

# Segmental repetition of neuronal phenotype sets in the chick embryo hindbrain

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## SUMMARY

The neurons within the segmented hindbrain of the early chick embryo have been mapped with the neuronal tracers HRP and fluorescent lysinated dextran. We have categorised neurons according to their axonal pathways and have then compared rhombomeres with respect to the number and class of neurons present. The results indicate that most rhombomeres are similar in that they contain the same set of basic neuronal types but differ in that particular neuronal types are more abundant in some rhombomeres than others. The data support the concept that the hindbrain develops according to 'variations on a segmental theme' rather than 'each segment is unique'. Many of the cell types occupy distinct mediolateral domains that are probably established by both the differential migration of some neuronal classes and the spatial segregation of distinct precursors. The caudal rhombomeres 7 and 8 are

exceptional in that they do not have the full set of basic neuronal types and also contain two additional medial cell types that are not present rostrally. The mechanisms that may generate the regional diversity apparent in the more mature hindbrain are discussed.

Abbreviations: HRP, horseradish peroxidase; LRD, lysinated rhodamine dextran; LFD, lysinated fluorescein dextran; r, rhombomere; mlf, medial longitudinal fasciculus; llt, lateral longitudinal tract; **idm**, ipsilateral descending mlf; **cdm**, contralateral descending mlf; **iam**, ipsilateral ascending mlf; **cam**, contralateral ascending mlf; **idl**, ipsilateral descending llt; **cdl**, contralateral descending llt; **ial**, ipsilateral ascending llt; **cal**, contralateral ascending llt

Key words: hindbrain, rhombomeres, neuron, axon pathway, chick embryo

## INTRODUCTION

Recently both cellular and molecular studies have shown that the hindbrain develops from an overtly segmented region of neuroepithelium (for review see Lumsden, 1990). At the cellular level, neuronal differentiation follows a segmental pattern (Lumsden and Keynes, 1989), with neurons in even-numbered segments (rhombomeres) differentiating in advance of those in odd-numbered rhombomeres. Adjacent pairs of rhombomeres are found to contribute motor neurons to particular branchiomotor nuclei (i.e. rhombomeres 2 and 3 together form the trigeminal motor nucleus, while rhombomeres 4 and 5 form the facial motor nucleus). Cell-lineage analysis (Fraser et al., 1990) has shown that each rhombomere behaves as a developmental compartment, such that once the rhombomeres appear, neuroepithelial cells no longer move between rhombomeres. At the molecular level, the analysis of patterns of gene expression has suggested that the positional identity of the rhombomeres may be imposed by a combinatorial code of homeobox genes that encode transcription factors (reviewed in Graham, 1992). Thus the cellular and genetic partitioning of the hindbrain may represent important mechanisms by which neuronal organisation and diversification are established.

The developing hindbrain will eventually contain a multitude of neuronal nuclei and many different cell types. The mechanisms that control the differentiation of these diverse cell groups remain largely unknown. Recent studies on the chick hindbrain have examined the development of particular systems (reticulospinal, vestibulospinal and vestibulo-ocular) at relatively late stages when morphological segmentation is no longer conspicuous, and suggest that a neuron's phenotype may be determined by its position within the neuroepithelium (Glover and Petrusdottir, 1991). Other studies suggest that lineage may play an important role in phenotypic determination (Lumsden, Clarke, Keynes and Fraser, unpublished data). A prerequisite for understanding these control mechanisms is first to describe in detail all the basic cell types and their positional organisation within the hindbrain throughout the early stages of development. Comprehensive accounts of both ascending and descending neurons in the developing hindbrain are not yet available for any species. In the zebrafish, however, detailed descriptions of the developing, segmentally arranged reticulospinal neurons and their axons have been made (Mendelson, 1986a,b; Metcalfe et al., 1986). These studies suggest that similar neurons in different rhombomeres represent segmental homologues and that each rhombomere contains a unique set of the different neuronal

classes. Embryonic reticulospinal and vestibulospinal neurons have also been described in *Xenopus* embryos (van Mier and ten Donkelaar, 1984; Nordlander et al., 1985), but have not yet been related to the rhombomeric pattern of hindbrain organisation. To date, our understanding of the early hindbrain neuroanatomy of birds and mammals has been limited by the difficulties of interpreting silver-stained (Windle and Austin, 1936) and neurofilament-stained material (Lumsden and Keynes, 1989). Beyond the earliest stages of development, when the number of differentiated neurons is still small, these techniques do not reveal the morphology of individual neurons. We have therefore examined neuronal populations in the chick embryo hindbrain using horseradish peroxidase and lysinated fluorescent dextrans as neuronal tracers. We have concentrated on stages when segmentation is prominent and have studied both ascending and descending pathways. We determine that, up to stage 20, the neuronal composition of each rhombomere is remarkably similar. Segmental variation is apparent when the relative numbers of cells within the different neuronal classes is considered, but abrupt phenotypic changes are rare. Regional variation within the hindbrain thus appears to be generated by imposing local specialisations upon an initially uniform plan.

