the neural tube adjacent to the mes–r1 deletion that do not express *Wnt-1*.

This conclusion is consistent with the idea that the neural tube is a mosaic of subunits that develop with some independence. Transverse interneuromeric boundaries coincide with zones that restrict the movements of clones of labeled cells in chick, including r2 (Fraser et al., 1990) and p1 (Figdor and Stern, 1993). These clonal domains also maintain their regulatory environment to a degree: appropriate local *Hox* gene expression is maintained following the transplantation of rhombomeres to more rostral positions (Guthrie et al., 1992), although *Hox* expression can be modified by long caudalward transplants (Grapin-Botton et al., 1995). Thus, relatively autonomous cellular differentiation patterns in neuromeres may be the result of restricted clonal movements and maintenance of region-specific regulatory gene expression.

The general conclusion that neuromeres develop autonomously must be qualified by the notable exception of r1, which does not itself express *Wnt-1* but is dependent on *Wnt-1* expression in the adjacent mes (McMahon et al., 1992; Parr et al., 1993). The deletion of r1 suggests cross talk between mes and r1, which could be mediated either by *Wnt-1* or by some other long-range signal under *Wnt-1* control. Consistent with this suggestion of cross talk, surgical manipulations in chick demonstrate that the isthmus, the mes–r1 border region, has clear inductive patterning properties (Marin and Puelles, 1994). Conclusions about autonomy must also be qualified by the observation that the presumptive mes and r1 are initially present and soon deleted (McMahon et al., 1992). Thus, the possibility remains that mes and r1 may have already emitted the necessary patterning signals before they disappeared.

The absence of *Wnt-1* causes a complete deletion of a large region of the neural tube, with the loss of multiple contiguous segments, thus creating novel juxtapositions between the remaining segments. This phenotype is observed for other genes in the vertebrate central nervous system (CNS) and in *Drosophila*, suggesting a general genetic theme. In the mouse, similar phenotypes result from the loss of function of two other genes, *En-1* and *Hoxa-1*. In the *En-1* phenotype, most of the dorsal midbrain and the cerebellum are missing in advanced embryos, and both nIII and nIV are missing on E10.5 (Wurst et al., 1994). The deletion is clearly less severe than that in *Wnt-1*, however, because *En-2* expression is retained at the dorsal mes–r1 border and the cephalic flexure has a fairly normal curve. The partial overlap of the *Wnt-1* and *En-1* mutant

phenotypes suggests that some of the *Wnt-1* deletion can be accounted for by the loss of *En-1* function; the remaining deletion could be dependent on *En-2* or mediated by other downstream targets. In the *Hoxa-1*$^{-/-}$ phenotype, both neuronal and molecular markers indicate that r5 is deleted and r4 is markedly reduced (Carpenter et al., 1993; Dolle et al., 1993; Mark et al., 1993).

These large segmental deletions are reminiscent of the "gap" class of genes in *Drosophila*, at least on the phenotypic level, where a typical loss-of-function phenotype includes the deletion of several adjacent epidermal segments. Gaplike phenotypes have also been described in the fly anterior CNS involving mutations in two genes: *orthodenticle* mutations result in the specific deletion of the first brain segment, and *empty spiracles* mutations result in the deletion of the second and third brain segments (Hirth et al., 1995). The conclusion that related genes may function in defining large regions of both mammal and fly CNS has been further strengthened by the finding that mutations in a mouse *orthodenticle* homologue, *Otx-2*, result in a large deletion in the brain, a truncation of CNS structures rostral to r3 (Acampora et al., 1995; Matsuo et al., 1995). In contrast to *Wnt-1* mutants, however, mesoderm and ectoderm defects are apparent during gastrulation, and the *Otx*-dependent brain regions are never formed. This last point emphasizes that, although a general conclusion is that blocks of multiple neuromeres are genetically defined as a unit, the developmental mechanisms of this genetic specification are likely diverse, differing not only between different genes in the vertebrate brain but also between vertebrates and invertebrates. We raise two additional notes of caution. First, some of the vertebrate genes that produce gaplike phenotypes are related not to gap genes of *Drosophila* but rather to those of other classes, including segment-polarity (*Wnt-1, En*) and homeotic (*Hoxa-1*) gene families. Second, the idea that vertebrate neuromeres are segments has received mixed support (reviewed in Guthrie, 1995); because neuromeres and insect CNS segments arise by clearly different developmental mechanisms (bulges in a neuroectodermal tube and delamination of individual cells to form a subectodermal array of neuroblasts, respectively), the two structures are unlikely to be homologous.

