Development 126, 2727-2737 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV1393

The zebrafish *detour* gene is essential for cranial but not spinal motor neuron induction

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[†]We dedicate this paper to the memory of Pascal Haffter, a dear friend and colleague

Accepted 13 April; published on WWW 19 May 1999

SUMMARY

The zebrafish *detour* (*dtr*) mutation generates a novel neuronal phenotype. In *dtr* mutants, most cranial motor neurons, especially the branchiomotor, are missing. However, spinal motor neurons are generated normally. The loss of cranial motor neurons is not due to aberrant hindbrain patterning, failure of neurogenesis, increased cell death or absence of *hh* expression. Furthermore, activation of the Hh pathway, which normally induces branchiomotor neurons, fails to induce motor neurons in the *dtr* hindbrain. Despite this, not all Hh-mediated regulation of hindbrain development is abolished since the regulation of a neural gene by Hh is intact in the *dtr* hindbrain. Finally, *dtr* can function cell autonomously to induce branchiomotor neurons. These results suggest that *detour* encodes a component of the Hh signaling pathway that is essential for the induction of motor neurons in the hindbrain but not in the spinal cord and that *dtr* function is required for the induction of only a subset of Hh-mediated events in the hindbrain.

Key words: Zebrafish, Hindbrain, Rhombomere, Cranial motor neuron, Spinal cord, *detour*, *sonic hedgehog*, *protein kinase A*, Hedgehog signaling

INTRODUCTION

Two sets of mechanisms have evolved to generate specific neuronal types at particular locations in the vertebrate embryo (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). One set of mechanisms initiated by secreted factors belonging to the hedgehog (Hh) and bone morphogenetic protein (BMP) families controls neuronal patterning along the dorsoventral axis of the neural tube (Tanabe and Jessell, 1996; Liem et al., 1997; Ingham, 1998). Explant studies in chick demonstrated that sonic hedgehog (Shh) induces different ventral cell types depending upon the rostrocaudal location of the explanted tissue in the neural tube: midbrain, hindbrain or spinal cord explants gave rise to floor plate and motor neurons, whereas forebrain explants generated forebrain-specific ventral cell types (Roelink et al., 1995; Ericson et al., 1995). These studies suggest that a second set of mechanisms, acting along the rostrocaudal axis, may control the identity of the neurons generated by the dorsoventral patterning mechanisms. In fact, paraxial mesoderm from posterior locations grafted onto the rostral neural tube induced caudal neural tube markers in the rostral neural tube, suggesting that caudally restricted secreted factors may play a role in controlling the identity of neurons induced by Shh and BMPs (Muhr et al., 1997; Ensini et al., 1998; Woo and Fraser, 1997; Bang et al., 1997).

Little is known about the genes that generate rostrocaudal differences in the ventral cell types induced by Shh. For example, motor neurons innervate different muscles depending upon their rostrocaudal position. The well-studied branchiomotor neurons are located at characteristic positions along the rostrocaudal axis of the hindbrain and innervate different peripheral targets (Lumsden and Keynes, 1989; Gilland and Baker, 1993; Chandrasekhar et al., 1997). Therefore, they are an attractive system to examine dorsoventral and rostrocaudal patterning mechanisms. Earlier studies showed that Shh (Krauss et al., 1993) and tiggy-winkle hedgehog (Twhh; Ekker et al., 1995) can induce branchiomotor neurons in the zebrafish embryo (Chandrasekhar et al., 1998). Interestingly, deletion of *shh* in zebrafish (Schauerte et al., 1998) only eliminates a subset of branchiomotor neurons, suggesting the action of redundant and complex inducing mechanisms (Chandrasekhar et al., 1998). As a part of our efforts to elucidate these mechanisms, an immunohistochemical screen was performed on the Tübingen zebrafish mutants (Haffter et al., 1996) to identify mutations affecting branchiomotor neuron development. We found that the *detour* (*dtr*) mutation, which was originally identified due to defects in midline development and retinotectal axon guidance (Brand et al., 1996; Karlstrom et al., 1996), generated a novel neuronal phenotype in the hindbrain. In *dtr* mutants, motor neurons are specifically missing from the hindbrain, and possibly the midbrain, but not the spinal cord. We show that, except for the

motor neuron defect, hindbrain development and neurogenesis are unaffected in *dtr* mutant embryos. Activation of the hedgehog (Hh) signaling pathway in *dtr* mutants by overexpressing *shh* or dominant negative protein kinase A (*dnPKA*) does not induce branchiomotor neurons or motor neuron-specific genes in the hindbrain. Mosaic analysis shows that the *dtr* branchiomotor neuron phenotype is cell autonomous. Interestingly, Hh-regulated expression of *netrin1a*, which is normally expressed in the ventral CNS (Lauderdale et al., 1997, 1998), is intact in the *dtr* hindbrain. Together, these results suggest that *detour* may encode a component of the hedgehog (Hh) signaling pathway downstream of protein kinase A and that some essential events leading to cranial motor neuron induction are different from those leading to spinal motor neuron induction or to *netrin1a* gene activation in the hindbrain.

MATERIALS AND METHODS

Animals

Zebrafish were reared and maintained as described in Westerfield (1995). Embryos were collected from pairwise matings and allowed to develop at 28.5°C. Throughout the text, the developmental age of the embryos corresponds to the hours elapsed since fertilization (hours post fertilization, HPF). Embryos were transferred to water containing 0.2 mM phenylthiourea between 18 and 22 HPF to prevent pigmentation (Burrill and Easter, 1994).

The mutant strains used (*dtr*^{te370a}, *dtr*^{ts269} and *dtr*^{tm276b}) were ENU-generated, and were originally identified on the basis of their midline and retinotectal phenotypes (Brand et al., 1996; Karlstrom et al., 1996). Though all three mutant alleles exhibited branchiomotor neuron defects, most of the data presented here were obtained from the *dtr*^{te370a} mutant, which showed the most severe defects.

RNA injections

Synthesis of full-length RNA and injection of RNA into embryos were carried out as described previously (Chandrasekhar et al., 1998). The plasmids containing full-length cDNAs were provided by D. Turner (CS2-*βgal*), S. Ekker (T7TS-*shh*; Ekker et al., 1995) and A. Ungar (*dnPKA*; Ungar and Moon, 1996).

Immunohistochemistry and in situ hybridization

Whole-mount immunohistochemistry with the islet (39.4D5; Korzh et al., 1993; 1:500 dilution), zn5 (Trevarrow et al., 1990; 1:10 dilution), anti-acetylated tubulin (Piperno and Fuller, 1985; 1:500 dilution), Hu (rabbit polyclonal at 1:500 dilution; Linda Hansen, University of Oregon) and 3A10 (Hatta, 1992; 1:500 dilution) antibodies was performed as described previously (Chandrasekhar et al., 1997, 1998). For Hu immunolabeling, a RITC-conjugated secondary antibody (Jackson Immunochemicals) was used. Synthesis of the digoxigenin-labeled probe and whole-mount in situ hybridization were carried out as described previously (Chandrasekhar et al., 1997). In all comparisons, at least five wild-type and five mutant embryos were examined.

Cell transplantation

1-cell-stage embryos (donor) from wild-type AB strain parents were injected with 2:1 mixture of 25 mg/ml biotinylated dextran and 50 mg/ml rhodamine dextran (Molecular Probes). At the blastula stage, donor and host embryos were dechorionated and aligned in embryo-sized agarose wells in a Petri dish. A few cells were removed from donor embryos with a polished glass pipette and transplanted into the unlabeled host embryos obtained from a *dtr*^{te370a} /+ incross. Transplanted host embryos containing fluorescent cells in the hindbrain were fixed at 30-36 HPF and processed for *tag1* or *isl1* in situ hybridization to identify mutant embryos. Following in situ hybridization, embryos were fixed overnight, washed several times in the incubation buffer used for immunohistochemistry (Chandrasekhar et al., 1997), incubated overnight in streptavidin-peroxidase solution (Vector Labs) and finally incubated in diaminobenzedine/H_2O_2 to

