

Independent assignment of antero-posterior and dorso-ventral positional values in the developing chick hindbrain

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Background: Cell patterning in the developing central nervous system seems to involve a coordinate system of positional information, in which specific fates are assigned to multipotent precursor cells by positional signals acting on the antero-posterior and dorso-ventral axes of the neural tube. Before neurons differentiate in the hindbrain, it becomes subdivided antero-posteriorly into a series of developmental compartments, the rhombomeres. When the rhombomeres are delineated from each other by interfaces at which cell mixing is transiently restricted, they are determined for expression of specific selector *Hox* genes that may encode aspects of their individual identity. To assess whether the phenotypic identities of the rhombomeres are also determined at this stage, we have analyzed the capacity of individual rhombomeres to realize specific neuronal fates when grafted heterotopically along both antero-posterior and dorso-ventral axes.

Results: When rhombomere 4 (r4) is grafted unilaterally to the r2 position, both facial motor neurons and contralateral vestibulo-acoustic efferent neurons differentiate, as

normal, in the ventral region of the graft. These aspects of phenotypic identity therefore appear to have been determined at or before the time of grafting. When r4 is grafted to the r2 position with its dorso-ventral polarity inverted, both types of neuron again develop, but in the ventral region of the graft, in a position appropriate to the dorso-ventral pattern of the host, rather than their original dorso-ventral position. The change in fate of these cells is restricted, however, to the repertoire characteristic of the antero-posterior position of origin, in this case r4.

Conclusions: Cells seem to 'know' details of their presumptive fate before more general features. At this stage of development, precursor cells in r4 seem to have been assigned an 'r4 fate', but remain multipotent in their choice of r4-specific cell type. Precursor cells seem to be committed to their fates according to position on an orthogonal grid, the coordinates of which are set (or read) independently and sequentially. Thus, at the 7–10 somite stage, dorso-ventral positional values are still labile, whereas antero-posterior values are already fixed.

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Background

During development of the vertebrate central nervous system, a very large variety of neurons appear, each in a characteristic position with respect to the principal axes of the system. A correct pattern of cell specification is crucial for later events when, for example, these various neurons establish connections with each other to form complex functional networks. But how the pattern of cell specification is controlled is, for the moment, an unanswered question. As a convenient working hypothesis, however, it can be proposed that spatial order is organized on a Cartesian grid of positional information [1], the coordinates of which correspond to the antero-posterior and dorso-ventral axes of the early neural tube. Fates are assigned to multipotent precursor cells according to their position on both axes — as if the cells were reading their latitude and longitude in the neuroepithelium with a sextant and a chronometer.

The hindbrain offers several advantages for studying the early stages of neural pattern formation. First, its early development is marked by the process of segmentation, suggesting the operation of a simplifying principle of organization [2]. Second, a sizeable repertoire of identified neuronal cell types is formed during this period

[3–5]. Third, there is a pronounced segmental variation in cell pattern on the antero-posterior axis, by contrast with the spinal cord where most cell types form continuous columns along the antero-posterior axis. Fourth, the hindbrain neuroepithelium can be viewed as a virtually flat sheet of cells in which the antero-posterior and dorso-ventral (lateral, latero-medial) axes are straight lines, orthogonal to each other. At early stages, there is no appreciable pattern on the third (inside–outside) axis — all neurons are disposed in a mantle layer, one to three cells deep, at the outer surface [3].

The segmental pattern of the chick embryo hindbrain emerges between Hamburger and Hamilton (HH) [6] stages 9– and 12, and is virtually complete as the first neurons differentiate [2]; the initially cylindrical neural tube is progressively subdivided by constrictions in its wall [7] to form a series of eight varicosities along its length. These rhombomeres are lineage-restricted compartments, as evidenced by the non-mixing of cells across their interfaces [8]. Compartments provide a way of setting aside blocks of cells that have distinct cell states, allowing each a degree of autonomy during the period of cell specification [9]. The formation of compartments also allows the adjacent blocks to interact with each other — to establish third cell states at the boundaries, for

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example [2,7]. The containment of groups of neuroepithelial cells in rhombomeres persists up to at least stage 17 [10], suggesting a critical period for precursor specification lasting about 24 hours.

Two levels of organization can be distinguished in the neuronal pattern of the segmented hindbrain (at stage 17–20), one involving neurons of the reticular formation and the other involving the motor formations. Eight identified types of reticular neurons [3] are repeated as 'segmental homologues' through sequential rhombomeres (Fig. 1) such that each rhombomere contains a more or less complete set [3,11]. Later in development, local variations are played on this segmental theme as certain cell types become more numerous in particular rhombomeres [12], suggesting the differential production of some cell types and/or the selective elimination of others. Motor neurons also develop in each rhombomere but they, by contrast to the reticular neurons, are organized from the start into discrete specification groups in different rhombomeres: the somatic motor nuclei of cranial nerves IV and XII develop in r1 and r8, respectively, whereas the branchiomotor nuclei of nerves V, VII and IX, and the somatic motor nucleus of VI, develop in r2+r3, r4+r5, r6+r7 and r5+r6, respectively [4]. Additionally, the efferent nucleus of the VIIth nerve (vestibulo-acoustic) develops in ventral r4 [5]. A subpopulation of these r4-specific neurons, the contralateral vestibulo-acoustic (CVA) neurons, have a distinctive behaviour: their axons extend out of the exit point in dorsal r4, later to connect with hair cells of the inner ear, while their cell bodies migrate in the opposite direction, across the floor plate, to form a contralateral nucleus.

Thus, the neurons of the efferent cranial nerves develop in single rhombomeres or in adjacent pairs (Fig. 1), suggesting that, in addition to a reiterated common identity, each rhombomere has a unique identity. It is therefore reasonable to consider rhombomeres as specification units, and to ask how their individual identity is conferred. The clustered *Hox* genes are considered prime candidates for this role [13], on account of their appropriate spatio-temporal expression and their sequence similarity with the *Antennapedia*-class of *Drosophila* homeotic genes, which encode segment identity in the fly [14]. Genes at the 3' ends of the *Hox-a* and *Hox-b* clusters are expressed in overlapping or nested domains in the hindbrain, where their anterior limits of expression coincide with rhombomere boundaries [15]. The expression of *Hox* genes in a nested pattern may depend on a retinoid signal that emanates from Hensen's node and forms a posterior-to-anterior gradient in the plane of the neuroepithelium [16–19]. A gradient positional signal of this sort may accompany the planar element of 'neural induction' [20,21].