## MATERIALS AND METHODS

Fertilised eggs were obtained from a local supplier (Poyndon Farm, Waltham Cross, Herts), and incubated to Hamburger & Hamilton stages 17 to 21. Embryos were then removed from the egg and placed in chick embryo saline (137 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM sodium phosphate, 5 mM Hepes, 11 mM glucose, pH 7.4; Glover and Petursdottir, 1988) at room temperature. Embryos were pinned onto Sylgard-covered dishes: dorsal side up for tracer applications direct to the neuroepithelium or ventral side up for applications to the nerve roots or peripheral ganglia. For ganglia or nerve root applications, the mesenchyme surrounding the tissue was teased away using pins, while for neuroepithelium applications the thin roof of the hindbrain was removed to allow tracer application through the ventricular surface. For both horseradish peroxidase (HRP, Boehringer grade 1) and fluorescent dextran (D-1817 and D-1820, Molecular Probes Inc.) applications, the dyes were dissolved in a very small quantity of distilled water and then recrystallised onto the tips of fine tungsten needles. These dye-laden needles were then simply pushed into the area of tissue to be labelled. The number of cells labelled could be roughly controlled by the size of the needles used. Excess dye was washed away by squirting fresh saline at the application site. Embryos were then incubated in aerated chick embryo saline at room temperature for between 3 and 6 hours.

For HRP histochemistry, embryos were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) for 1 to 2 hours, then thoroughly washed in several changes of PBS, before being incubated in 0.08% diaminobenzidine in PBS for 30 minutes and then 0.08% diaminobenzidine plus 0.05% hydrogen peroxide for a further 10 minutes. For the fluorescent dextran applications, embryos were fixed in 3.5% paraformaldehyde in PBS for 1 to 2 hours. In most cases, brains and rostral spinal cord were dissected free of surrounding mesenchyme and meninges and mounted as whole mounts, pial surface up, in 90% glycerol/10% PBS and coverslipped. Ten of the HRP-processed brains were subsequently sectioned transversely at 50 µm using a vibratome.

Five specimens were retrogradely labelled with fluorescent dextrans as above and then processed for immunohistochemistry. After fixation in 3.5% paraformaldehyde the tissue was washed extensively in PBS before incubation for 3 days at 4°C in the primary monoclonal antibody DM2 diluted 1 in 100 (kind gift of Dr S. Chang). A FITC-conjugated second antibody (Boehringer) diluted 1 in 50 was used overnight at 4°C to visualise the first antibody. These double-labelled specimens were either viewed as whole-mounted hindbrains or sectioned transversely at 75 µm on a vibratome before viewing on a confocal microscope.

Fluorescent tracers were visualised using standard filter sets (Zeiss 09 & 15) on a fluorescence microscope and using laser scanning confocal microscopy (BioRad MRC 500). The analysis was performed on more than 150 HRP preparations and 50 fluorescent dextran preparations. Neuronal positions and morphologies were drawn with the aid of a camera lucida.

## RESULTS

In the following description, we use the terms basal and alar to define cell positions. However, since a precise border between alar and basal plates is usually not visible in our preparations, we have simply divided the lateral neural plate into basal and alar halves. We use the terms medial and lateral as relative circumferential positions such that most medial is adjacent to the floor plate and most lateral is adjacent to the roof plate (Fig. 1).