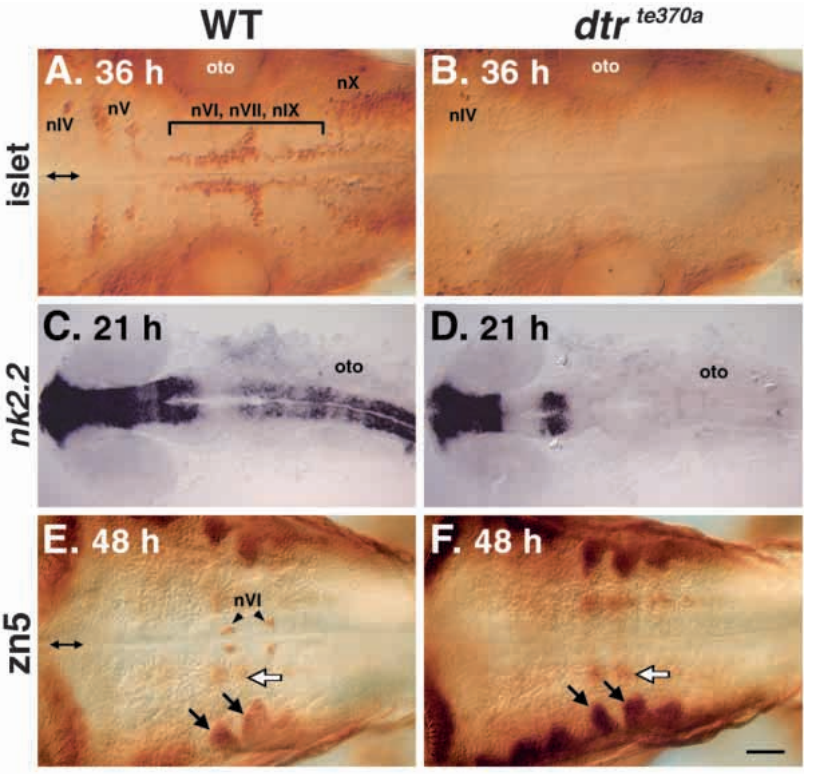

Fig. 1. Cranial motor neurons are missing in *dtr*^{te370a} embryos. All panels depict dorsal views, with rostral to the left, of the hindbrain of whole-mounted embryos analyzed either by islet (A,B) or zn5 (E,F) immunohistochemistry, or by *nk2.2* in situ hybridization (C,D). Double arrows (A,E) mark the midline. (A) In a 36 HPF wild-type sibling, the islet antibody labels the trigeminal motor (nV) neurons in r2 and r3, the abducens (nVI), the facial motor (nVII) and the glossopharyngeal motor (nIX) neurons in r4, r5, r6 and r7, and the vagal motor (nX) neurons in the caudal hindbrain. The antibody also labels the presumptive trochlear (nIV) neurons in r1. (B) In a *dtr*^{te370a} homozygote, all cranial motor neurons, except the putative nIV neurons, are missing. (C) In a 21 HPF wild-type sibling, *nk2.2* is expressed in the ventral CNS throughout the forebrain, the rostral midbrain and the hindbrain. (D) In a *dtr*^{te370a} homozygote, *nk2.2* expression is missing throughout the hindbrain and in the rostralmost midbrain. (E) In a 48 HPF wild-type sibling, the zn5 antibody labels the abducens neurons (nVI) in r5 and r6, and some unidentified cells just laterally (white arrow). The labeled cells located most laterally (black arrows) are the hindbrain commissural neurons. (F) In a *dtr*^{te370a} homozygote, the nVI neurons are missing. However, the hindbrain commissural neurons (black arrows) and the unidentified zn5-labeled cells (white arrow) are unaffected. oto, otocyst. Scale bar, 40 μm (A,B,E,F), 100 μm (C,D).

visualize the donor cells. Out of ~150 transplanted host embryos with labeled hindbrain cells, two wild-type and three mutant embryos with donor-derived branchiomotor neurons were obtained.

Quantification of neuronal populations

Neuronal populations were counted in strongly labeled, well-mounted preparations. Hindbrain neurons were quantified in dorsally mounted preparations and spinal neurons were quantified in laterally mounted preparations.

RESULTS

Cranial motor neurons are missing in *detour*^{te370a} embryos

The branchiomotor neurons in *detour* mutants are greatly reduced in number or completely absent. Branchiomotor neurons were visualized by whole-mount islet antibody labeling. In 36 HPF (hours post fertilization) wild-type siblings, the cranial motor neurons occupy characteristic positions within the hindbrain (Fig. 1A; Chandrasekhar et al., 1997). The trigeminal motor neurons (nV) are located in rhombomeres 2 and 3 (r2, r3), and the vagal motor neurons (nX) in the caudalmost hindbrain. At 36 HPF, the abducens (nVI), facial (nVII) and glossopharyngeal (nIX) motor neurons are located in r4-r7 and they are not yet fully segregated (Fig. 1A). By 48 HPF, the nVI neurons are restricted to r5 and r6, the nVII neurons to r6 and r7, and the nIX neurons to r7 (Chandrasekhar et al., 1997). In embryos homozygous for the *dtr*^{te370a} allele, all branchiomotor neurons are absent (Fig. 1B). In embryos homozygous for the *dtr*^{ts269} or *dtr*^{tm276b} alleles, the branchiomotor neurons are greatly reduced in number (Table 1). On the basis of the severity of the branchiomotor neuron phenotype, the three *detour* alleles form an allelic series *dtr*^{te370a}>*dtr*^{ts269}>*dtr*^{tm276b}.

We further analyzed branchiomotor neuron development in *dtr* mutants by studying the expression of *nk2.2* (Barth and Wilson, 1995) because its mouse homolog, *Nkx2.2*, is expressed in progenitors of the nX, and probably of the nV, nVII and nIX, motor neurons (Ericson et al., 1997; Osumi et al., 1997). In 21 HPF wild-type siblings, *nk2.2* is expressed in the ventral CNS throughout the brain, except the floor plate and a small region at the mid-hindbrain boundary (Fig. 1C; Barth and Wilson, 1995). In situ hybridization with a mixed probe