The requirement for *Wnt-1* in neuronal development

The early disappearance of neuromeres suggests that the *Wnt-1*$^{-/-}$ neuronal deficits on E9.5 are secondary effects of a deletion of neuronal precursors because the onset of the first neural tube defect in our mutants coincides with the onset of neuronal differentiation. *En* labeling is lost in mesencephalic cells as early as the 5-somite stage (about E8.5), but the deletion of these cells does not occur until the 7-somite stage 3–4 hours later (McMahon et al., 1992). Using immunoreactivity for β-tubulin as a marker, the first neurons also appear on E8.5 in dorsal rostral mes (Easter et al., 1993). Thus, although a few early neurons may be formed and then deleted, the action of *Wnt-1* must be primarily on the much more numerous epithelial and preneuronal cells that make up the wall of the neural tube. Recent work has shown that *Wnt-1* is mitogenic in the CNS, strongly suggesting that the neuronal defects are due at least in part to the failure of neuronal precursors to proliferate (Dickinson et al., 1994).

Not all of the effects of *Wnt-1* may be attributed to its proliferative activity, however. The absence of *Sim-2* expression in a subset of ventral forebrain cells in the mutant is not associated with an obvious large structural deletion, raising the possibility that *Wnt-1* directly controls *Sim-2* expression in these cells rather than being required for their generation or survival. More studies with additional markers of these cells will be required to determine whether this interpretation is correct.

Mutations in the *wingless* (*wg*) gene, a closely related *Drosophila* member of the *Wnt* family, also indicate a similar function in neuronal development, although the Wg gene product acts on a very different scale, signaling only a few cell diameters. *wg* mutations cause the loss of specific neurons by altering expression patterns of genes (including *engrailed*) in neuroectodermal cells before neuroblast delamination, thus preventing the initial formation of some neuroblasts and misspecifying the developmental fates of others (Chu-LaGraff and Doe, 1993). Defining the roles of additional mouse homologues of the molecular players in the *wg* signaling pathway (Noordermeer et al., 1994; Siegfried et al., 1994) may reveal how *Wnt-1* acts in the development of the embryonic mouse brain.

Axon guidance in *Wnt-1*$^{-/-}$ embryos

The large deletion in mutant embryos might be expected to perturb axon navigation through this region because the normal local environment, containing as-yet unidentified navigational cues, is missing. Axons extending caudally from p1, for example, would encounter r2 on crossing the caudal boundary of p1, in effect bypassing the normally intervening mes and r1. Because the first two tracts that form in the brain, the tmesV and the mlf, are composed of caudally directed longitudinal axons, we examined the mutants to see if these tracts were abnormal. The only defect was a reduction in the number of axons in both of the tracts. This reduction is most likely caused by the elimination of most of their somata of origin because both tracts arise from somata in both p1 and mes (Mastick and Easter, 1996). The trajectories of these axons appear normal as assessed by immunolabeling studies (in wholemounts and in transverse sections), which showed that the tracts are in the correct locations, and by dil-labeling studies, which showed that p1 neurons extend axons with normal caudal trajectories. Others have used surgical manipulations of the neural tube to demonstrate the autonomy of neuronal development: the longitudinal axon projections of Mauthner neurons conform to the local polarity in rostral–caudal inversions of the rhombencephalon in salamanders, although the axon trajectories do respond to the global embryonic polarity when encountering the surgically created novel borders with host tissue (Jacobson, 1964; Hibbard, 1965). In fact, based on silver-stained fibers in late embryos, the tmesV and mlf form in accordance with the local polarity of their cell bodies after rostral–caudal inversions in the axolotl neural tube (Jacobson, 1964).