Specific *Hox* gene products, expressed within the confines of a particular rhombomere in response to a positional signal, may determine the identity, or positional value, of that rhombomere. The identity of r4, for example, may be conferred by the expression of *Hoxa-1*,

Hoxb-1, *Hoxa-2* and *Hoxb-2*. The best characterized of these genes is *Hoxb-1* [22,23], the high level expression of which is confined to r4; expression is strongly up-regulated soon after the rhombomere becomes defined by its boundaries [24]. One requirement of a putative determinant is that its expression should be autonomous from the developmental stage at which regional commitment becomes irreversibly fixed. Thus, in a previous study, we

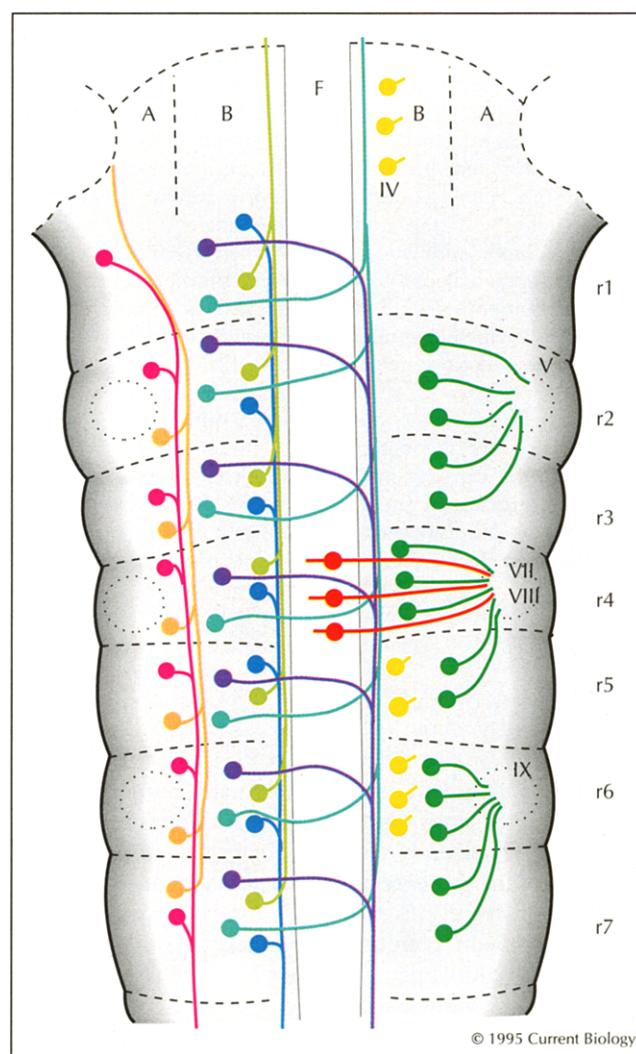


Fig. 1. Diagram showing the distribution of neuronal types in the chick hindbrain at stage 17–20. On the right side of the figure are shown the branchiomotor neurons (green), forming in the basal plate (B) of r2+r3 (Vth nerve, trigeminal), r4+r5 (VIth nerve, facial) and r6+r7 (IXth nerve, glossopharyngeal). Also shown on the right are the contralaterally migrating efferent neurons (red) of the VIIth nerve (vestibulo-acoustic), which are in the floor plate (F) at r4 level at stage 19–20. Somatic motor neurons (yellow) form in r1 (IVth nerve, trochlear), r5+r6 (VIIth nerve, abducens) and r8 (XIIth nerve, hypoglossal). Cranial nerve entry/exit points associated with r2, r4 and r6 are shown as dotted circles (data from [4]). On the left side are shown the reticular neurons. Each rhombomere contains cells of each of the six classes shown. Basal plate cell types project either ipsilaterally or contralaterally, and their axons either ascend or descend in the medial longitudinal fasciculus, at the border between basal and floor plates. More laterally located cells in the alar plate (A) have projections that either ascend or descend in the lateral longitudinal tract (data from [3]).

transplanted the presumptive r4 region, in embryos at HH stage 9– (6 somites), into the more anterior position of r2, and probed for *Hoxb-1* transcripts. We found that *Hoxb-1* was expressed in the ectopic r4 as strongly as in the normal r4, whereas reciprocal grafts of presumptive r2 placed in the r4 position did not express *Hoxb-1*. Preliminary analysis, by retrograde axonal tracing of branchiomotor nerve nuclei, indicated that the phenotypes of the ectopic rhombomeres developed according to their original position. We concluded that both *Hox* expression and segment identity are independent of position in the neuroepithelium from as early as the 6 somite stage [24].

Cell pattern on the antero-posterior axis changes step-wise between rhombomeres. On the dorso-ventral axis, by contrast, there is a continuous gradation from one cell type to another between ventral (lateral, medial) and dorsal (lateral, lateral) poles of all rhombomeres [25], a pattern that has many similarities with that of the spinal cord, and which may therefore be controlled by similar mechanisms. The control of dorso-ventral cell pattern in the spinal cord has been studied extensively by Jessell and colleagues [26–28]; their explant co-culture and transplantation studies have shown that vertical signalling from the notochord is responsible for establishing elements of ventral pattern (floor plate cells and motor neurons), that similar signalling properties are induced in the floor plate, and that the signal from the notochord and floor plate involves the secreted protein Hedgehog [29,30]. Signalling from the dorsal pole, involving the secreted protein Dorsalin-1 [31], may be set up by interactions between the neural tube and surface ectoderm (T.M. Jessell, personal communication).