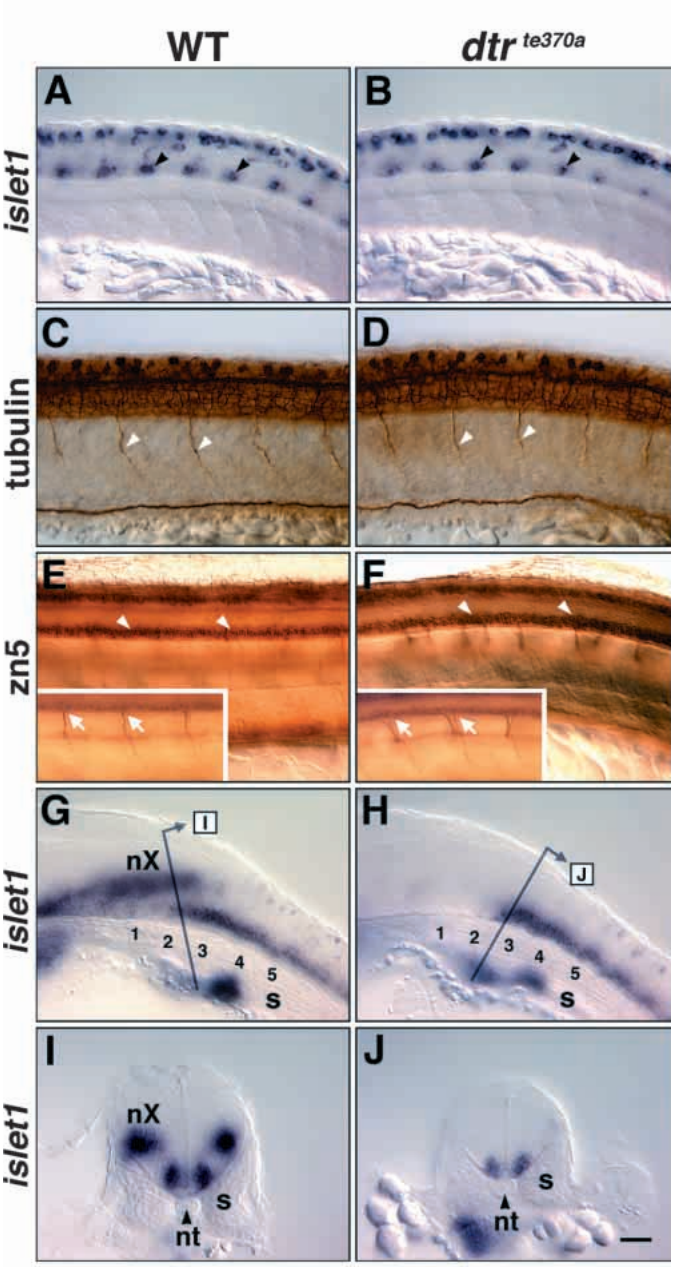

Fig. 2. Spinal motor neurons are generated normally in *dtr*^{te370a} embryos. Panels A-H depict lateral views, with rostral to the left and dorsal up, of the trunk of whole-mounted embryos analyzed either by anti-tubulin (C,D) or zn5 (E,F) immunohistochemistry, or by *islet1* in situ hybridization (A,B,G,H). The right-angled arrows in G and H indicate the approximate location and orientation of the transverse sections shown in I and J, respectively, obtained from different embryos. (A) In a 21 HPF wild-type sibling, the two to three *isl1*-expressing cells (arrowheads) in the ventral spinal cord in every hemisegment are the primary motor neurons. The *isl1*-expressing cells in the dorsal spinal cord are the Rohon-Beard neurons. (B) In a *dtr*^{te370a} homozygote, the primary motor neurons (arrowheads) and Rohon-Beard neurons appear normal in number and location. (C) In a 24 HPF wild-type sibling, the primary motor axons exit the spinal cord, with one motor root per hemisegment (arrowheads). (D) In a *dtr*^{te370a} homozygote, the number and appearance of the primary motor axons exiting the spinal cord (arrowheads) are unaffected. (E) In a 48 HPF wild-type sibling, the zn5-labeled secondary motor neurons are located in the ventral fourth of the spinal cord (arrowheads). Inset depicts a more lateral focal plane showing the secondary motor axons (arrows) exiting the spinal cord and extending ventrally into the somites. (F) In a *dtr*^{te370a} homozygote, the secondary motor neurons (arrowheads) appear normal in number. However, many secondary motor axons (Inset, arrows) exit the spinal cord at ectopic locations. (G) In a 30 HPF wild-type sibling, the caudalmost nX neurons overlap the rostralmost spinal motor neurons located at the level of somites 2 and 3. (H) In a *dtr*^{te370a} homozygote, the nX neurons are missing, but the rostralmost spinal motor neurons are still present. (I) Transverse section through the caudal hindbrain in a wild-type sibling shows that the nX neurons and the spinal motor neurons overlap rostrocaudally, but occupy distinct dorsolateral locations. (J) Transverse section through the caudal hindbrain in a *dtr*^{te370a} homozygote reveals only the rostralmost spinal motor neurons. The apparent difference in *isl1* expression in spinal motor neurons between I and J results from the different thicknesses of the sections, which were done by hand. s, somite; nt, notochord. Scale bar, 40 μm.

revealed that the *nk2.2* and the motor neuron-specific *islet1* (Inoue et al., 1994; Appel et al., 1995) genes are coexpressed in branchiomotor neurons (data not shown). In *dtr*^{te370a} homozygotes, *nk2.2* expression is specifically missing in the rostral midbrain and throughout the hindbrain (Fig. 1D). These observations suggest that branchiomotor neuron progenitor cells may fail to form in *dtr*^{te370a} mutants.

Since branchiomotor neurons, which innervate the pharyngeal arches, are missing in *dtr*^{te370a} mutants, we determined whether hindbrain motor neurons that innervate the extraocular muscles (nIII, nIV, nVI; Gilland and Baker, 1993) are also affected in mutant embryos. The fate of the nVI motor neurons was determined by examining 48 HPF embryos labeled with the zn5 antibody, which recognizes the Dm-Grasp protein (Kanki et al., 1994; Fashena, 1996). The zn5 antibody strongly labels many neurons including the abducens (nVI) and hindbrain commissural neurons, but not the branchiomotor neurons (Trevarrow et al., 1990; Chandrasekhar et al., 1997). In 48 HPF wild-type siblings, the nVI neurons are located medially in r5 and r6 (Fig. 1E). In *dtr*^{te370a} homozygotes, the nVI neurons are missing (Fig. 1F). The nVI motor neurons are also greatly reduced in *dtr*^{ts269} and *dtr*^{tm276b} mutant embryos (data not shown). Examination of 30 HPF embryos processed for *islet1* in situ hybridization revealed that some *isl1*-expressing cells in the midbrain, tentatively identified as oculomotor neurons (nIII), are missing in *dtr*^{te370a} homozygotes (see Fig. 5A,B). Interestingly, islet-labeled cells in r1, likely to be trochlear motor neurons (nIV; Fig. 1A), are unaffected in *dtr*^{te370a} mutants (Fig. 1B) as well as in *dtr*^{ts269} and *dtr*^{tm276b} mutants (data not shown). These results demonstrate that all cranial motor neurons (branchial and extraocular), with the possible exception of nIV, are severely reduced or absent in *detour* mutants.

Spinal motor neurons are unaffected in *detour* mutant embryos

Since *detour* mutants contained few or no cranial motor neurons, we wondered whether spinal motor neurons were also decreased in mutant embryos. However, both primary and secondary spinal motor neurons were generated normally in *dtr* mutants.

Primary spinal motor neurons were studied by examining *islet1* expression in the spinal cords of 21 HPF embryos obtained from a *dtr*^{te370a} /+ incross. In wild-type siblings, 2-3 ventral cells in every hemisegment express *isl1*, corresponding to the primary motor neurons (Fig. 2A; Appel et al., 1995). In *dtr*^{te370a} homozygotes, which are missing *isl1*-expressing cells in the hindbrain, the pattern of *isl1* expression in the ventral spinal cord is indistinguishable from that of wild-type embryos (Fig. 2B). This result demonstrates that the formation of primary spinal motor neurons is unaffected in *dtr*^{te370a} mutants. To further analyze primary and secondary motor neuron development in the *dtr* mutant spinal cord, embryos were processed for whole-mount immunohistochemistry with either an antibody against acetylated tubulin or the zn5 antibody. Both antibodies label a variety of neuronal cell types and their processes in the central and peripheral nervous systems of the zebrafish embryo (Chitnis and Kuwada, 1990; Trevarrow et al., 1990; Kanki et al., 1994; Beattie et al., 1997). In 24 HPF wild-type siblings, anti-tubulin antibody labels motor axons, mostly primary, exiting the spinal cord in every segment (Fig. 2C; Eisen et al., 1986; Myers et al., 1986). In 24 HPF *dtr*^{te370a} homozygotes, the primary motor axons extend normally (Fig. 2D), suggesting that primary spinal motor neuron development is unaffected in mutant embryos. In 48 HPF wild-type siblings, zn5 immunolabeling reveals a continuous column of cells in the ventral spinal cord, corresponding to the secondary motor neurons (Fig. 2E; Kanki et al., 1994; Beattie et al., 1997). The column of spinal motor neurons in *dtr*^{te370a} homozygotes is indistinguishable from that in wild-type siblings suggesting that the number of secondary motor neurons is unaffected (Fig. 2F). However, not all aspects of secondary motor neuron development are normal since motor axons often emerge from the spinal cord at ectopic sites (Fig. 2F, inset).

The differential effect of the *dtr* mutation on cranial versus spinal motor neurons is especially clear in the caudalmost hindbrain. At 30 HPF, the wild-type CNS at the level of somites 2 and 3 contains *isl1*-expressing cells corresponding to the caudalmost nX motor neurons and the rostralmost spinal motor neurons (Fig. 2G). In *dtr*^{te370a} homozygotes, the nX motor neurons are completely missing, but the spinal motor neurons are still present at the level of somites 2 and 3 (Fig. 2H), indicating that the CNS at this position can support motor neuron formation in mutant embryos. A transverse section of wild-type siblings at the level of somite 2 reveals spinal motor neurons immediately adjacent to the floor plate cells, and the nX neurons more dorsolaterally (Fig. 2I). By contrast, a transverse section at the same position in *dtr*^{te370a} mutant embryos reveals only spinal motor neurons (Fig. 2J), demonstrating the specific absence of nX neurons in the region of overlap.

To further verify that the *dtr* mutation had no effect on spinal motor neuron generation, spinal neurons were quantified in 36 HPF embryos labeled with the islet antibody (Table 1). In all three *dtr* alleles, the islet-labeled cells in the ventral spinal cord, representing mostly primary and secondary motor neurons (Korzh et al., 1993; Inoue et al., 1994; Appel et al., 1995), are normal in number. These observations demonstrate that spinal motor neuron generation is essentially normal in *dtr* mutants and that the pathways leading to motor neuron development in the spinal cord and the hindbrain are independent to some extent.

Table 1. Severity of motor neuron phenotypes in three *detour* (*dtr*) alleles*

	Number of islet antibody-labeled neurons	
Phenotype	In hindbrain (nV, nVI, nVII, nIX)‡	In ventral spinal cord (motor neurons)§
WT (te370a)	185.7±2.9	27.8±0.7
dtr ^{te370a}	2.3±2.5	26.3±2.6
Ratio	0.01	0.95
WT (ts269)	221.3±42.2	31.2±1.1
dtr ^{ts269}	19.3±1.5	30.6±4.0
Ratio	0.09	0.98
WT (tm276b)	213±8.9	26.4±2.8
dtr ^{tm276b}	28.7±1.2	27.9±0.4
Ratio	0.13	1.06

*n=3 embryos for each phenotype.