### Axon positions and pathways

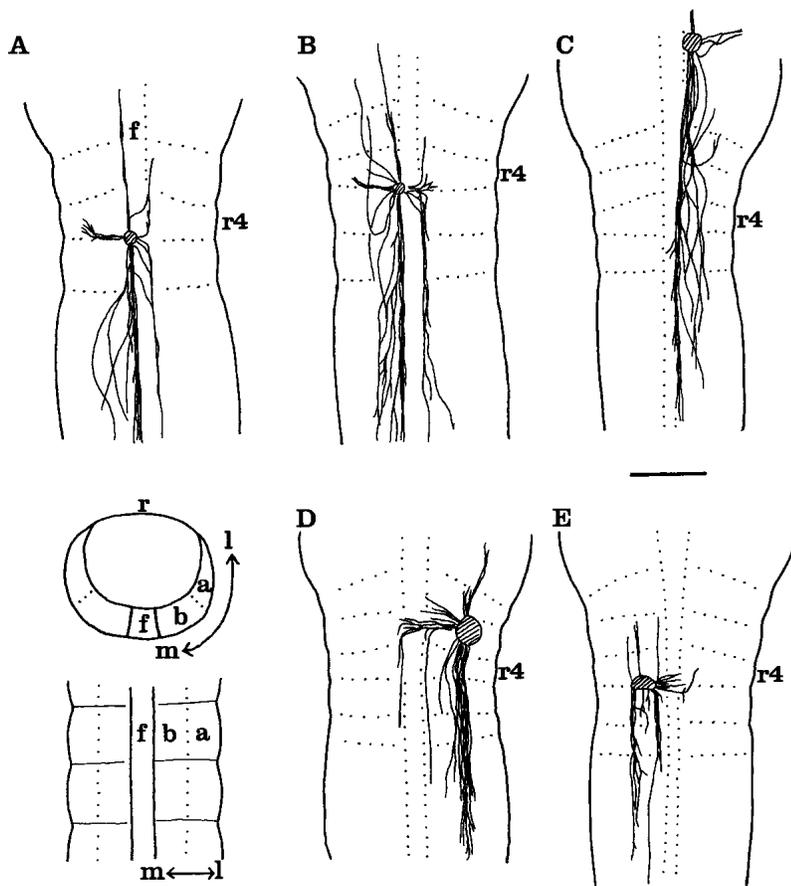
Single stabs of HRP into the developing hindbrain resulted in strong staining of retrogradely filled neurons together with their dendrites and proximal axon, and of the anterogradely filled distal portion of axons and their growth cones. At early stages of development, neuronal types are most easily defined by the projection of their axons; thus it is necessary first to define and describe the major axonal tracts that run through the developing hindbrain.

### Medial HRP applications

HRP applied immediately lateral to the floor plate fills predominantly axons that run either rostrally or caudally in the medial longitudinal fasciculus (mlf). The mlf is the most developmentally advanced axon tract at these early stages; the more lateral axon tracts are thin and more dispersed.

Axons filled by mlf applications are not restricted to the mlf throughout their entire length. Rather, many axons are seen to have grown into more lateral regions of the marginal zone (Fig. 1A-C). This lateral growth usually occurs at relatively shallow angles, although in some preparations fairly abrupt changes in direction are seen. Most laterally shifted axons do not reach the lateral longitudinal tract (llt, see later).

From the HRP stab site, some axons that cross the floor plate are filled. Most anterogradely filled axons cross directly from the stab site, although a few have been seen distant from the stab (Fig. 1A). The angle of crossing can be quite variable; this is most noticeable at stages 16-18 (Fig. 1A), but is less striking at subsequent stages when later axons tend to cross the floor plate at angles close to 90 degrees. Having crossed, most axons turn and either ascend or descend in the contralateral mlf. As in the ipsi-



**Fig. 1.** Principal axonal pathways illustrated in camera lucida (CL) drawings of five flat-mounted embryo hindbrains at stages 18 (A) and 20 (B-E). HRP was applied (hatched area) to the mlf (A-C) or to more lateral axons (D,E). Only anterogradely filled axons have been drawn, i.e. those filled between the point of HRP application and their growth cones. Retrogradely labelled cell bodies and axons have been omitted for clarity. In A, B and C note the lateral shift of some descending axons away from the mlf and in D the medial shift of some axons away from the lt. In E HRP was applied slightly more medially than in D and has labelled a few axons in the mlf as well as in the lt, they are separated by a region containing few labelled axons. In all specimens most axons that cross the floor plate (f) turn to run into the contralateral mlf and do not reach the contralateral lt. U-turns apparent in B and C are rare events. r4 is rhombomere 4. Rostral is up. Bar is 400  $\mu$ m for A and 500  $\mu$ m for B to E. Inset diagram illustrates our usage of the terms alar (a), basal (b), floor plate (f) and roof plate (r), and our definition of medial (m) and lateral (l) in both transverse section and in an opened-out flat-mounted preparation.

lateral mlf, some lateral displacement of contralateral axons away from the mlf is apparent.