Although the navigational cues used by tmesV and mlf axons are unknown, these axons are clearly able to compensate for their altered environment. Some mesencephalic or r1-derived neural tube may be retained in the dorsal mutant brain (recall our arguments to the contrary above), possibly providing a less abrupt transition zone for tmesV axons, but it is clear that these regions are deleted ventrally. The faithful tract formation in the absence of mes and r1 implies that any navigational cues that lie in the region between p1 and r2 are dispensable for these axons. We can therefore conclude that no essential chemoattrac-

tive cue or unique local marked pathway is present in mes or r1. However, we can hypothesize several other classes of guidance cues that could act independently of mes and r1. Long-range diffusible cues, such as a gradient of diffusible chemoattractant emitted by some caudal structure (e.g., Tessier-Lavigne et al., 1988) or a repellent from a rostral source, would not be interrupted by the deletion of intermediate neural tube and would allow caudalward axon guidance independent of precise healing of the deletion. Long-range diffusible cues from either the ventral or the dorsal neural tube, such as the netrins expressed by the floor plate (Tessier-Lavigne et al., 1988; Kennedy, et al., 1994; Skarnes et al., 1995), would also be uninterrupted by the transverse deletion and could provide positional information in the form of a dorsal–ventral gradient, in which longitudinal axons navigate caudally by following a particular concentration within that gradient. Local cell surface cues, such as cell adhesion molecules, could provide longitudinal paths, but these paths must be continuous along the rostral–caudal axis, so that r2 is functionally equivalent to mes and r1, and the labeled pathways must be precisely aligned at the novel border, as implied by the smooth caudal trajectories of axons. Similarly, longitudinal physical channels could provide guidance (Singer et al., 1979), but these would also have to be precisely aligned to guide axons accurately. Axons projecting caudally from p1 may continue caudally because of intrinsic ballistic momentum, tending to extend straight independently of additional guidance. Distinguishing among these possibilities will require defining the axon guidance mechanisms used by tmesV and mlf axons.

Because the initial establishment of two major longitudinal tracts was successful in forming an early axonal scaffold, the guidance cues may be sufficient for later ascending and descending axons that normally traverse mes and r1; thus, these connections should be properly made. However, axons whose target lies in the deleted region will be unable to make the proper connections, with deleterious effects on brain function. Optic axons would be a major population with missing targets, assuming that sufficient navigational cues remain to guide them toward the superior colliculus in the dorsal mes, although alteration or loss of their synapses would be unlikely to be the cause of the perinatal death of *Wnt-1*$^{-/-}$ animals.

ACKNOWLEDGMENTS

We thank Celeste Malinoski for excellent technical assistance and David Siebert for assistance with cryostat sections. Critical comments on the manuscript were made by Cynthia Mastick, James Lauderdale, and David Cameron. This work was supported by postdoctoral traineeships to G.S.M. from NSF (9014275) and NIH (5T32HD07274 and 1F32NS09701), to C.M.F. from the Damon Runyon-Walter Winchell Cancer Research Fund and the Howard Hughes Medical Institute, by research grants from NIH (EY00168 to S.S.E., Jr., and HD30249 to A.P.M.), and by the Howard Hughes Medical Institute (M.T.L.).

LITERATURE CITED

Acampora, D., S. Mazan, Y. Lallemand, V. Avantaggiato, M. Maury, A. Simeone, and P. Brulet (1995) Forebrain and midbrain regions are deleted in *Otx-2*$^{-/-}$ mutants due to a defective anterior neuroectoderm *specification during gastrulation. Development 121:*3279–3290.

Bergquist, H., and B. Kallen (1954) Notes on the early histogenesis and morphogenesis of the central nervous system in vertebrates. J. Comp. Neurol. *100:*627–659.

Bulfone, A., L. Puelles, M.H. Porteus, M.A. Frohman, G.R. Martin, and J.L.R. Rubenstein (1993) Spatially restricted expression of *Dlx-1*, *Dlx-2* (*Tes-1*), *Gbx-2*, and *Wnt-3* in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. J. Neurosci. *13:*3155–3172.

Carpenter, E.M., J.M. Goddard, O. Chisaka, N.R. Manley, and M.R. Capecchi (1993) Loss of $^{Hox\text{-}A1}$(*Hox-1.6*) function results in the reorganization of the murine hindbrain. Development *118:*1063–1075.

Chu-LaGraff, Q., and C.Q. Doe (1993) Neuroblast specification and formation regulated by *wingless* in the *Drosophila* CNS. Science *261:*1594–1597.

Crews, S.T., J.B. Thomas, and C.S. Goodman (1988) The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product. Cell *52:*143–151.

Dickinson, M.E., R. Krumlauf, and A.P. McMahon (1994) Evidence for a mitogenic effect of *Wnt-1* in the developing mammalian central nervous system. Development *120:*1453–1471.