‡The number corresponds to the total number of labeled cells in rhombomeres 2-7.

§Labeled cells were counted on one side in the ventral spinal cord in 3 contiguous segments at the level of the tip of the yolk tube. The number shown corresponds to the number of cells per hemisegment.

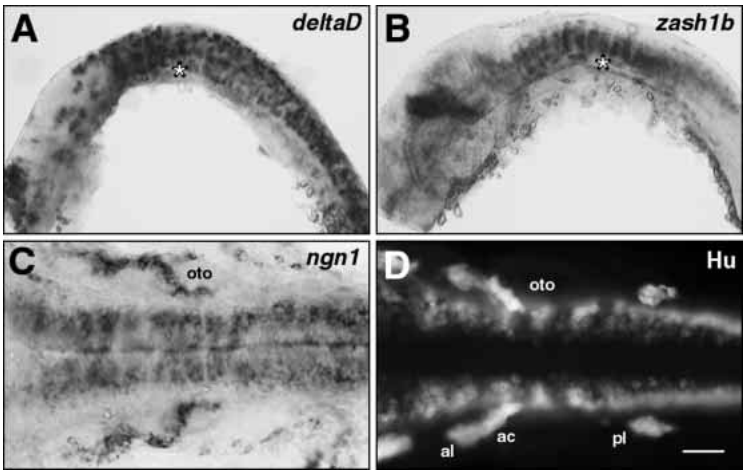

Fig. 3. Neurogenesis occurs normally in *dtr*^{te370a} mutants. (A,B) Lateral views, and (C,D) dorsal views, with rostral to the left, of whole-mounted embryos analyzed either by in situ hybridization (A-C) or by immunohistochemistry (D). The embryos used in each experiment were obtained from a *dtr*^{te370a}/+ incross, and all embryos exhibited similar labeling indicating that these markers were expressed in a similar fashion in wild-type and mutant embryos. A representative embryo is shown in each case. The asterisk (A,B) indicates the location of the otocyst. (A) At 18 HPF, when branchiomotor neurons are beginning to differentiate, *deltaD* is expressed extensively in the hindbrain at all dorsoventral levels. (B) At 24 HPF, *zash1b* is expressed strongly in the hindbrain, especially in dorsoventral columns of cells. (C) At 24 HPF, when branchiomotor neurons are normally still being generated, *neurogenin1* is expressed in a broad longitudinal column of cells located medially in the ventral hindbrain. (D) At 24 HPF, the Hu antibody labels cells within longitudinal columns located laterally within the ventral hindbrain. ac, acoustic ganglion; al, anterior lateral line ganglion; oto, otocyst; pl, posterior lateral line ganglion. Scale bar, (A,B) 100 μm, (C,D) 50 μm.

Hindbrain development is normal in *detour* mutants

Various explanations were considered for the hindbrain-specific motor neuron phenotype of *detour* mutants. First, cranial motor neurons may not be generated because hindbrain patterning in *detour* mutants may be abnormal. Second, there may be a failure of neurogenesis in the mutant hindbrain leading to the absence of not only cranial motor neurons, but also other neuronal types. Third, hindbrain motor neuron progenitors may die prior to differentiation in mutant embryos. Finally, motor neuron inducers like *shh* (Krauss et al., 1993) and *twhh* (Ekker et al., 1995) may not be expressed properly in *dtr* mutant embryos. Our data rule out these hypotheses.

To determine whether neurogenesis was affected in *dtr* mutant embryos, we examined the expression of *zash1b*, *deltaD*, *neurogenin1* and the *Hu* family, which are expressed extensively in the developing nervous system including the hindbrain (Allende and Weinberg, 1994; Henion et al., 1996; Kim et al., 1996; Blader et al., 1997; Haddon et al., 1998). No defects in the expression of these genes can be found in any 18 HPF or 24 HPF embryos collected from *dtr*^{te370a} /+ incrosses (Fig. 3; data not shown), suggesting that neurogenesis occurs normally in *dtr*^{te370a} homozygotes.

Hindbrain patterning in *dtr* mutants was studied by examining the expression of a number of genes that are normally expressed in specific subsets of rhombomeres. A receptor tyrosine kinase gene (*rtk1*/*EphA4*; Xu et al., 1995) is expressed in r1, r3 and r5 in both wild-type siblings and *dtr*^{te370a} mutants (Fig. 4A,B). Likewise, the transcription factor genes, *krox20* (Oxtoby and Jowett, 1993) and *valentino* (Moens et al., 1998), are expressed normally in *dtr*^{te370a} homozygotes, in r3 and r5, and in r5 and r6, respectively (data not shown). These results demonstrate that rhombomere formation is not affected in *dtr* mutants.

Since neurogenesis and hindbrain patterning appeared to be unaffected in *dtr* mutants, it is possible that the absence of branchiomotor neurons reflected a general failure of central and peripheral neurons to differentiate in the mutant hindbrain. To address this, whole-mount immunolabeling with the 3A10 (Hatta, 1992) and zn5 (Trevarrow et al., 1990) antibodies was used to examine the development of two other types of central neurons, the reticulospinal and hindbrain commissural neurons, respectively. In 36 HPF wild-type siblings, the 3A10 antibody labels the Mauthner reticulospinal neurons and their crossing axons (Fig. 4C; Hatta, 1992). In *dtr*^{te370a} homozygotes, the number, location and axonogenesis of Mauthner cells are unaffected (Fig. 4D). In 36 HPF wild-type siblings, the zn5 antibody labels hindbrain commissural neurons and their axons at the rhombomere boundaries (Fig. 4E; Trevarrow et al., 1990). In *dtr*^{te370a} mutants, the sizes of the commissural neuron clusters, dorsoventral position, rhombomeric location and axonogenesis are similar to those in wild-type siblings (Fig. 4F). Development of peripheral neurons at the level of the hindbrain was studied by examining the expression of *tag1*, which encodes an Ig superfamily adhesion molecule (Warren et al., 1999). In 20 HPF embryos obtained from a *dtr*^{te370a} /+ incross, *tag1*-expressing presumptive nVII neurons (Fig. 4G) are specifically missing in *dtr*^{te370a} mutants (Fig. 4H). However, *tag1* is expressed in a similar fashion in wild-type and mutant embryos in the neurons of the trigeminal, acoustic, and anterior and posterior lateral line sensory ganglia (Fig. 4G, H). These data demonstrate that the generation and differentiation of particular hindbrain central and peripheral neurons, with the exception of the cranial motor neurons, is normal in *dtr*^{te370a} homozygotes.

Since branchiomotor neurons were absent despite apparently normal neurogenesis in *dtr*^{te370a} mutants (Fig. 3), it is possible that presumptive branchiomotor neuron progenitors died prior to differentiation in mutant embryos. Because new branchiomotor neurons are normally added continually between 18 and 36 HPF (Chandrasekhar et al., 1997), we performed acridine orange labeling of live embryos (Brand et al., 1996) obtained from *dtr*^{te370a} /+ incrosses to examine cell death in the hindbrain. Qualitatively similar patterns of cell death are found in the hindbrains of all embryos at 15, 18 or 24 HPF (data not shown). Together, the absence of increased cell death and the absence of *nk2.2*-expressing cells in the *dtr*^{te370a} hindbrain (Fig. 1D) suggest that motor neuron progenitors are not generated in the mutant hindbrain.