Not all axons to cross the floor plate turn into the contralateral mlf. At stage 20 and later some axons grow beyond the mlf and turn longitudinally at more lateral positions. Both ascending and descending lateral turns are seen, but these axons have seldom extended far in the longitudinal direction.

The mlf also contains axons that descend, ipsilaterally, from cells in the midbrain and forebrain. These axons will eventually reach the cervical cord, thus most of the mlf applications label axons that extend from these neurons.

At the level of the HRP stab site a well-defined group of axons that grow laterally towards and out of the cranial nerve exit points are always filled from medial HRP applications (Fig. 1A,B). These are the axons from motor neurons and sensory efferent neurons. In even-numbered rhombomeres these axons are oriented fairly directly towards the exit points, while in odd-numbered rhombomeres they are directed first laterally to the level of the sensory tracts and then turn rostrally towards the exit point in the adjacent rhombomere. More detailed descriptions of efferent axon pathways have been made elsewhere (Lumsden and Keynes, 1989; Guthrie and Lumsden, 1992; Simon and Lumsden, unpublished data).

#### Mid-lateral HRP applications

HRP applied to mid-lateral positions (i.e. just medial to the

level of cranial root exit points) fills caudally directed axons that descend to and through the hindbrain from the midbrain tectum (tectobulbar and tectospinal axons) as well as ascending and descending axons from hindbrain neurons (Fig. 1D). Axons from the tectum run immediately medial to the sensory trigeminal axons described above (Fig. 2A) and, together with the similarly positioned axons from hindbrain neurons, they form a tract that we shall refer to as the lateral longitudinal tract (llt). In r1 the llt axons occupy a wider band of neuroepithelium than elsewhere. Here the descending tectal axons appear to curve laterally in flat whole-mount preparations and many of them follow unusual looping pathways as they run through the isthmic region (data not shown). This looping behaviour is also, but more rarely, seen in more caudal rhombomeres. As the llt widens in r1, it is apparent that some of the lateral axons from hindbrain neurons run medial to those from the tectum. This mediolateral organisation of the llt may also be present, but less conspicuous, throughout the hindbrain.