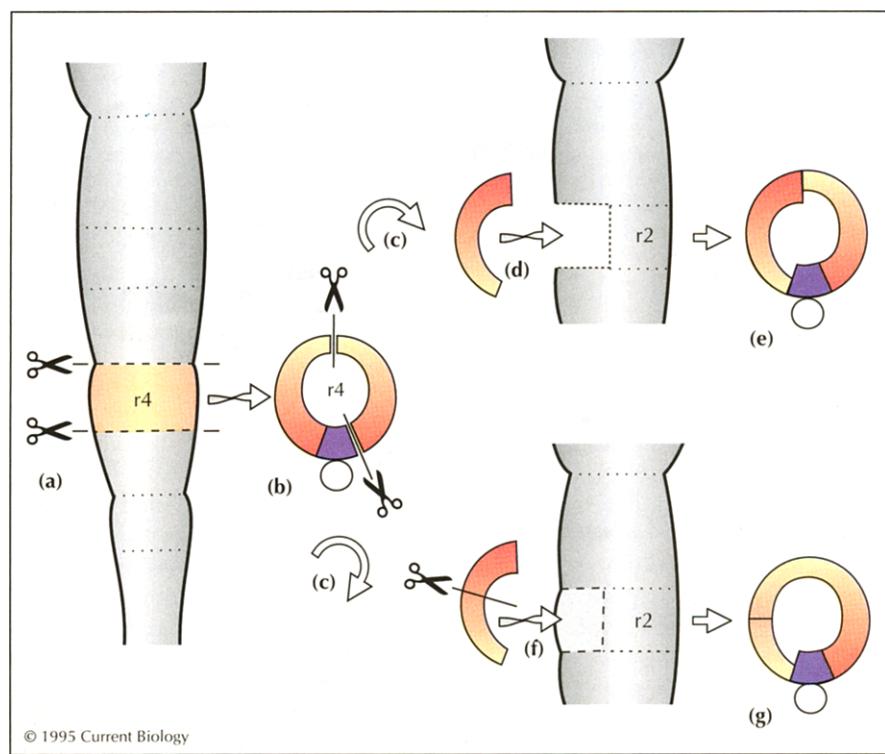
Fig. 2. Scheme of rhombomere transplantation experiments. (a) At stage 9–10, the hindbrain is a cylindrical tube and the boundaries between rhombomeres (dotted lines in (a), (d) and (f)) are visible as constrictions in the wall. A rhombomere, for example, r4, was isolated by cutting along its boundaries. (b) After removing adherent mesenchyme and surface ectoderm, the r4 (shown in transverse plane) was subdivided to obtain the lateral plate, free of floor-plate cells (purple zone above notochord). (c) The lateral plate was inverted on its dorso-ventral axis and (d) inserted into a slot formed in the contralateral hindbrain of an isochronic host by removal of the lateral plate of presumptive r2. The host floor plate was left *in situ*. (e) The graft was positioned so that its alar plate contacted the host floor plate (shown in transverse plane). (f) For alar plate grafts, the lateral plate was subdivided, inverted and inserted into a window made by removal of the host r2 basal plate. (g) On the operated side, there are two alar plates with the dorsal edge of the r4 donor in contact with the host r2 floor plate (shown in transverse plane).

When spinal notochord is grafted alongside the anterior hindbrain, ectopic serotonergic neurons characteristic of the basal hindbrain are formed [27], suggesting that some differential restriction of potential exists between different antero-posterior levels of the neural tube. This finding raises the question of whether dorso-ventral position with respect to the ventral midline (notochord/floor plate) signalling region can affect cell fate within a rhombomere territory that is already determined with respect to antero-posterior position. In addition to experiments in which we have shifted rhombomeres on the antero-posterior axis, we describe here further experiments that combine this antero-posterior displacement with either dorso-ventral inversion or transposition, such that cells are shifted on both axes simultaneously (Fig. 2). We find that cells are not committed to dorso-ventral fate at a stage at which they are no longer labile with respect to *Hox* gene expression and have a determined antero-posterior fate. Our results confirm and extend the morphological studies by earlier workers [32–34], who found that the antero-posterior axis of the amphibian neural plate is determined before the dorso-ventral (lateral-medial) axis. We conclude that neuronal determination involves at least two steps, and that fate selection by multipotent precursors depends on independent and sequential assignment of positional values on antero-posterior and dorso-ventral axes.

Results and discussion

Alteration of antero-posterior position

To alter the antero-posterior position of a rhombomere without reorienting its dorso-ventral axis, we transplanted