Finally, we determined whether the expression of genes

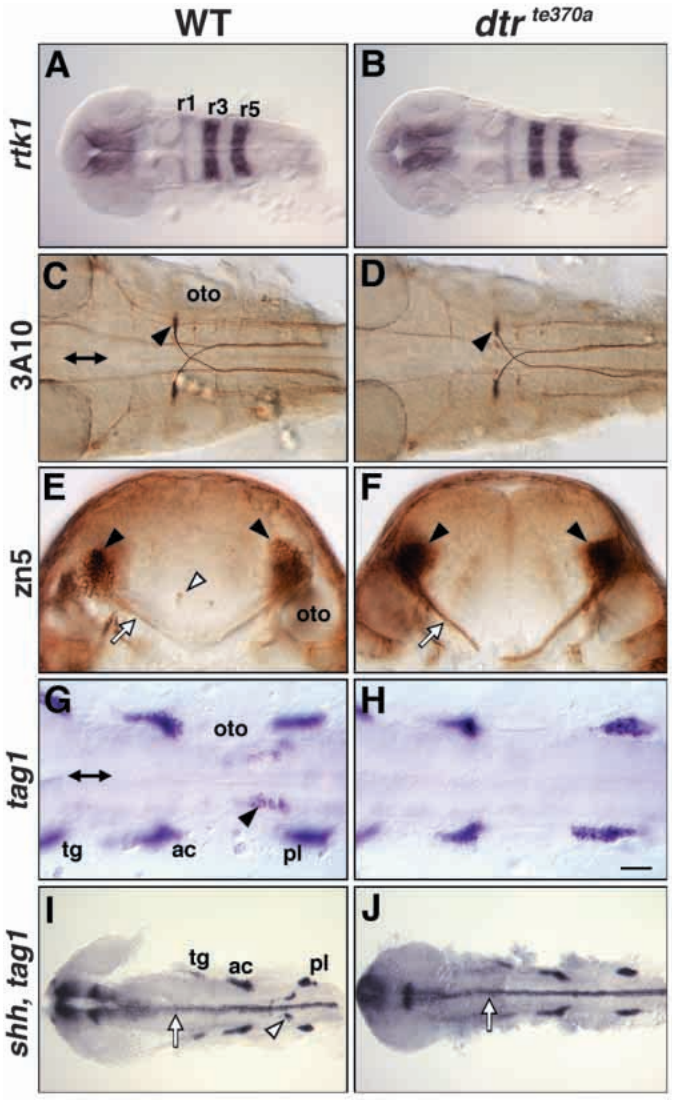

Fig. 4. Hindbrain patterning and differentiation of many neurons are normal in *dtr*^{te370a} mutants. All panels depict dorsal views (except E, F), with rostral to the left, of whole-mounted embryos analyzed either by immunohistochemistry (C-F) or by in situ hybridization (A,B,G-J). Double arrows (C,G) mark the midline. (A) In a 28 HPF wild-type sibling, *rtk1* (*EphA4*) is expressed in r1, r3 and r5. (B) In a *dtr*^{te370a} homozygote, *rtk1* expression is normal. (C) In a 36 HPF wild-type sibling, the 3A10 antibody labels the Mauthner cells (arrowhead) and their axons, which cross the midline and extend caudally into the spinal cord. (D) In a *dtr*^{te370a} homozygote, the Mauthner cells (arrowhead) and their axons are unaffected. (E) In a 36 HPF wild-type sibling, a transverse section at the level of rhombomere 5 reveals zn5 antibody-labeled commissural neurons (black arrowheads) and their axons (arrow), and the abducens motor neurons medially (white arrowhead). (F) In a *dtr*^{te370a} homozygote, the commissural neurons (arrowheads) and their axons (arrow) are unaffected, but the abducens motor neurons are missing. The difference in staining intensity between E and F results from the different thicknesses of the sections, which were done by hand. The break in the commissural axons in F is due to a crack in the tissue. (G) In a 20 HPF wild-type sibling, the *tag1*-expressing presumptive nVII neurons (arrowhead) span r5, r6 and r7. The prominent patches of labeling located laterally, rostral and caudal to the otocyst (oto), represent *tag1* expression in cranial sensory ganglia. (H) In a *dtr*^{te370a} homozygote, the presumptive nVII neurons are missing, but the cranial ganglia are unaffected. (I) In a 24 HPF wild-type sibling, *shh* is expressed in the floor plate (arrow) throughout the midbrain and hindbrain, and in the ventral neuroectoderm in the forebrain. *tag1* expression in the nVII neurons (arrowhead) and in the cranial ganglia is also evident. (J) In a *dtr*^{te370a} homozygote, the nVII neurons are missing. However, *shh* is expressed normally in the floor plate (arrow), and the cranial ganglia are unaffected. ac, acoustic ganglion; oto, otocyst; tg, trigeminal ganglion; pl, posterior lateral line ganglion. Scale bar, (A,B,I,J) 100 μm, (C,D) 50 μm, (E,F) 25 μm, (G,H) 40 μm.

encoding the branchiomotor neuron inducing signals, *shh* and *twhh* (Chandrasekhar et al., 1998), is reduced in the *dtr* mutant hindbrain. Embryos were processed for in situ hybridization with a mixed probe containing *tag1* (Warren et al., 1999) and *shh* (Krauss et al., 1993). In 24 HPF wild-type siblings, *tag1* is expressed in the nVII neurons (Fig. 4I; Warren et al., 1999), while *shh* is expressed in the floor plate cells in the hindbrain (Fig. 4I; Krauss et al., 1993; Chandrasekhar et al., 1998). In *dtr*^{te370a} homozygotes, the *tag1*-expressing cells corresponding to the nVII neurons are missing, but *shh* is expressed normally in the ventral neuroectoderm at all rostrocaudal levels, including the floor plate cells in the hindbrain (Fig. 4J). When 18 HPF embryos from a *dtr*^{te370a} /+ incross are processed for *twhh* in situ hybridization, all embryos express *twhh* normally in the floor plate cells (data not shown; Ekker et al., 1995), indicating that *twhh* expression is also unaffected in *dtr*^{te370a} homozygotes. These results demonstrate that the elimination of branchiomotor neurons in *dtr* mutants is not due to the absence or reduced expression of *shh* or *twhh*. However, they do not rule out that some downstream component of the Hh signaling pathway may be affected in *dtr* mutants.

Activation of the Hh signaling pathway does not generate branchiomotor neurons in *detour* mutant embryos

Since the above experiments did not reveal any defects in neurogenesis or neural patterning in the mutant hindbrain, we investigated whether *dtr* may specifically function in a pathway leading to cranial motor neuron induction. It was shown previously that Shh induces branchiomotor neurons (Chandrasekhar et al., 1998). Studies in vertebrates and invertebrates demonstrated that activation of the Hh signaling pathway, which is inhibited by protein kinase A (PKA), leads to the activation of the Gli family of transcription factors (Ingham, 1998). Although the *dtr* branchiomotor phenotype is not due to defective *shh* or *twhh* expression, it is still possible that *dtr* functions downstream in the Hh signaling pathway. Therefore, we tested whether activation of the Hh pathway by overexpressing either *shh* or a dominant negative protein kinase A (*dnPKA*) could restore branchiomotor neurons in *dtr* mutant embryos. No branchiomotor neurons were generated in *dtr* mutants following *shh* or *dnPKA* overexpression.

Synthetic full-length RNA encoding β-galactosidase (lacZ), or Shh, or dnPKA, was injected into 1- to 4-cell-stage embryos obtained from *dtr*^{te370a} /+ incrosses. To assay branchiomotor neuron induction, injected embryos were fixed at 30 HPF and processed for *isl1* in situ hybridization. In *lacZ*-injected wild-type embryos, the branchiomotor neurons are present only at their characteristic locations (Fig. 5A; Table 2). In *lacZ*-

