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

Anand Chandrasekhar\(^1,\ast\), Heike E. Schauerte\(^2\), Pascal Haffter\(^2,\dagger\) and John Y. Kuwada\(^1\)

\(^1\)Department of Biology, University of Michigan, 830 N. University, Ann Arbor, MI 48109-1048, USA
\(^2\)Max-Planck-Institut für Entwicklungsbiologie, Spemannstrasse 35/III, Tübingen, 72076, Germany

\(\ast\)Author for correspondence: Division of Biological Sciences, 205 Lefevre Hall, University of Missouri, Columbia, MO 65211, USA (e-mail: anandc@missouri.edu)

\(\dagger\)We dedicate this paper to the memory of Pascal Haffter, a dear friend and colleague

**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 pigment formation. Embryos were transferred to water containing 0.2 mM phenylthiourea between 18 and 22 HPF to prevent pigment formation (Burrill and Easter, 1994).

The mutant strains used (dtr<sup>pc370a</sup>, dtr<sup>ts269</sup> and dtr<sup>tm276b</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>pc370a</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 (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 FITC-conjugated secondary antibody (Jackson Immunoreagents) 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>pc370a</sup>/+ incross. Transplanted host embryos containing fluorescent cells in the hindbrain were fixed at 30-36 HPF and processed for tagl and isl1 in situ hybridization to identify mutant embryos. For Hu immunolabeling, a FITC-conjugated secondary antibody (Jackson Immunoreagents) 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.

**Fig. 1.** Cranial motor neurons are missing in dtr<sup>pc370a</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>pc370a</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>pc370a</sup> 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 r3 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>pc370a</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, 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).
revealed that the nk2.2 and the motor neuron-specific isl1 (Inoue et al., 1994; Appel et al., 1995) genes are coexpressed in branchiomotor neurons (data not shown). In dtr<sup>te370a</sup> 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<sup>te370a</sup> mutants.

Since branchiomotor neurons, which innervate the pharyngeal arches, are missing in dtr<sup>te370a</sup> 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 (Trevorrow 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<sup>te370a</sup> homozygotes, the nVI neurons are missing (Fig. 1F). The nVI motor neurons are also greatly reduced in dtr<sup>ts269</sup> and dtr<sup>tm276b</sup> mutant embryos (data not shown). Examination of 30 HPF embryos processed for isl1 in situ hybridization revealed that some isl1-expressing cells in the midbrain, tentatively identified as oculomotor neurons (nIII), are missing in dtr<sup>te370a</sup> homozygotes (see Fig. 5A,B). Interestingly, islet-labeled cells in r1, likely to be trochlear motor neurons (nIV; Fig. 1A), are unaffected in dtr<sup>te370a</sup> mutants (Fig. 1B) as well as in dtr<sup>ts269</sup> and dtr<sup>tm276b</sup> 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 isl1 expression in the spinal cords of 21 HPF embryos obtained from a dtr<sup>te370a</sup>/+ 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<sup>te370a</sup> 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<sup>te370a</sup> 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; Trevorrow 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<sup>te370a</sup> 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<sup>te370a</sup> 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<sup>te370a</sup> 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<sup>te370a</sup> 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>
<thead>
<tr>
<th>Phenotype</th>
<th>Number of islet antibody-labeled neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (te370a)</td>
<td>185.7±2.9 (nV, nVI, nVII, nX)‡</td>
</tr>
<tr>
<td>dtr&lt;sup&gt;te370a&lt;/sup&gt;</td>
<td>2.3±2.5</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.01</td>
</tr>
<tr>
<td>WT (ts269)</td>
<td>221.3±42.2</td>
</tr>
<tr>
<td>dtr&lt;sup&gt;ts269&lt;/sup&gt;</td>
<td>19.4±1.5</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.09</td>
</tr>
<tr>
<td>WT (tm276b)</td>
<td>212.8±2.9</td>
</tr>
<tr>
<td>dtr&lt;sup&gt;tm276b&lt;/sup&gt;</td>
<td>28.7±2.2</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.13</td>
</tr>
</tbody>
</table>

§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.

*†n=3 embryos for each phenotype.
‡The number corresponds to the total number of labeled cells in rhombomeres 2-7.
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 dtrte370a/+ incrosses (Fig. 3; data not shown), suggesting that neurogenesis occurs normally in dtrte370a 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 (rtkI/EphA4; Xu et al., 1995) is expressed in r1, r3 and r5 in both wild-type siblings and dtrte370a mutants (Fig. 4A,B). Likewise, the transcription factor genes, knox20 (Oxtoby and Jowett, 1993) and valentineto (Moenes et al., 1998), are expressed normally in dtrte370a 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 branchiomatic 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 dtrte370a 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 dtrte370a mutants, the sizes of the commissural neuron clusters, dorsalventral 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 dtrte370a/+ incross, tagl-expressing presumptive nVII neurons (Fig. 4G) are specifically missing in dtrte370a 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 dtrte370a homozygotes.

Since branchiomatic neurons were absent despite apparently normal neurogenesis in dtrte370a mutants (Fig. 3), it is possible that presumptive branchiomatic neuron progenitors died prior to differentiation in mutant embryos. Because new branchiomatic 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 dtrte370a/+ 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 dtrte370a hindbrain (Fig. 1D) suggest that motor neuron progenitors are not generated in the mutant hindbrain.

Finally, we determined whether the expression of genes...
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 tagl (Warren et al., 1999) and shh (Krauss et al., 1993). In 24 HPF wild-type siblings, tagl 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 dtrte370a homozygotes, the tagl-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. 4I). When 18 HPF embryos from a dtrte370a +/ 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 dtrte370a 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 dtrte370a +/+ 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 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 contrast 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 isl1 and tagl 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 detox 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, tagl and probably nk2.2 expression), but not the activation of net1a expression.

**detox can function cell autonomously to induce branchiomotor neurons**

If detox 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 dtrts269ts/+ incross, donor cells can differentiate into branchiomotor neurons. The transplanted cells express tagl 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 dtrts269ts homozygotes (n=3 embryos), the donor cells are still able to differentiate into branchiomotor neurons (Fig. 6D). 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 tagl or isl1. These observations

![Fig. 6. detox 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 tagl (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 tagl (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.](image-url)
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* 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* mutants. 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* 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* 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 *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 *rkl* 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* mutants. Finally, the absence of *nk2.2* expression in the ventral CNS of *dtr* homoyzogotes 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 (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; Eckert 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 Shh 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,

Role of *detour* in motor neuron induction
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, Kathy Krull, Jim Lauderdale, Cecilia Moens, Tatjana Piotrowski, Bill Trevorrow, David Turner, Anne Ungar, Jim Warren and Eric Weinberg for expression plasmids, probes, and antibodies, Yong Huang for help with RNA injection, and Fengyu 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.).

### REFERENCES


**Barth, K. A. and Wilson, S. W.** (1995). Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone

---

**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 isll 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

---

**REFERENCES**


Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone
of neuronal differentiation in the embryonic forebrain. Development 121, 1755-1768.