Dolle, P., T. Lufkin, R. Krumlauf, M. Mark, D. Duboule, and P. Chambon (1993) Local alterations of *Krox-20* and *Hox* gene expression in the hindbrain suggest lack of rhombomeres 4 and 5 in homozygote null *Hoxa-1* (*Hox-1.6*) mutant embryos. Proc. Natl. Acad. Sci. USA *90:*7666–7670.

Easter, S.S. Jr., L.S. Ross, and A. Frankfurter (1993) Initial tract formation in the mouse brain. J. Neurosci. *13:*285–299.

Fan, C.M., E. Kuwana, A. Bulfone, C.F. Fletcher, N.G. Copeland, N.A. Jenkins, S. Crews, S. Martinez, L. Puelles, J.L.R. Rubenstein, and M. Tessier-Lavigne (1996) Expression patterns of two murine homologs of *Drosophila single-minded* suggest possible roles in embryonic patterning and in the pathogenesis of Down syndrome. Mol. Cell. Neurosci. *7:*1–16.

Figdor, M.C., and C.D. Stern (1993) Segmental organization of embryonic diencephalon. Nature *363:*630–634.

Fraser, S., R. Keynes, and A. Lumsden (1990) Segments in the chick embryo hindbrain are defined by cell lineage restrictions. Nature *344:*431–435.

Fritzsch, B., D.H. Nichols, Y. Echelard, and A.P. McMahon (1995) Development of midbrain and anterior hindbrain ocular motoneurons in normal and *Wnt-1* knockout mice. J. Neurobiol. *27:*457–469.

Godemont, P., J. Vanselow, S. Thanos, and F. Bonhoeffer (1987) A study in developing visual systems with a new method of staining neurones and their processes in fixed tissue. Development *101:*697–713.

Grapin-Botton, A., M.A. Bonnin, L.A. McNaughton, R. Krumlauf, and N.M. Le Douarin (1995) Plasticity of transposed rhombomeres: *Hox* gene induction is correlated with phenotypic modifications. Development *121:*2707–2721.

Guthrie, S (1995) The status of the neural segment. Trends Neurosci. *18:*74–79.

Guthrie, S., M. Butcher, and A. Lumsden (1991) Patterns of cell division and interkinetic nuclear migration in the chick embryo hindbrain. J. Neurobiol. *22:*742–754.

Guthrie, S., I. Muchamore, A. Kuroiwa, H. Marshall, R. Krumlauf, and A. Lumsden (1992) Neuroectodermal autonomy of *Hox-2.9* expression revealed by rhombomere transpositions. Nature *356:*157–159.

Hibbard, E. (1965) Orientation and directed growth of Mauthner's cell axons from duplicated vestibular nerve roots. Exp. Neurol. *13:*289–301.

Hirth, F., S. Therianos, T. Loop, W.J. Gehring, H. Reichert, and K. Furukubo-Tokunaga (1995) Developmental defects in brain segmentation caused by mutations of the homeobox genes *orthodenticle* and *empty spiracles* in *Drosophila*. Neuron *15:*769–778.

Honig, M.G., and R.I. Hume (1986) Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. J. Cell. Biol. *103:*171–187.

Hunt, P., M. Gulisano, M. Cook, M.-H. Sham, A. Faiella, D. Wilkinson, E. Boncinelli, and R. Krumlauf (1991) A distinct *Hox* code for the branchial region of the vertebrate head. Nature *353:*861–864.

Jacobson, C.-O. (1964) Motor nuclei, cranial nerve roots, and fibre pattern in the medulla oblongata after reversal experiments on the neural plate of axolotl larvae. I. Bilateral operations. Zool. Bidrag. Uppsala *36:*73–161.

Kennedy, T.E., T. Serafini, J.R. de la Torre, and M. Tessier-Lavigne (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell *78:*425–435.

Lee, M.K., J.B. Tuttle, L.I. Rebhun, D.W. Cleveland, and A. Frankfurter (1990) The expression and postranslational modification of a neuron-

specific beta tubulin isotype during chick embryogenesis. Cell. Motil. Cytoskel. *17:*118–132.

Lumsden, A., and R. Keynes (1989) Segmental patterns of neuronal development in the chick hindbrain. Nature *337:*424–428.

Marin, F., and L. Puelles (1994) Patterning of the embryonic avian midbrain after experimental inversions: A polarizing activity from the isthmus. Dev. Biol. *163:*19–37.

Mark, M., T. Lufkin, J.L. Vonesch, E. Ruberte, J.C. Olivo, P. Dolle, P. Gorry, A. Lumsden, and P. Chambon (1993) Two rhombomeres are altered in *Hoxa-1* mutant mice. Development *119:*319–338.