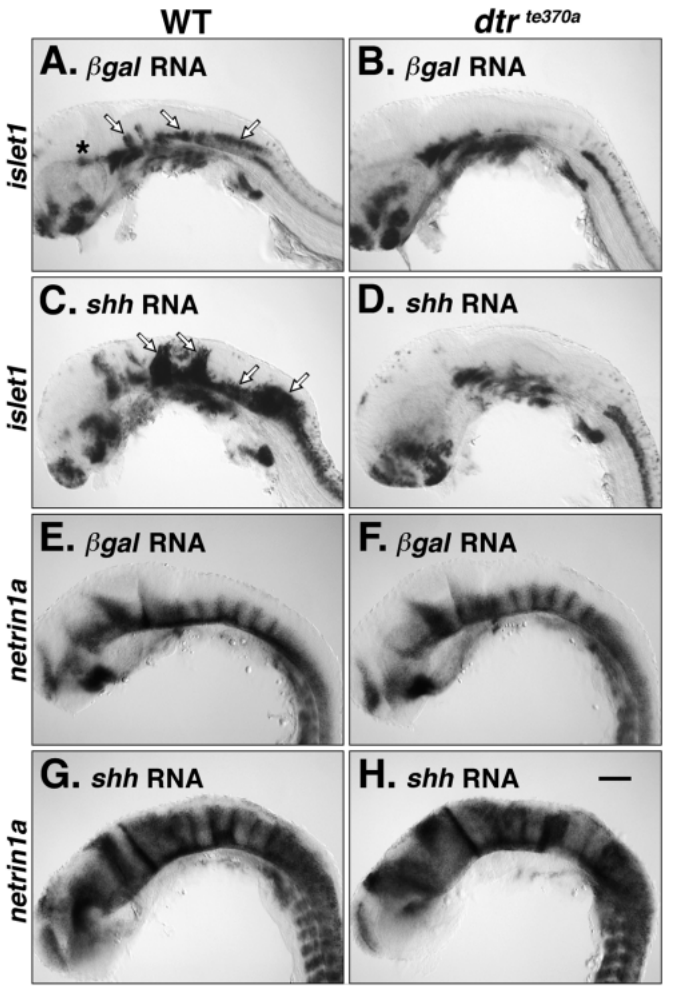

Fig. 5. *shh* overexpression does not induce branchiomotor neurons in *dtr*^{te370a} embryos. All panels show lateral views of the head, with rostral to the left and dorsal up, in embryos analyzed by *islet1* (*isl1*; A-D) or *netrin1a* (*net1a*; E-H) in situ hybridization. Embryos at the 1- to 4-cell stage were injected with full-length *lacZ* (A,B,E,F) or *shh* (C,D,G,H) RNA, and fixed at 30 HPF. (A) In a *lacZ*-injected wild-type sibling, the clusters of nV, nVII and nX neurons (arrows) are evident. The asterisk denotes putative oculomotor (nIII) neurons in the midbrain. (B) In a *lacZ*-injected *dtr*^{te370a} homozygote, the branchiomotor, as well as the putative oculomotor, neurons are missing. (C) In a *shh*-injected wild-type sibling, a large number of ectopic *isl1*-expressing cells are induced throughout the hindbrain (arrows), which have been shown to be branchiomotor neurons (Chandrasekhar et al., 1998). (D) In a *shh*-injected *dtr*^{te370a} homozygote, no *isl1*-expressing cells are induced in the hindbrain. (E) In a *lacZ*-injected wild-type sibling, *net1a* is expressed in the ventral half of the CNS throughout the embryo and in dorsoventral columns at rhombomere boundaries. (F) In a *lacZ*-injected *dtr*^{te370a} homozygote, *net1a* expression is normal. (G) In a *shh*-injected wild-type sibling, *net1a* expression in the CNS is expanded dorsally at all rostrocaudal levels. Supernumerary muscle pioneer cells expressing *net1a* are also evident in the somites, as described previously. (H) In a *shh*-injected *dtr*^{te370a} homozygote, *net1a* is ectopically expressed in the CNS and in the somites, in a similar fashion to injected wild-type embryos. Scale bar, 100 μm.

injected *dtr* mutants, no *isl1*-expressing cells are found in the hindbrain, indicating the absence of branchiomotor neurons (Fig. 5B; Table 2). Following *shh* or *dnPKA* injection, a large number of *isl1*-expressing cells are generated at ectopic locations in the hindbrain of many wild-type embryos (Fig. 5C; Table 2; Chandrasekhar et al., 1998). The pattern of ectopic cells generated is similar between *shh*- and *dnPKA*-injected embryos (data not shown), consistent with previous studies (Hammerschmidt et al., 1996; Ungar and Moon, 1996). In contrast to *shh* or *dnPKA* injection in wild-type embryos, no ectopic *isl1*-expressing cells are generated in the hindbrain following *shh* or *dnPKA* injection in *dtr* mutants (Fig. 5D; Table 2). Interestingly, *shh* or *dnPKA* overexpression generates ectopic spinal motor neurons in mutant embryos, as in wild-type embryos (compare Fig. 5C and D to A and B). Therefore, it appears that the Hh signaling pathway is intact in the mutant spinal cord. However, there was variability in the number of spinal motor neurons generated in these embryos because *isl1* expression is downregulated in the spinal cord by 30 HPF, the time point analyzed. To overcome this difficulty, some *dnPKA*-injected embryos were processed for in situ hybridization with *tag1*, which is strongly expressed in the spinal motor neurons at 30 HPF (Warren et al., 1999; Chandrasekhar et al., 1998). As observed for *isl1*, *dnPKA* overexpression leads to the generation of ectopic *tag1*-expressing cells in the hindbrain (branchiomotor neurons) in wild-type, but not in *dtr* mutant embryos (Table 2). Furthermore, consistent with the *isl1* data, *dnPKA* overexpression leads to the generation of ectopic spinal motor neurons in both wild-type and *dtr* mutant embryos indicating that the *dtr* mutation specifically blocks Hh-mediated induction of motor neurons in the hindbrain, but not in the spinal cord. These results, in conjunction with our previous finding that Shh and Twhh can induce branchiomotor and spinal motor neurons (Chandrasekhar et al., 1998), suggest that the *dtr* gene product may function downstream of PKA in the Hh-mediated pathway for branchiomotor neuron induction, but not spinal motor neuron induction.

Since *shh* and *dnPKA* RNA injection in *dtr* mutants failed to induce ectopic expression of motor neuron markers (*isl1* and *tag1*) in the hindbrain, we wondered whether expression of other *hh*-regulated genes was also blocked in the mutant hindbrain following *shh* or *dnPKA* injection. This was addressed by examining the expression of *netrin1a* (*net1a*), which encodes a putative growth cone guidance molecule (Lauderdale et al., 1997). *net1a* is normally expressed in the ventral CNS and is ectopically expressed at all rostrocaudal levels, including the hindbrain, following *shh* or *twhh* overexpression in wild-type embryos (Lauderdale et al., 1998). In *lacZ*-injected wild-type embryos, *net1a* is expressed normally in the muscle pioneer cells in the somites and in the ventral CNS, including in dorsoventral stripes at rhombomere boundaries in the hindbrain (Fig. 5E; Table 2; Lauderdale et al., 1997). In *lacZ*-injected *dtr* mutants, *net1a* expression is similar to that in wild-type siblings (Fig. 5F; Table 2). Thus the *dtr* mutation does not affect the normal Hh-mediated expression of *net1a*. In *shh*- or *dnPKA*-injected wild-type embryos, *net1a* expression is expanded dorsally at all rostrocaudal levels including the hindbrain and is found in supernumerary muscle pioneer cells in the somites (Fig. 5G; Table 2; Lauderdale et al., 1998). Interestingly, *net1a* is ectopically expressed in both the CNS and the somites of *shh*- or *dnPKA*-injected *dtr* mutant embryos, in a similar fashion to injected wild-type embryos (Fig. 5H; Table 2). This is in sharp

Table 2. Activation of the *shh* signaling pathway in *dtr* mutants does not generate ectopic branchiomotor neurons*

Injected RNA	Probe used	No. of expts	% embryos with ectopic expression in hindbrain‡		% embryos with ectopic expression in spinal cord‡	
			Wild-type§	Mutant§	Wild-type§	Mutant§
lacZ	*islet1*	2	0% (43)¶	0% (19)	not scored (n.s)	n.s.
shh	*islet1*	1	80% (20)	0% (8)	n.s.	n.s.
dnPKA	*islet1*	2	42% (50)	0% (15)	n.s.	n.s.
dnPKA	*tag1*	2‖	34% (79)	0% (16)	96% (79)	94% (16)
lacZ	*netrin1a*	1	0% (14)	0% (3)	0% (14)	0% (3)
shh	*netrin1a*	1	79% (19)	100% (6)	100% (19)	100% (6)
dnPKA	*netrin1a*	1‖	100% (20)	100% (8)	100% (20)	100% (8)

*The *dtr*^{te370a} allele was used for all injections, except where noted.

‡Any wild-type embryos containing *islet1*-, *tag1*-, or *netrin1a*-expressing cells outside their normal domains of expression were considered to have ectopic expression (Chandrasekhar et al., 1998; Lauderdale et al., 1998). For mutant embryos, the generation of any *islet1*- or *tag1*-expressing cells was scored as ectopic expression. Ectopic *netrin1a* expression in the somites, which is normally expressed only in the muscle pioneers (Lauderdale et al., 1997), was included in the analysis of the spinal cord.

§Mutant embryos were identified on the basis of their curly trunks (Brand et al., 1996). Using this criterion, mutant identification was extremely reliable (at least 99%; data not shown)

¶Number in parentheses represents number of embryos analyzed.

‖The *dtr*^{ts269} allele was used in one experiment.

contrast to *isl1* and *tag1* that are ectopically expressed in the spinal cord, but not in the hindbrain, of *shh*- or *dnPKA*-injected mutant embryos.

