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

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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 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<sup>pec370a</sup>, dtr<sup>pec269</sup> and dtr<sup>isa276m</sup>) 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<sup>pec370a</sup> 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 (TTTS-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 (Trevorrow 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 FITC-conjugated secondary antibody (Jackson Immunchemicals) 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<sup>pec370a</sup> /+ incross. Transplanted host embryos containing fluorescent cells in the hindbrain were fixed at 30–36 HPF and processed for tag1 or is1l 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 diaminobenzidine/H<sub>2</sub>O<sub>2</sub> to

![Fig. 1](image-url) Cranial motor neurons are missing in dtr<sup>pec370a</sup> 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<sup>pec370a</sup> 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<sup>pec370a</sup> homozygote, nk2.2 expression is missing throughout the hindbrain 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<sup>pec370a</sup> 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.

**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 isil 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 dtrte370a 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 dtrte370a mutants.

Since branchiomotor neurons, which innervate the pharyngeal arches, are missing in dtrte370a 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 dtrte370a homozygotes, the nVI neurons are missing (Fig. 1F). The nVI motor neurons are also greatly reduced in dtrte269 and dtrtm276b mutant embryos (data not shown). Examination of 30 HPF embryos processed for islet1 in situ hybridization revealed that some islet1-expressing cells in the midbrain, tentatively identified as oculomotor neurons (nIII), are missing in dtrte370a homozygotes (see Fig. 5A,B). Interestingly, islet-labeled cells in r1, likely to be trochlear motor neurons (nIV; Fig. 1A), are unaffected in dtrte370a mutants (Fig. 1B) as well as in dtrte269 and dtrtm276b 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 dtrte370a/+ incross. In wild-type siblings, 2-3 ventral cells in every hemisegment express islet1, corresponding to the primary motor neurons (Fig. 2A; Appel et al., 1995). In dtrte370a homozygotes, which are missing islet1-expressing cells in the hindbrain, the pattern of islet1 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 dtrte370a mutants. To further analyze primary and secondary motor neuron development in the detour 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 both 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 dtrpe370a 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 dtrpe370a 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 detour 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 isll-expressing cells corresponding to the caudalmost nX motor neurons and the rostralmost spinal motor neurons (Fig. 2G). In dtrpe370a 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 dtrpe370a 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 detour 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 detour alleles, the islet-labeled cells in the ventral spinal cord, representing mostly primary and secondary motor neurons (Korzhe 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 detour mutants and that the pathways leading to motor neuron development in the spinal cord and the hindbrain are independent to some extent.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Number of islet antibody-labeled neurons in hindbrain (nV, nVI, nVII, nIX)</th>
<th>Number of islet antibody-labeled neurons in ventral spinal cord (motor neurons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (te370a)</td>
<td>185 ±2.9</td>
<td>27.6 ±0.7</td>
</tr>
<tr>
<td>dtrte370a</td>
<td>2.3 ±1.7</td>
<td>26.3 ±2.6</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>WT (ts269)</td>
<td>221 ±24.2</td>
<td>31.2 ±1.1</td>
</tr>
<tr>
<td>dtrtm276b</td>
<td>19.3 ±1.5</td>
<td>30.4 ±3.0</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.09</td>
<td>0.98</td>
</tr>
<tr>
<td>WT (tm276b)</td>
<td>212 ±8.9</td>
<td>26.4 ±2.8</td>
</tr>
<tr>
<td>dtrtm276b</td>
<td>28.7 ±1.2</td>
<td>27.9 ±1.4</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.13</td>
<td>1.06</td>
</tr>
</tbody>
</table>

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.
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 twi (Ekker et al., 1995) may not be expressed properly in detour mutant embryos. Our data rule out these hypotheses.

To determine whether neurogenesis was affected in detour 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 detour+/+ incrosses (Fig. 3; data not shown), suggesting that neurogenesis occurs normally in detour homozygotes.

Hindbrain patterning in detour 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 detour mutants (Fig. 4A,B). Likewise, the transcription factor genes, krox20 (Oxtoby and Jowett, 1993) and valentino (Moens et al., 1998), are expressed normally in detour homozygotes, in r3 and r5, and in r5 and r6, respectively (data not shown). These results demonstrate that rhombomere formation is not affected in detour mutants.