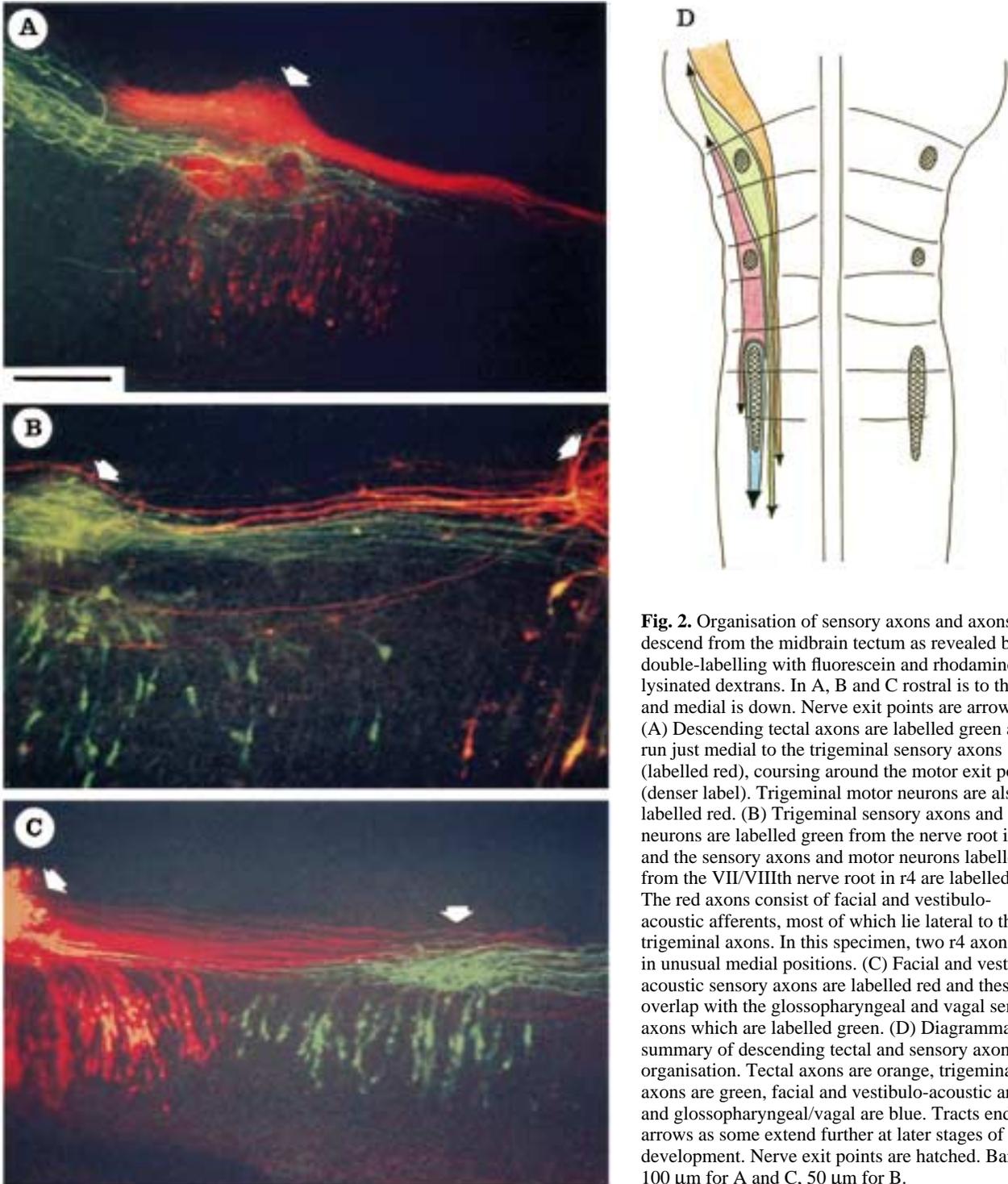
Mid-lateral HRP stabs often fill a few axons that run into the region between the llt and the mlf (Fig. 1E). This area contains relatively few longitudinally running axons at these stages, and those present there appear to have grown slightly laterally or slightly medially from the mlf or llt, respectively. These axons are called intermediate axons by Glover and Petursdottir (1991). A distinct population of axons has not been defined for this region.

Mid-lateral HRP stabs also fill axons directed medially towards and across the floor plate. Those which cross the floor plate turn to enter either the contralateral mlf (Fig. 1D) or, more rarely, the contralateral llt.

**Cranial nerve root and alar HRP applications**

HRP stabs into the alar plate of the hindbrain epithelium fill two classes of neuron. Running medially towards the floor-plate are the axons and growth cones of alar plate neu-

rons. At the stages examined here these axons are short, most have growth cones close to or within the floor plate and few show extensive longitudinal growth. The second class of axons filled are the central processes of primary sensory neurons of the cranial ganglia. These axons run in defined longitudinal tracts and their organisation is best analysed not from dye applications to the neuroepithelium but from applications to individual nerve roots or pairs of roots. With the combined use of fluorescein and rhodamine



**Fig. 2.** Organisation of sensory axons and axons that descend from the midbrain tectum as revealed by double-labelling with fluorescein and rhodamine lysinated dextrans. In A, B and C rostral is to the left and medial is down. Nerve exit points are arrowed. (A) Descending tectal axons are labelled green and run just medial to the trigeminal sensory axons (labelled red), coursing around the motor exit point (denser label). Trigeminal motor neurons are also labelled red. (B) Trigeminal sensory axons and motor neurons are labelled green from the nerve root in r2, and the sensory axons and motor neurons labelled from the VII/VIIIth nerve root in r4 are labelled red. The red axons consist of facial and vestibulo-acoustic afferents, most of which lie lateral to the trigeminal axons. In this specimen, two r4 axons lie in unusual medial positions. (C) Facial and vestibulo-acoustic sensory axons are labelled red and these overlap with the glossopharyngeal and vagal sensory axons which are labelled green. (D) Diagrammatic summary of descending tectal and sensory axon organisation. Tectal axons are orange, trigeminal axons are green, facial and vestibulo-acoustic are red and glossopharyngeal/vagal are blue. Tracts end with arrows as some extend further at later stages of development. Nerve exit points are hatched. Bar is 100 µm for A and C, 50 µm for B.

conjugated dextrans the organisation of the sensory tracts becomes apparent and is summarised in Fig. 2.