These results demonstrate that *shh*- or *dnPKA*-mediated upregulation of some neural genes like *net1a* is normal in the hindbrain of *dtr* mutants. However, since *shh*- or *dnPKA*-mediated induction of branchiomotor neurons is blocked in *dtr* mutants, the *detour* gene product in the hindbrain may normally function downstream of PKA in the Hh signaling pathway leading to the induction of branchiomotor neurons (and *isl1*, *tag1* and probably *nk2.2* expression), but not the activation of *net1a* expression.

detour can function cell autonomously to induce branchiomotor neurons

If *detour* encodes a downstream component of the Hh signaling pathway, it should function cell autonomously to induce branchiomotor neurons. We tested and verified this prediction through cell transplantation experiments.

When biotinylated dextran-labeled wild-type cells are transplanted into wild-type host embryos obtained from a *dtr*^{te370a} /+ incross, donor cells can differentiate into branchiomotor neurons. The transplanted cells express *tag1* and extend axons that follow trajectories characteristic of the nV and nVII neurons (Fig. 6A; 2 donor-derived nV neurons, 1 embryo; 12 donor-derived nVII neurons, 2 embryos; Chandrasekhar et al., 1997). When labeled wild-type cells are transplanted into *dtr*^{te370a} homozygotes (n=3 embryos), the donor cells are still able to differentiate into branchiomotor neurons (Fig. 6B-D). The donor wild-type cells differentiate into nVII (Fig. 6B; 3 donor-derived neurons, 1 embryo), nV (Fig. 6C; 3 donor-derived neurons, 1 embryo) and nIX (Fig. 6D; 1 donor-derived neuron) neurons. Some of these donor-derived neurons express *tag1* or *isl1*. These observations

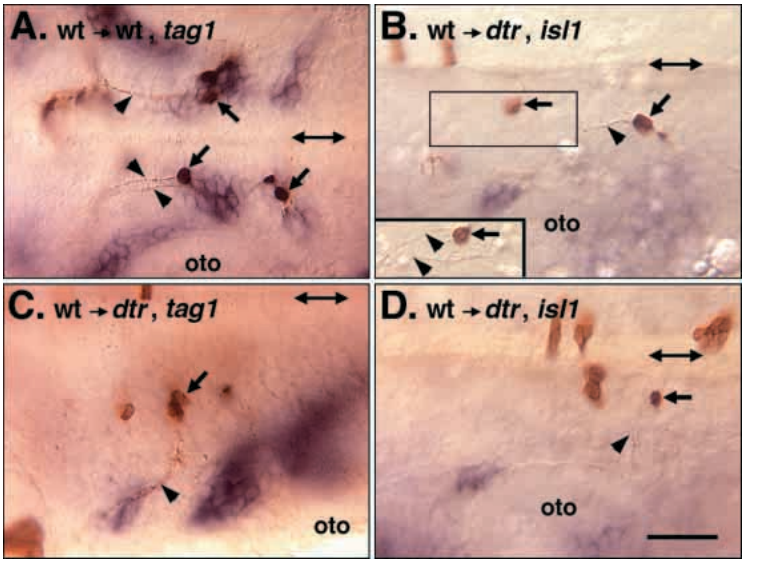

Fig. 6. *detour* functions cell autonomously in branchiomotor neuron induction. All panels show dorsal views of the hindbrain, with rostral to the left, in whole-mounted embryos processed for *tag1* (A,C) or *islet1* (B,D) in situ hybridization. Double arrows in each panel mark the midline. Donor wild-type cells and their axons are labeled brown. Embryos in B-D were identified as mutant host embryos because their hindbrains did not contain the characteristic clusters of branchiomotor neurons expressing *tag1* (C) or *islet1* (B,D). (A) When wild-type cells are transplanted into a wild-type host embryo, the donor cells within the nVII neuronal clusters (arrows) extend axons (arrowheads) anteriorly that exit the hindbrain in r4 into the hyoid arch, as described previously (Chandrasekhar et al., 1997). (B) In a *dtr* host embryo, donor wild-type cells (arrows) in r4 and r6 extend axons (arrowhead) anteriorly. The boxed area is depicted in a different focal plane (inset) to show that the axons (arrowheads) turn laterally exiting the hindbrain in r4 into the hyoid arch, in a manner characteristic of nVII axons. (C) In another *dtr* host embryo, donor wild-type cells (arrow) in r2 extend axons (arrowhead) laterally that exit the hindbrain in r2 into the mandibular arch, in a manner characteristic of nV axons. (D) In a third *dtr* host embryo, a donor wild-type cell (arrow) in r6 extends an axon (arrowhead) laterally that exits the hindbrain in r6, in a manner characteristic of nIX axons. oto, otocyst. Scale bar, 40 μm.

demonstrate that *dtr* can function cell autonomously in branchiomotor neuron induction and lend support to its proposed role as a downstream component in the Hh signaling pathway.

DISCUSSION

The *detour* mutation leads to the loss of cranial motor neurons

The *detour* mutation was originally identified due to defects in midline development and retinotectal axon guidance (Brand et al., 1996; Karlstrom et al., 1996). This report demonstrates that, in addition to these defects, the *dtr* mutation leads to the drastic reduction or absence of cranial motor neurons. All branchiomotor neurons (nV, nVII, nIX and nX), which innervate jaw and gill muscles (Chandrasekhar et al., 1997), are missing in dtr^{te370a} mutants. The abducens motor neurons (nVI), which innervate one group of extraocular muscles (Trevarrow et al., 1990; Gilland and Baker, 1993), are also missing in dtr^{te370a} homozygotes. Our data further suggest that the oculomotor neurons (nIII) in the midbrain may also be absent, while the trochlear motor neurons (nIV) may be unaffected in dtr^{te370a} mutants. However, our identification of these two neuronal populations (nIII, nIV), which also innervate extraocular muscles, is tentative.

In light of these results, it is possible that specific populations of cells are missing from the ventral CNS at all rostrocaudal levels in *dtr* mutants. Consistent with this, lateral floor plate cells are missing in the spinal cord of *dtr* mutants (J. Odenthal, F. J. M. van Eeden, C. Fricke, P. Haffter, P. W. Ingham and C. Nüsslein-Volhard, unpublished data). It will be of interest to determine whether specific populations of ventral forebrain cells are eliminated in *dtr* mutants. These putative missing cells may normally provide essential guidance cues for the post optic commissural and retinotectal axons, since both groups of axons fail to cross the midline in *dtr* mutants (Karlstrom et al., 1996).

Despite the absence of most cranial motor neurons in dtr^{te370a} mutants, commissural and reticulospinal neurons are generated normally in the mutant hindbrain. Surprisingly, motor neurons in the spinal cord, which like the branchiomotor neurons express *isl1* and *tag1* (Chandrasekhar et al., 1997), are also generated in normal numbers in mutant embryos. Thus there is a very selective defect in neuronal specification in *dtr* mutants. The mutation affects the generation of motor neurons only in the hindbrain, and possibly the midbrain, but not in the spinal cord.

detour and the Hh signaling pathway

Given the unique neuronal phenotype of *detour* embryos, we initially hypothesized that the *dtr* mutation may affect early events in brain development such as neurogenesis or hindbrain formation. However, the normal expression of marker genes like *zash1b*, *deltaD*, *neurogenin1*, *Hu* family, *krox20*, *valentino* and *rtk1* suggests that neurogenesis and hindbrain patterning are unaffected in *dtr* mutant embryos. It is possible that progenitor cells are generated but fail to differentiate into branchiomotor neurons and therefore die in *dtr* mutants. However, this appears unlikely for a number of reasons. First, increased cell death is not observed in the *dtr* hindbrain. Second, a putative branchiomotor neuron progenitor gene *nk2.2* is not expressed in dtr^{te370a} mutants. Finally, the absence of *nk2.2* expression in the ventral CNS of dtr^{te370a} homozygotes is not accompanied by an obvious reduction in the size of the CNS in the mutant hindbrain suggesting that there is no significant loss of ventral CNS tissue. Given these observations, it is possible that branchiomotor progenitor cells in *dtr* mutants transfate into other hindbrain neuronal types, thus increasing their numbers. However, there is no obvious increase in other major neuronal types in the hindbrain: the commissural and reticulospinal neurons appear normal in *dtr* mutants. Since not all cell types were assayed, it is not known whether other cell types such as glia increase in number, nor whether branchiomotor neuron progenitors fail to express *nk2.2* but still persist in the *dtr* hindbrain.