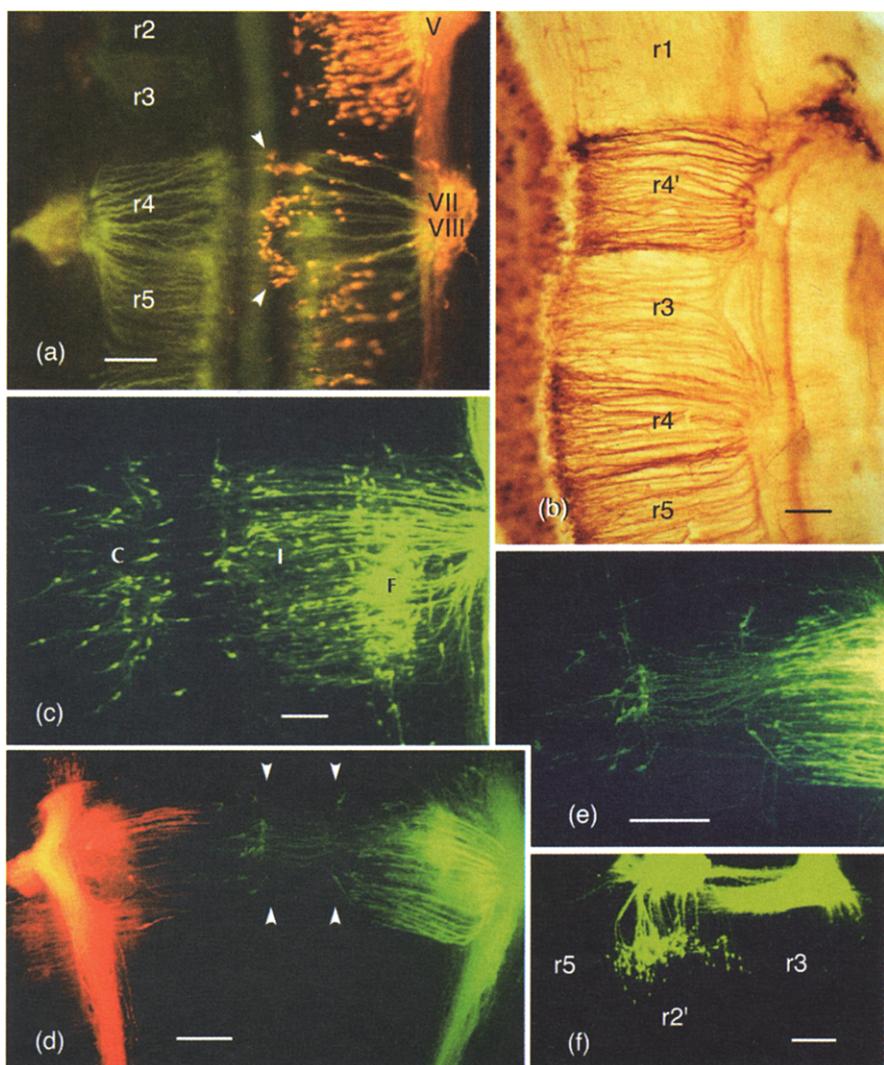


Fig. 3. Alteration of antero-posterior position. (a) Normal stage 19 embryo; whole-mount hindbrain double-labelled with a rhodamine-lysine-dextran back-label from the Vth and VII/VIIIth nerve exit points on the right side (yellow) and SC1/DM-GRASP antibody (green). Note that both the floor plate cells and the (young) motor neurons are stained by SC1/DM-GRASP, but whereas the facial nerve (and more posterior) motor neurons are stained heavily, the trigeminal motor neurons in rhombomeres 2 and 3 (dextran-filled on right side; V) are very lightly stained by the antibody. The difference in staining intensity between trigeminal and facial motor neurons, which reflects a marked difference in the level of transcription [38] between the two subpopulations, provides a comparative control within all experimental embryos. At this stage, CVA neurons have migrated into the floor plate from the ventral extremity of r4 (antero-posterior extent marked by arrowheads). Scale bar = 100 µm. (b) r4-to-r2 embryo, 28 hours after grafting (stage 17); whole-mount hindbrain stained with the SC1/DM-GRASP antibody. The floor plate and motor neurons are stained; markedly different staining intensities differentiate between the motor neurons of the host r3 (trigeminal) and r4 (facial). According to this criterion, motor neurons developing in the graft (r4') are of facial type. When r4 is grafted in place of r2, trigeminal motor axons in the rostral half of r3 usually exit via the exit point in the graft, whereas those in the caudal half of r3 exit abnormally via the host r4 exit point (see [37]). Scale bar = 100 µm. (c) Normal stage 26 embryo; whole-mount hindbrain labelled with a Dil injection into the VII/VIIIth nerve

exit point. The facial motor nucleus (F) and both ipsilateral (I) and contralateral (C) vestibulo-acoustic efferent nuclei are labelled; the CVA neurons migrate posteriorly after crossing the floor plate to reside in the contralateral r4 and r5. Confocal micrograph in false colour. Scale bar = 100 µm. (d) r4-to-r2 embryo, 3 days after grafting (stage 26); whole-mount hindbrain labelled by carbocyanine axon-tracing dyes applied to the nerve roots at trigeminal level. Tracing from the nerve root on the operated side reveals CVA efferent neurons (green axon label) that are specific to r4. These cells have emerged from the ventral extremity of the graft and crossed the host floor plate (floor plate border marked by arrowheads) into the contralateral, unoperated side (r2) before being filled with the tracer dye. No CVA neurons can be detected from the control side (red axon label). Motor axons and neurons have also been traced into r3 on the operated side; these trigeminal neurons share the same exit point as the facial neurons in the graft. Confocal micrograph in false colour. Scale bar = 100 µm. (e) Higher power view of (d), showing details of the ectopic commissure and CVA neurons. Compare with the normal embryo shown in (c). Scale bar = 100 µm. (f) r2-to-r4 embryo, whole-mount, as in (d). No CVA neurons can be traced by retrograde labelling from the nerve roots at the graft level to the opposite side (Dil visualized in green). CVA neurons do not normally form in r2 and cannot be induced in r2 tissue by grafting it into the r4 position (r2'). Confocal micrograph in false colour. Scale bar = 100 µm.

either the left or right half of r4 from stage 9–10 (7–10 somite) embryos to the same side of equivalent stage hosts in which a recipient site had been made in a more anterior or more posterior position by the removal of either r2 or r6 (r4-to-r2 or r4-to-r6), leaving the host floor plate in place. The half-rhombomeres were dissected cleanly away from their floor plate and adherent mesenchyme or surface ectoderm cells before grafting.

After incubation to stage 17–19, r4-to-r2 animals were immunostained as whole mounts with the SC1/DM-GRASP antibody, which recognizes a glycoprotein of

the immunoglobulin superfamily that is expressed by both motor neurons and floor plate cells [35,36]. Antibody staining revealed that motor neurons had formed in donor tissue alongside the host floor plate. These were characteristic of facial (normal r4) motor neurons in that they had straight, rather than arcuate, axonal trajectories to the exit point in the alar plate and stained heavily with SC1/DM-GRASP antibody — in contrast to trigeminal (normal r2+r3) motor neurons, which stain lightly or not at all; compare Figure 3a (normal animal) with Figure 3b (r4-to-r2). The marked difference in staining intensity between trigeminal and

Table 1. Numbers and treatment of grafts.

Graft Donor→host	Number of grafts	Number analyzed	SC1/DM (*)	Dil/DiO (†)	Whole-mount <i>in situ</i>
r4→r2	19	8	4 (4)	4 (4)	nd
r4→r6	30	6	nd	6 (4, ns)	nd
r2→r4	6	4	nd	4 (0)	nd
r3→r4	2	1	nd	1 (ns)	nd
r5→r4	5	1	nd	1 (ns)	nd
r6→r4	16	6	nd	6 (0)	nd
r5→r3	2	1	nd	1 (ns)	nd
r4inv→r2	85	62	20 (20)	17 (12)	25 (<i>Pax</i>)
r2inv→r4	8	4	nd	4 (0, ns)	nd
r3inv→r4	14	7	nd	7 (0, ns)	nd
r5inv→r4	5	5	nd	5 (0, ns)	nd
r4a→r2b	117	70	16 (16)	26 (10)	28 (<i>Hox</i>)
Total	310	175	40	82	53

* Numbers of animals with SC1/DM-GRASPS++ motor neurons in ventral region of graft are shown in parentheses. The preceding number in the column is the number of embryos stained with antibody.

† Numbers of animals with CVA neurons are shown in parentheses. The preceding number in the column refers to the number of embryos traced with carbocyanine dye.

ND: not done; NS: not shown.

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facial motor neurons [37], which reflects different levels of transcription [38], provides a comparative control within all experimental embryos.

Incubation of operated embryos to later stages allowed us to examine them for the formation of r4-specific CVA neurons [5] in r4→r2 and r4→r6 grafts. These specialized cholinergic efferent neurons are first detectable in normal embryos by retrograde axonal tracing from the combined VIIth/VIIIth nerve exit point at stage 16, when their cell bodies lie in the ipsilateral basal plate, directly adjacent to the floor plate. During subsequent stages, as their growth cones extend from the exit point in r4 towards the otic epithelium, the cell bodies of these neurons migrate in the opposite direction, crossing the r4 floor plate during stages 19–21 (Fig. 3a) and forming a dispersed subpopulation in the contralateral basal plate during stages 24–27 (Fig. 3c) [5]. Novel ectopic commissures in operated embryos were sought by retrograde axonal tracing with carbocyanine dyes from the nerve exit points alongside either the trigeminal ganglion (r4→r2 grafts) or the superior ganglion (r4→r6 grafts) at stage 26–27.