Martinez, S., and R.-M. Alvarado-Mallart (1989) Rostral cerebellum originates from the caudal portion of the so-called "mesencephalic" vesicle: A study using chick/quail chimeras. Eur. J. Neurosci. *1:*549–560.

Mastick, G.S., and S.S. Easter, Jr. (1996) Initial organization of neurons and tracts in the embryonic mouse fore- and midbrain. Dev. Biol. *173:*79–94.

Matsuo, I., S. Kuratani, C. Kimura, N. Takeda, and S. Aizawa (1995) Mouse *Otx-2* functions in the formation and patterning of rostral head. Genes Dev. *9:*2646–2658.

McMahon, A.P. (1992) The *Wnt* family of developmental regulators. Trends Genet. *8:*236–242.

McMahon, A.P., and A. Bradley (1990) The *Wnt-1* (*int-1*) proto-oncogene is required for development of a large region of the mouse brain. Cell *62:*1073–1085.

McMahon, A.P., A.L. Joyner, A. Bradley, and J.A. McMahon (1992) The midbrain–hindbrain phenotype of *Wnt-1*−/*Wnt-1*− mice results from stepwise deletion of *engrailed*-expressing cells by 9.5 days postcoitum. Cell *69:*581–595.

Moody, S.A., M.S. Quigg, and A. Frankfurter (1987) Development of the peripheral trigeminal system in the chick revealed by an isotype-specific *anti-beta-tubulin* monoclonal antibody. J. Comp. Neurol. *279:*567–580.

Noordermeer, J., J. Klingensmith, N. Perrimon, and R. Nusse (1994) *dishevelled* and *armadillo* act in the Wingless signalling pathway in *Drosophila*. Nature *367:*80–83.

Nusse, R., and H.E. Varmus (1992) *Wnt* genes. Cell *69:*1073–1087.

Parr, B.A., M.J. Shea, G. Vassileva, and A.P. McMahon (1993) Mouse *Wnt* genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. Development *119:*247–261.

Puelles, L., and J.L.R. Rubenstein (1993) Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. Trends Neurosci. *16:*472–479.

Puelles, L., J.A. Amat, and M. Martinez-de-la-Torre (1987) Segment-related, mosaic neurogenetic pattern in the forebrain and mesencephalon of early chick embryos: I. Topography of AChE-positive neuroblasts up to stage HH18. J. Comp. Neurol. *266:*247–268.

Siegfried, E., E.L. Wilder, and N. Perrimon (1994) Components of *wingless* signalling in *Drosophila*. Nature *367:*76–80.

Singer, M., R.H. Nordlander, and M. Egar (1979) Axonal guidance during embryogenesis and regeneration in the spinal cord of the newt: The blueprint hypothesis of neuronal pathway patterning. J. Comp. Neurol. *185:*1–22.

Skarnes, W.C., J.E. Moss, S.M. Hurtley, and R.S.P. Beddington (1995) Capturing genes encoding membrane and secreted proteins important for mouse development. PNAS USA *92:*6592–6596.

Tessier-Lavigne, M., M. Placzek, A.G.S. Lumsden, J. Dodd, and T.M. Jessell (1988) Chemotropic guidance of developing axons in the mammalian central nervous system. Nature *336:*775–778.

Thomas, J.B., S.T. Crews, and C.S. Goodman (1988) Molecular genetics of the *single-minded* locus: A gene involved in the development of the *Drosophila* nervous system. Cell *52:*133–141.

Thomas, K.R., and M.R. Capecchi (1990) Targeted disruption of the murine *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature *346:*847–850.

Wilkinson, D.G. (1992). In Situ Hybridization: A Practical Approach. Oxford, UK: Oxford University Press.

Wilkinson, D.G., and R. Krumlauf (1990) Molecular approaches to the segmentation of the hindbrain. Trends Neurosci. *13:*335–339.

Wilkinson, D.G., J.A. Bailes, and A.P. McMahon (1987) Expression of the proto-oncogene *int-1* is restricted to specific neural cells in the developing mouse embryo. Cell *50:*79–88.

Wurst, W., A.B. Auerbach, and A.L. Joyner (1994) Multiple developmental defects in *Engrailed-1* mutant mice: An early mid-hindbrain deletion and pattern defects in forelimbs and sternum. Development *120:*2065–2075.