In the absence of evidence for general defects in neurogenesis or hindbrain patterning in *dtr* mutants, we tested the hypothesis that *dtr* encodes a downstream component of the Hh signaling pathway. Numerous observations are consistent with this proposed role for *dtr*. First, we showed previously that Shh induces branchiomotor neurons and that loss of *shh* expression leads to loss of branchiomotor neurons (Chandrasekhar et al., 1998). Therefore, the *dtr* mutation, which blocks branchiomotor neuron generation, could interfere with the Hh signaling pathway. Second, lateral floor plate cells are absent in both *dtr* (J. Odenthal, F. J. M. van Eeden, C. Fricke, P. Haffter, P. W. Ingham and C. Nüsslein-Volhard, unpublished data) and *sonic you* (*syu*) mutants (Schauerte et al., 1998). Since *syu* encodes Shh, the lateral floor plate phenotypes of *syu* and *dtr* mutants suggest that these two genes act in a common pathway. Therefore, *dtr* may function downstream of the Shh signal (ie., *syu*). Third, since the expression of motor neuron inducers, *shh* (Krauss et al., 1993) and *twhh* (Ekker et al., 1995), in the axial tissues is unaffected in *dtr* mutants (this report; see also Brand et al., 1996), the loss of branchiomotor neurons in *dtr* mutants suggests that *dtr* might encode a downstream component of the Hh signaling cascade. Fourth, activation of the Hh signaling pathway by overexpression of *shh* (Krauss et al., 1993; Ekker et al., 1995; Chandrasekhar et al., 1998) or *dnPKA* (Ungar and Moon, 1996; Hammerschmidt et al., 1996) does not induce branchiomotor neurons in *dtr* mutants, even though ectopic spinal motor neurons are induced in overexpressing mutant embryos (this report). This is consistent with *dtr* encoding a downstream component of the Hh pathway and indicates that the response to Hh signaling is blocked in the *dtr* hindbrain, but not in the spinal cord. The result also suggests that *dtr* may be required for Hh-mediated induction of motor neurons only in the hindbrain. Finally, mosaic analysis shows that *dtr* can function cell autonomously to induce branchiomotor neurons (this report). Taken together, these results suggest that *detour* encodes a component of the Hh signaling cascade and functions downstream of protein kinase A in this pathway.

Some aspects of the *dtr* mutant phenotype are also found in other mutants such as *chameleon* (*con*), *iguana* (*igu*), *you-too* (*yot*) and *syu* (Brand et al., 1996; Karlstrom et al., 1996; Schauerte et al., 1998). Mutations at these loci also lead to defective retinotectal pathfinding and to the absence of lateral floor plate cells (Brand et al., 1996; Karlstrom et al., 1996; Schauerte et al., 1998; J. Odenthal, F. J. M. van Eeden, C. Fricke, P. Haffter, P. W. Ingham and C. Nüsslein-Volhard,

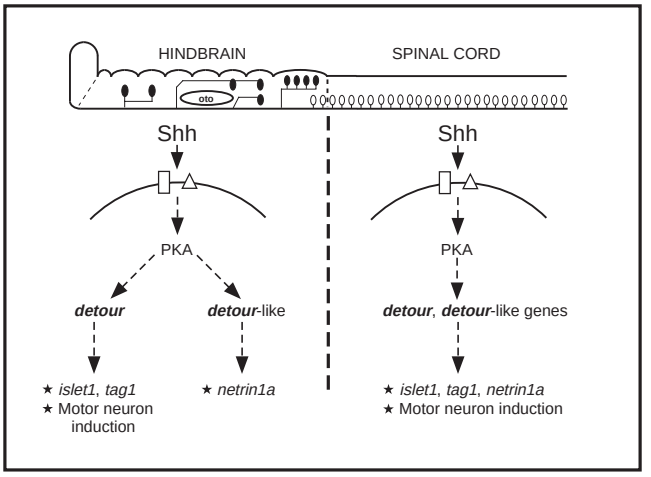

Fig. 7. Schematic of model for *detour* functioning as a downstream component in the hedgehog (Shh) signaling pathway. The neural tube comprising the hindbrain and spinal cord is shown in a lateral view with rostral to the left. The branchiomotor neurons in the hindbrain are depicted as filled ovals and the spinal motor neurons are depicted as unfilled ovals. The dashed arrows represent putative events comprising the signaling cascade. Within the hindbrain domain, the signaling cascade initiated by Shh branches downstream of protein kinase A (PKA) into *detour*-dependent and *detour*-independent pathways. The *detour*-dependent pathway leads to the upregulation of *islet1* and *tag1*, and to motor neuron induction. The *detour*-independent pathway may be mediated by putative *detour*-like genes and leads to the upregulation of *netrin1a* (and probably *neurogenin1*). Within the spinal cord domain, the signaling cascade downstream of PKA is linear, with *detour* and putative *detour*-like genes functioning in a redundant manner to mediate the effects of Shh on the expression of *islet1*, *tag1* and *netrin1a*, and on motor neuron induction.

unpublished data). Since *syu* encodes Shh, these results suggest that the *con*, *igu* and *yot* loci may also encode components of the Hh pathway. Therefore, it will be of interest to examine the branchiomotor neuron phenotypes of *con*, *igu* and *yot* mutants.

detour function is essential for a subset of Hh-mediated events in the hindbrain

We show that motor neuron development is affected in the *dtr* hindbrain, but not in the *dtr* spinal cord, and that activation of the Hh pathway induces spinal but not hindbrain motor neurons in *dtr* mutants. Therefore, it is possible that *dtr* encodes a Hh pathway component that is required for all Hh-regulated events only in the hindbrain but not in the spinal cord, while a second *dtr*-like gene product is required for all Hh-regulated events only in the spinal cord but not in the hindbrain. However, this hypothesis appears unlikely for two reasons. First, expression of the Hh-regulated genes, *net1a* (Lauderdale et al., 1997, 1998) and *neurogenin1* (Blader et al., 1997), is normal in the *dtr* mutant hindbrain. This suggests that Hh-induced *net1a* and *neurogenin1* expression in the hindbrain do not require *dtr* function. Second, *net1a*, unlike *isl1* and *tag1*, is expressed ectopically in a similar fashion in the hindbrains of both wild-type and *dtr* mutant embryos following *shh* or *dnPKA* overexpression. This result again indicates that Hh-mediated induction of *net1a* expression in the hindbrain is independent of *dtr* function. Therefore, Hh-mediated inductions of branchiomotor neurons and *net1a* expression in the hindbrain do not lie in the same linear pathway; some signaling events leading to branchiomotor neuron induction (and *nk2.2*, *tag1* and *isl1* gene activation) are normally uncoupled from those leading to *net1a* gene activation in the hindbrain (Fig. 7). This dissociation must occur downstream of PKA because *dnPKA* overexpression in wild-type embryos can induce both ectopic branchiomotor neurons and ectopic *net1a* expression.

The *detour* mutant phenotype also shows that some signaling events leading to motor neuron induction in the hindbrain are normally uncoupled from those leading to motor neuron induction in the spinal cord, since spinal motor neuron induction (and *tag1* and *isl1* gene expression) is completely normal in the *dtr* mutant spinal cord. Therefore, while it appears unlikely that exactly two *dtr*-like genes function in non-overlapping domains, namely the hindbrain and spinal cord, to regulate all Hh-induced events (see above), it is possible that two or more *dtr*-like genes may be expressed in overlapping domains and regulate Hh-mediated induction of subsets of events in the hindbrain or spinal cord (Fig. 7). Thus the Hh signaling cascade, which controls dorsoventral patterning of the CNS (Tanabe and Jessell, 1996), may be branched and may vary in complexity along the rostrocaudal axis of the CNS. This putative complexity and heterogeneity of Hh pathways, in combination with a requirement for sequential Hh signaling (Ericson et al., 1996), and a Hh response threshold gradient (Ericson et al., 1997), could potentially generate a large number of different cell types within the developing CNS.

We thank Mary Halloran and Wataru Shoji for critically reading the manuscript, Bruce Appel, Patrick Blader, Ajay Chitnis, Stephen C. Ekker, Paul Henion, Trevor Jowett, Rolf Karlstrom, Cathy Krull, Jim Lauderdale, Cecilia Moens, Tatjana Piotrowski, Bill Trevarrow, David Turner, Anne Ungar, Jim Warren and Eric Weinberg for expression plasmids, probes, and antibodies, Yong Huang for help with RNA injection, and Fengyun Su and Yumei Lai for fish care. A. C. is grateful to Christiane Nüsslein-Volhard for encouragement and support, and for providing laboratory facilities to conduct some of the experiments described here. The 39.4D5 islet and the 3A10 antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, under contract N01-HD-7-3263 from the NICHD. This work was supported by a Human Frontiers Science Programme Short-term Fellowship and start-up funds from the University of Missouri (A. C.), and by grants from the Muscular Dystrophy Association and NINDS NS24848 (J. Y. K.).

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