Since neurogenesis and hindbrain patterning appeared to be unaffected in detour 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., 1999) 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 detour 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 detour 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 tagl, which encodes an Ig superfamily adhesion molecule (Warren et al., 1999). In 20 HPF embryos obtained from a detour+/+ incross, tagl-expressing presumptive nVII neurons (Fig. 4G) are specifically missing in detour mutants (Fig. 4H). However, tagl 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 detour homozygotes.

Since branchiomotor neurons were absent despite apparently normal neurogenesis in detour 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 detour+/+ 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 detour hindbrain (Fig. 1D) suggest that motor neuron progenitors are not generated in the mutant hindbrain.

Finally, we determined whether the expression of genes...
Hindbrain patterning and differentiation of many neurons are normal in dtr<sup>te370a</sup> 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 24 HPF wild-type sibling, tkr1 (EphA4) is expressed in r1, r3 and r5. (B) In a dtr<sup>te370a</sup> homozygote, tkr1 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<sup>te370a</sup> 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<sup>te370a</sup> 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-labeled presumptive nVII neurons (arrowhead) span r3, 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<sup>te370a</sup> 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<sup>te370a</sup> 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.

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<sup>te370a</sup> l<sup>+</sup> 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-
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 tagl, 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 tagl-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 tagl) 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 contrast to previously described data, indicating a role for Detour in the regulation of expression of Hh-regulated genes in the developing nervous system.
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<sup>ts269</sup>/+* 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<sup>ts269</sup>* 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

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

<table>
<thead>
<tr>
<th>Injected RNA</th>
<th>Probe used</th>
<th>No. of expts</th>
<th>% embryos with ectopic expression in hindbrain‡</th>
<th>% embryos with ectopic expression in spinal cord‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lacZ</em></td>
<td><em>islet1</em></td>
<td>2</td>
<td>0% (±43)†</td>
<td>0% (19)</td>
</tr>
<tr>
<td><em>shh</em></td>
<td><em>islet1</em></td>
<td>1</td>
<td>80% (20)‡</td>
<td>0% (8)</td>
</tr>
<tr>
<td><em>dnPKA</em></td>
<td><em>tag1</em></td>
<td>2</td>
<td>42% (50)‡</td>
<td>0% (15)</td>
</tr>
<tr>
<td><em>lacZ</em></td>
<td><em>netrin1a</em></td>
<td>1</td>
<td>34% (79)‡</td>
<td>0% (16)</td>
</tr>
<tr>
<td><em>shh</em></td>
<td><em>netrin1a</em></td>
<td>1</td>
<td>0% (14)‡</td>
<td>0% (3)</td>
</tr>
<tr>
<td><em>dnPKA</em></td>
<td><em>netrin1a</em></td>
<td>1</td>
<td>79% (19)‡</td>
<td>100% (6)</td>
</tr>
</tbody>
</table>

*The *dtr<sup>ts269a</sup>* allele was used for all injections, except where noted.
‡Any wild-type embryos containing *islet1*- or *tag1*- or *netrin1a*-expressing cells outside their normal domains of expression were considered to have ectopic expression (Chandrasekhar et al., 1997, 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<sup>ts206</sup>* allele was used in one experiment.

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**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 neurons. (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<sup>te370a</sup> mutants. The abducens motor neurons (nVI), which innervate one group of extraocular muscles (Trevorrow et al., 1990; Gilland and Baker, 1993), are also missing in dtr<sup>te370a</sup> 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<sup>te370a</sup> 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<sup>te370a</sup> 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 isil and tagl (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 rtkl 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<sup>te370a</sup> mutants. Finally, the absence of nk2.2 expression in the ventral CNS of dtr<sup>te370a</sup> 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 translocate 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 (i.e., 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, 1996).
branchiomotor neurons and \textit{net1a} expression in the hindbrain do not lie in the same linear pathway; some signaling events leading to branchiomotor neuron induction (and \textit{nk2.2}, \textit{tag1} and \textit{isl1} gene activation) are normally uncoupled from those leading to \textit{net1a} gene activation in the hindbrain (Fig. 7). This dissociation must occur downstream of PKA because \textit{dnPKA} overexpression in wild-type embryos can induce both ectopic branchiomotor neurons and ectopic \textit{net1a} expression.

The \textit{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 \textit{tag1} and \textit{isl1} gene expression) is completely normal in the \textit{dtr} mutant spinal cord. Therefore, while it appears unlikely that exactly two \textit{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 \textit{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.

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