In many cases, we found that a vestibulo-acoustic efferent commissure had formed ectopically when r4 was transplanted in place of either r2 or r6 (Fig. 3d,e; Table 1). When the CVA axons of r4→r2 grafts were traced anterogradely into the contralateral periphery, we saw that their growth cones leave the central nervous system through the exit point in ectopic r4, now in association with the trigeminal or glossopharyngeal ganglia, not through the host's r4 exit point in association with their proper innervation target, the otic vesicle. Once in the periphery, furthermore, they were found to project not towards the otic vesicle but into the mandibular process,

a normal target for trigeminal motor neurons (data not shown). The number of cells constituting the ectopic commissure was in the range of 10 to 20, approximately half the number normally detectable by retrograde tracing in r4 at this stage (compare Fig. 3c and Fig. 3e) [5]. In reciprocal experiments, in which the host r4 was replaced by r2 (Fig. 3f; also by r3, r5 and r6, Table 1), the vestibulo-acoustic commissure was never found, suggesting the absence of this cell type. In the case of r5→r4 and r6→r4 grafts, furthermore, an abducens nucleus characteristic of the original position of the graft, and identifiable by its ventral-exiting axons, developed in the ectopic r5 or r6 (data not shown; see Table 1).

These results show that at least two phenotypic characteristics of r4 are independent of position along the antero-posterior axis by stage 9–10, suggesting that the positional identity of r4 has already been established. This confirms and extends preliminary evidence [24] that rhombomere phenotype is determined at the same stage as is *Hoxb-1* expression. The development of reciprocal grafts of other rhombomeres into the r4 position shows that the positional independence of rhombomere identity also applies to other rhombomeres.

Alteration of both antero-posterior position and dorso-ventral orientation

The notochord and floor plate are known to be the sources of a ventralizing signal that elicits the formation of motor neurons in basal plate in a distance-dependent manner [27]. Neural tube cells differentiate as motor neurons when adjacent to notochord or floor plate, even when the latter are grafted ectopically. One possible cause of normal variation in the types of motor neurons in different rhombomeres might be some corresponding variation in the notochord/floor plate signal. It is possible

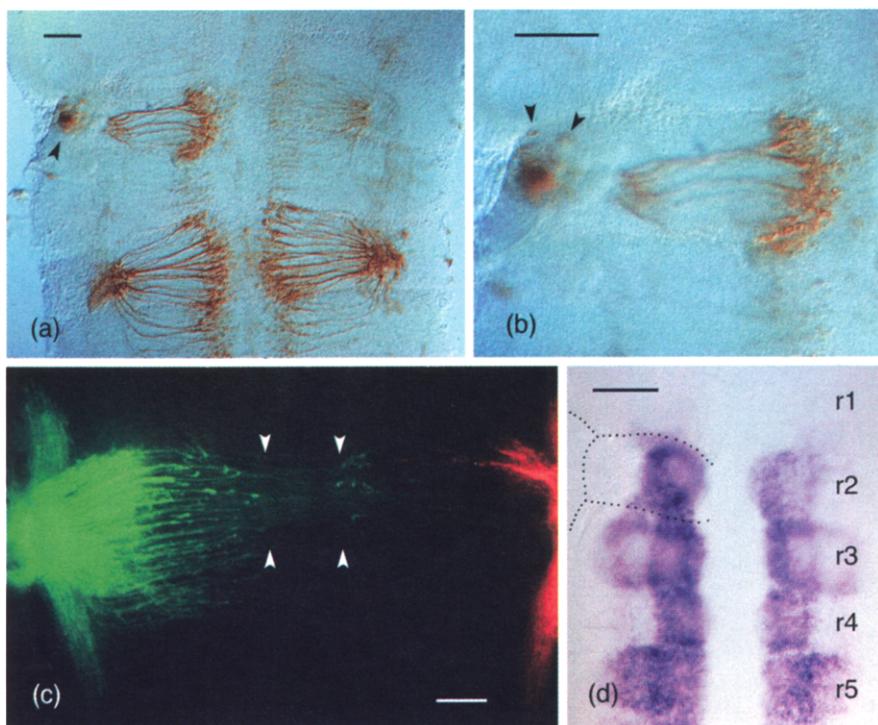
that r4-specific motor neuron fate, as a component of r4 identity, may have already been determined by a putative r4-specific midline signal at the time of grafting.

To reveal any antero-posterior position-dependent effect of the notochord and floor plate, we combined transplantation of r4 anteriorly or posteriorly with its rotation on the dorso-ventral axis by 180°, so as to expose the alar plate to the notochord/floor plate signal of a different antero-posterior level. In these experiments (Fig. 2a–e), the right or left side donor r4, prepared as before, was inverted dorso-ventrally and grafted to the contralateral side of the host in place of r2 or r6 (r4inv→r2 or r4inv→r6 grafts). Care was taken to ensure that the floor plate and dorsal ectoderm were left in place in the host and cleanly removed from the donor r4. In these experiments, therefore, it is the responding tissue rather than the ventral signalling region that is manipulated relative to the embryo as a whole, an approach previously used by Steding [39] to examine the regulation of dorso-ventral pattern in the chick spinal cord.

When incubated for 24 hours and stained with the SC1/DM-GRASP antibody, operated animals were seen to have formed an r4-type motor nucleus in the graft alongside the r2 notochord/floor plate (Fig. 4a,b; also in r4inv→r6 embryos, Table 1). The identification of these motor neurons in the former alar plate of the transplanted r4 was made, as before, on the basis of axon

trajectory and strong SC1/DM-GRASP immunoreactivity. In most cases, no motor neurons were detected in their formerly correct position, the basal plate, which now lay dorsal in the embryo and adjacent to both surface ectoderm and contralateral alar and roof plates. In some cases, however, where a small piece of donor floor plate remained attached to the graft, we saw a few motor neurons in the dorsal region (Fig. 4b). When animals were incubated for a further three days to stage 26–27, a contralateral efferent commissure was detectable by retrograde axonal tracing from the trigeminal or glossopharyngeal ganglion (Fig. 4c). Thus, while at the r2 or r6 position, r4-specific CVA neurons developed ectopically in r4 alar plate. In reciprocal experiments, where r2 was grafted with inverted dorso-ventral orientation in place of r4 (r2inv→r4, Table 1), motor neurons were again found ventrally by retrograde axonal tracing, but these stained weakly with SC1/DM-GRASP, suggesting a trigeminal identity; no CVA neurons were seen.

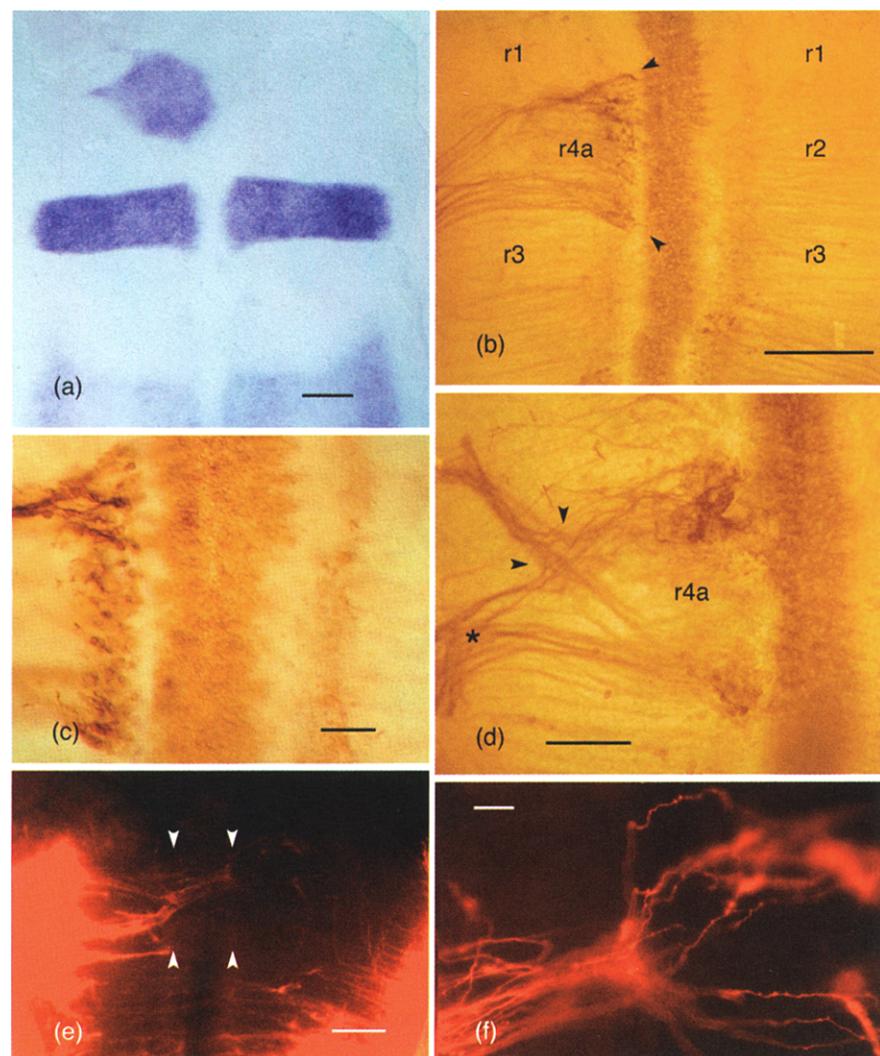
These results show that cell pattern on the dorso-ventral axis of the hindbrain is still labile with respect to polarizing signals from notochord/floor plate, and that the axis is capable of being (re)patterned according to orientation in the host environment. But the type of ectopic motor neurons produced depends on the antero-posterior level of origin of the graft and not on local signals at the graft site. Thus, the specification of facial motor neurons and CVA



emerged from the ventral extremity of the graft and crossed the host floor plate (borders marked by arrowheads) into the unoperated side. No CVA neurons can be detected from the control side (red axon label). Confocal micrograph in false colour. Scale bar = 100 µm. (d) r4inv→r2 embryo, 24 hours after grafting at stage 9+; whole-mount hindbrain hybridized *in situ* for Pax-6 transcripts. The dorso-ventral domain of Pax-6 expression in the graft (outlined by a broken line) matches that of the host (strong ventrally and absent dorsally), rather than the original dorso-ventral orientation of the donor tissue. The pattern of Pax-6 expression in the donor tissue has re-polarized after grafting. The dorsal extremity of the alar plate is marked by a solid line. Scale bar = 100 µm.

Fig. 4. Alteration of antero-posterior position with inverted dorso-ventral orientation. (a) r4inv→r2 embryo, 24 hours after grafting (stage 17); whole-mount hindbrain, antibody-stained for SC1/DM-GRASP. On the operated side (left), an r4 has been grafted with inverted dorso-ventral orientation at the r2 position, and its motor neurons stain with the same intensity as those of the host's r4s. Despite the inverted orientation of the graft, these facial-type motor neurons appear in the region of the graft that is adjacent to the floor plate of the host. Scale bar = 100 µm. (b) Higher-power view of the graft region in (a); a few motor neurons (indicated by arrowheads) are visible in association with a small fragment of SC1/DM-GRASP-positive donor floor plate/notochord that contaminated this graft (arrowheads in (a) and (b)). Scale bar = 100 µm. (c) r4inv→r2 embryo, 3 days after grafting (stage 26); whole-mount hindbrain after the application of carbocyanine axon-tracing dyes to the nerve root at trigeminal level. Tracing from the nerve root on the operated side (left) reveals contralateral vestibulo-acoustic efferent neurons (green axon label), which have

Fig. 5. Double-alar rhombomeres. (a) r4a→r2b embryo, grafted at stage 10 and fixed at stage 17; whole-mount hindbrain showing that high-level *Hoxb-1* expression, visualized by a digoxigenin-labelled riboprobe, is maintained in the ectopic r4 alar plate (upper left) as well as in the host r4. Low-level expression persists in r7 caudal. Scale bar = 100 µm. (b) r4a→r2b embryo, grafted at stage 9+ and fixed at stage 17; whole-mount hindbrain stained with SC1/DM-GRASP antibody. Facial type motor neurons have developed in the grafted r4 alar plate (antero-posterior extent marked by arrowheads) and have extended their axons out of the (trigeminal) exit point in the alar plate of the host r2. The motor neurons have formed in tissue that had originally been allocated as non-motor. Scale bar = 200 µm. (c) Higher-power view of the SC1/DM-GRASP-positive motor neurons in (b). Compare the density of staining on left (r4a) and right (r2) sides of the floor plate. Scale bar = 50 µm. (d) r4a→r2b embryo, as in (b), but grafted at stage 10+; whole-mount hindbrain showing the axons of SC1/DM-GRASP-positive motor neurons leaving the graft directly, through an exit point in the grafted r4 alar plate (arrowheads), as well as through the trigeminal exit point in the host r2 alar plate (*). Scale bar = 100 µm. (e) r4a→r2b embryo, fixed at stage 26; whole-mount hindbrain after application of carbocyanine dye to the trigeminal exit point in r2 on the operated side (left). CVA neurons are traced in the contralateral r2 basal plate, having developed in the grafted r4 alar plate before migrating across the floor plate (borders marked by arrowheads). Scale bar = 100 µm. (f) Higher-power view of the CVA neurons shown in (e). Scale bar = 25 µm.



neurons would not demand any putative rhombomere-specific specialization of the notochord or floor plate.

The re-polarization of the dorso-ventral axis that follows rhombomere inversion seems to involve further elements of the cell pattern; in addition to motor and CVA neurons, both ipsilateral and contralateral medial longitudinal fasciculus (MLF) reticular neurons (Fig. 1) were seen to have developed (abnormally) in the alar plate of the graft tissue that lay adjacent to the floor plate (data not shown). Identification of these cells, however, depends on their proximity to, and projection into, the MLF. In the absence of independent markers that might distinguish these basal plate cell types from reticular neurons that normally develop in the alar plate (Fig. 1), it remains possible that cells of unaltered phenotype had merely extended axons into a locally available longitudinal pathway.

Genes that may respond directly to dorso-ventral polarizing signals in the neural tube, and that may act as determinants of positional value and regional identity on the dorso-ventral axis, include members of the *Pax* family

[40]. *Pax-3* and *Pax-6*, for example, are expressed in domains that are continuous along the antero-posterior axis but sharply restricted down the dorso-ventral axis [41]. We showed previously [41] that in chick embryos, removal of the notochord, or implantation of an additional notochord alongside the spinal neural plate, rapidly and dramatically alters the dorso-ventral domains of expression of *Pax-3* and *Pax-6*, well in advance of the appearance of re-polarized tissue character in the operated region of the spinal cord. These manipulations suggested that signals from the ventral pole of the neural plate or early neural tube may normally regulate the establishment of the dorso-ventrally restricted expression domains of *Pax-3* and *Pax-6* in the spinal cord. Transcription factors of the *Pax* class are thus candidates for encoding positional value on the dorso-ventral axis of the hindbrain in an equivalent way to *Hox* genes on the antero-posterior axis.

In normal stage 9–10 hindbrain, *Pax-6* is expressed in the basal plate and ventral half of the alar plate. Later in development, expression weakens in the alar plate (r3, r5)

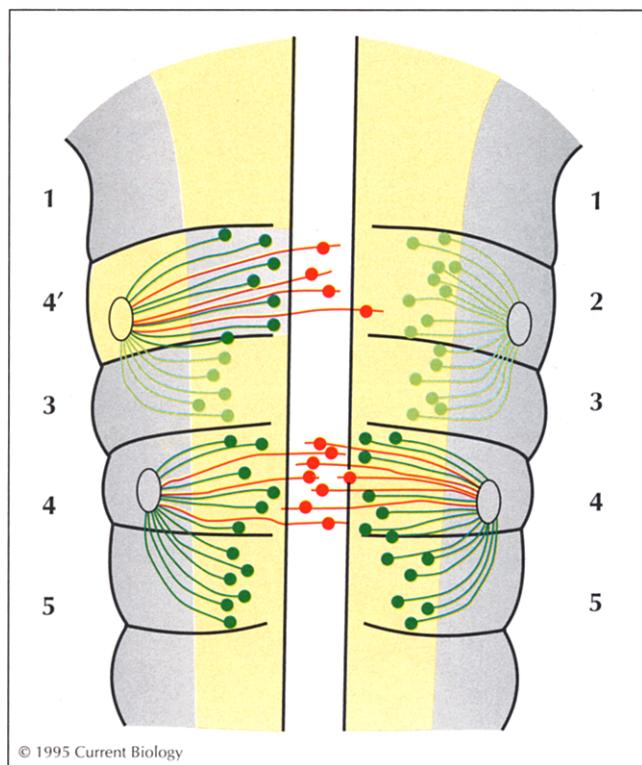


Fig. 6. Cartoon summary showing the result of grafting a right-side r4 in place of the left-side r2, with 180° dorso-ventral inversion of the transplant (r4'). The basal plate is shown in yellow. 1–2 days after grafting at stage 9/10, SC1/DM-GRASP-positive facial motor neurons (dark-green cell bodies) and contralateral vestibulo-acoustic efferent neurons (red cell bodies), both characteristic of r4 but not r2, develop at dorso-ventral positions in the graft that are normal for the host. Trigeminal neurons in r2 and r3 (light green) express SC1/DM-GRASP weakly.

or disappears from it altogether (r2, r4 and r6), as seen on the right (unoperated) side of the stage 15 embryo shown in Figure 4d. We found that, in r4inv→r2 operated embryos incubated for 24 hours, *Pax-6* expression in the graft closely matched the pattern of the host r4 and contralateral r2 — expression was confined to the ventral half of the neural epithelium, despite its dorsal (alar) origin. This compensatory alteration of *Pax-6* expression following tissue inversion reveals that *Pax-6* expression is not a determined property of the stage 9–10 alar neuroepithelium but is still labile. As in the spinal cord [41], the repolarization of *Pax-6* expression in response to inversion of positional signals anticipates a corresponding regulation of cell pattern on the dorso-ventral axis.

Double-alar rhombomeres

A possible, though unlikely, explanation for the appearance of ventral cell types in dorsal tissue would be that this results not from re-polarization of the pattern of cell specification but from altered or enhanced migration of cells that had already been specified before grafting — this is less unlikely for CVA neurons, which normally migrate towards the floor plate and may do so even from a distant location in the (now dorsal) basal plate. This possibility was tested by examining the capacity of alar plate

tissue alone to generate the basal plate cell phenotypes. In these experiments, the right or left side donor r4, prepared with the same precautions as before, was subdivided into basal and alar halves; the alar half was then inverted dorso-ventrally and grafted into a window in the contralateral r2 that had been prepared by removing the host basal plate (Fig. 2f,g). At the r2 level in these animals, there was thus a double alar plate and no basal plate (r4a→r2b grafts).

The results of these experiments were qualitatively similar to those involving inversion of the intact rhombomere (Table 1). In operated embryos incubated to stage 17–18, *Hoxb-1* expression was maintained in the partial rhombomere graft (Fig. 5a), as it is in whole rhombomere grafts [24]. The grafts contained SC1/DM-GRASP-positive neurons that had developed alongside the floor plate and had axons that projected out of the trigeminal exit point in the (r2) alar plate (Fig. 5b,c). In some examples, these stained axons seemed to leave the brain through a second, more ventral, exit point in the r4 part of the double-alar rhombomere (Fig. 5d). Application of carboxyfluorescein dye to the exit points in both alar r2 of the host and alar r4 of the graft at stage 26–27 revealed that CVA neurons had developed from the alar r4 and had migrated across the floor plate (Fig. 5e,f).

These results demonstrate that precursor cells in the alar plate, which never normally form motor neurons or CVA neurons, will do so when grafted into proximity to the floor plate/notochord. The alar plate cells that form these r4-type motor neurons must, therefore, have been multipotent at the time of grafting. Their repertoire of potentials is, however, restricted to (or inclusive of) r4-specific cell types; selection of a specific cell fate either has not yet occurred or is reversible by experimental repolarization of dorso-ventral signals.

Conclusions

The principal finding reported here is that when r4 is grafted to the r2 position with its dorso-ventral axis inverted, motor neurons fail to appear in their correct position — in the basal plate — but appear instead in the region originally allocated as alar plate. These ectopic neurons are of r4 type rather than r2 type and develop, abnormally, from alar plate precursors in the transplant. The change in dorso-ventral fate of these precursors is, however, restricted to the repertoire characteristic of the antero-posterior position of origin (Fig. 6).

Spatial order may be organized on a Cartesian grid of positional information, the coordinates of which correspond to the antero-posterior and dorso-ventral axes of the early neural tube. Multipotent precursor cells might be told their position with respect to both axes and then differentiate accordingly. Thus, immediately following closure of the hindbrain neural tube, the process of segmentation by internal compartmentation is accompanied

by specification of antero-posterior fate according to rhombomere position. By stage 10, rhombomeres have a determined antero-posterior identity, but this identity does not fully describe individual cell fates; rather, it defines a particular repertoire of potentials, defined by rhombomere position, and perhaps attendant on particular *Hox* gene products. Rhombomere identity must describe a uniform character of the compartment (such as an 'r4 fate'), including the equipotentiality of its constituent cells. The decision as to which cell type to become cannot have been finalized at this stage as the dorso-ventral axis is still mutable. Either the polarizing signals are produced later for the dorso-ventral axis or the response remains labile for longer.

Although these experiments cannot distinguish between these alternatives, the behaviour of the *Pax* genes does hint at protracted lability along the dorso-ventral axis. Either way, it appears that the two coordinates of positional signalling, antero-posterior and dorso-ventral, must be separate from each other, and the time windows within which individual precursors respond irrevocably to each signal occupy successive periods of development. In the context of positional information, cells seem to be committed to their fates according to position on an orthogonal grid, the coordinates of which are set independently and sequentially; dorso-ventral positional values are still labile when antero-posterior positional values have been fixed.

Materials and methods

Embryological methods

Fertile hens' eggs were obtained from a mixed flock (Poyndon Farm, Enfield) and incubated in a forced draft at 38 °C to stage 9–10 [6]. After windowing the egg shell, visibility of embryos was enhanced by sub-blastodermal injection of 0.1 ml dilute India ink (Pelikan Fount, 1:4 in Howard's Ringer). The vitelline membrane was removed over the hindbrain with sharp forceps and r2 removed unilaterally using needles flame-sharpened from 100 µm diameter pure tungsten wire. Care was taken to preserve the notochord, floor plate and superficial ectoderm at the site of excavation. Grafts were obtained by cutting an entire rhombomere from embryos pinned out in sylgard-coated dishes. Complete transections were made through the head at (for example) the r3/4 and presumptive r4/5 boundaries. The transverse head slices were then treated with dispase (Boehringer; 1 mg ml⁻¹ in L15 medium) for 10–15 minutes until the notochord, ectoderm and adherent cranial mesenchyme could be pulled cleanly away from the neural tube. Isolated r4s were then washed repeatedly in Howard's Ringer containing 10% fetal calf serum and then subdivided into left and right lateral plates (removing the floor plate) ready for grafting. Donor pieces were washed and then transferred in Howard's Ringer to prepared recipients. Grafts were moved into place, care being taken to ensure that the cut edge of the graft met precisely with that of the host's floor plate before bringing the rest of the graft into position. After gently layering 5 µl of Ringer over the exposed region of the embryo, eggs were resealed with electrical tape and incubated at 38 °C for a further period of 8 hours to 4 days.

Operations that replaced the r2 basal plate of the host with an r4 alar plate from a donor involved the following modifications. To prepare the graft site, a small aperture was made in the dorsal midline over the presumptive r2 by opening the dorsal seam of neural tube fusion and making a window in the ventral tube with fine tungsten needles. Donor tissue was obtained by subdividing r4 lateral plates (as above) into basal and alar halves. The alar r4 was transferred to the prepared host, as above, and manoeuvred into place.

Immunohistochemistry

After incubation to stage 17–20, operated embryos were fixed in 4% paraformaldehyde and examined for obvious defects. Normal-looking embryos were then blocked in 0.1% hydrogen peroxide and stained as whole mounts with the SC1/DM-GRASP antibody (kind gift of Elizabeth Pollerberg) using an indirect immunoperoxidase method, as described previously [4,37]. After final washing in phosphate buffered saline, hindbrains were dissected out intact and mounted flat in glycerol, pial side up, beneath a coverslip propped on pads of silicone grease.

In situ hybridization

Hoxb-1, *Pax-3* and *Pax-6* transcripts were detected in whole-mount stage 14–17 embryos using digoxigenin-labelled riboprobes, as described previously [41].

Retrograde axonal tracing

After incubation to stage 24–27, operated embryos were screened for obvious defects and axonal tracing performed either on the live embryo or, for carbocyanine dye tracing, after fixation in 4% paraformaldehyde. Embryos were pinned out on sylgard-bottomed dishes and dissected from the ventral side to reveal the cranial nerves and ganglia. A solution of Dil C₁₈ (1,1'-dioctadecyl-3,3',3'-tetramethyl indocarbocyanine; Molecular Probes D-282; 5 mg ml⁻¹ in dimethylformamide) or DiO (3,3'-dioctadecyl oxacarbocyanine perchlorate; Molecular Probes D-275; 10 mg ml⁻¹ in dimethylformamide) was pressure injected through a 2 µm-tip micropipette into the appropriate nerve root. Embryos were stored in paraformaldehyde in the dark for 3–5 days at room temperature before dissecting out their hindbrains and mounting, as above, in glycerol containing 2.5% DABCO anti-fade agent (Merck). Tracing with rhodamine-lysine-dextran was performed as described previously [3]; live embryos were kept in an oxygenated atmosphere at 32 °C for 3 hours before fixation, dissection and mounting.

Microscopy

Hindbrain whole-mounts were viewed and photographed either under bright-field/Nomarski optics or under epifluorescence optics using Zeiss #09 and #15 filter sets. Fluorescently labelled preparations were also optically sectioned on a BioRad MRC-600 confocal microscope equipped with a krypton–argon dual excitation laser (488/568 nm) that allows the complete separation of Dil and DiO emissions.

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