In rhombomeres 2 to 5, the most lateral axons are the facial and vestibulo-acoustic axons that enter r4 (Fig. 2B). Some of these afferents run caudally into r6 and rostrally as far as r2. In r6 and caudally, the glossopharyngeal afferents are the most lateral axons. There is a little rostrocaudal overlap with the caudal facial afferents (Fig. 2C). Just medial to facial and glossopharyngeal afferents are the trigeminal afferents (Fig. 2A). These form the longest primary afferent tract of the hindbrain, extending up into r1 and down into the cervical spinal cord. In r1 they are the most lateral axons as the facial axons do not extend beyond r2.

### Summary of axon positions and pathways

There are two principal longitudinal pathways through the hindbrain. Each one contains both ascending and descending axons originating from neurons located at all rostrocaudal levels of both sides of the hindbrain. Axon growth is predominantly aligned along either or both the circumferential and longitudinal axes of the neuroepithelium, with few axons taking oblique routes.

### Broad classes of neurons

We have identified neurons according to their position and the pathway selected by their axons. Because most neurons are still in the process of extending their axons at the stages examined, no comment can be made about the eventual length or target specificity of their processes. Similarly, with the exception of some of the early differentiating basal cells, most of the neurons have elaborated few if any dendritic processes. Thus, as development proceeds further, these basic neuronal classes may become subdivided according to the more mature phenotypic criteria of post-synaptic targets, dendritic morphology and neurotransmitter content. We have named the cell types with a simple three letter code that describes their axonal pathway. The first letter is either an **i** or a **c** to indicate an ipsilateral or a contralateral projection, the second letter is either an **a** or a **d** to indicate an ascending or a descending projection and the third letter is either an **m** or an **l** to indicate the projection is in the mlf or the llt. Thus an **iam** cell has

an ipsilateral ascending axon in the mlf and a **cdl** cell has a contralateral descending axon in the llt.

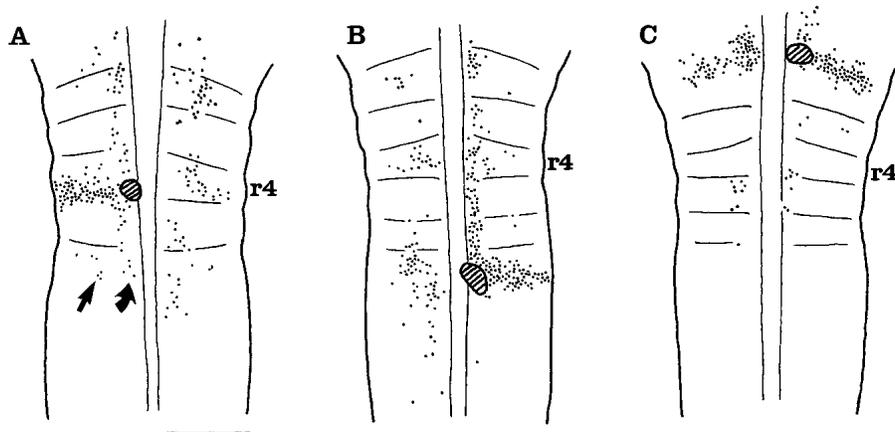
### Neurons with ipsilateral descending axons in the mlf (**idm** cells)

Together with cells with contralateral descending mlf axons, these are the earliest neurons to differentiate in the hindbrain (Lumsden and Keynes, 1989), and most will become the reticulospinal cells of older embryos (Glover and Petursdottir, 1991). When filled with HRP, these cells present the most mature morphologies of all classes labelled at stages 17-21. Examples are found at all levels of the developing hindbrain, although they differentiate first and are present in larger numbers, in the even-numbered rhombomeres (Figs 3A,B, 4; Table 1). Their cell bodies are largely restricted to the basal plate and most lie within its most medial half (Figs 3, 4). Their dendrites ramify within the marginal zone medial to the llt. For the majority of these cells, the elongated cell body, dendrites and proximal axon are all oriented circumferentially (i.e. mediolaterally in flat whole mounts, Fig. 4C,D), but the cell bodies of some of the neurons included in this category are aligned along the rostrocaudal axis, often with long rostral processes within the mlf (Fig. 4B,D). These rostrocaudally aligned neurons lie close to the floor plate just dorsal or dorsolateral to the mlf, their dendrites are largely restricted to the mlf and are

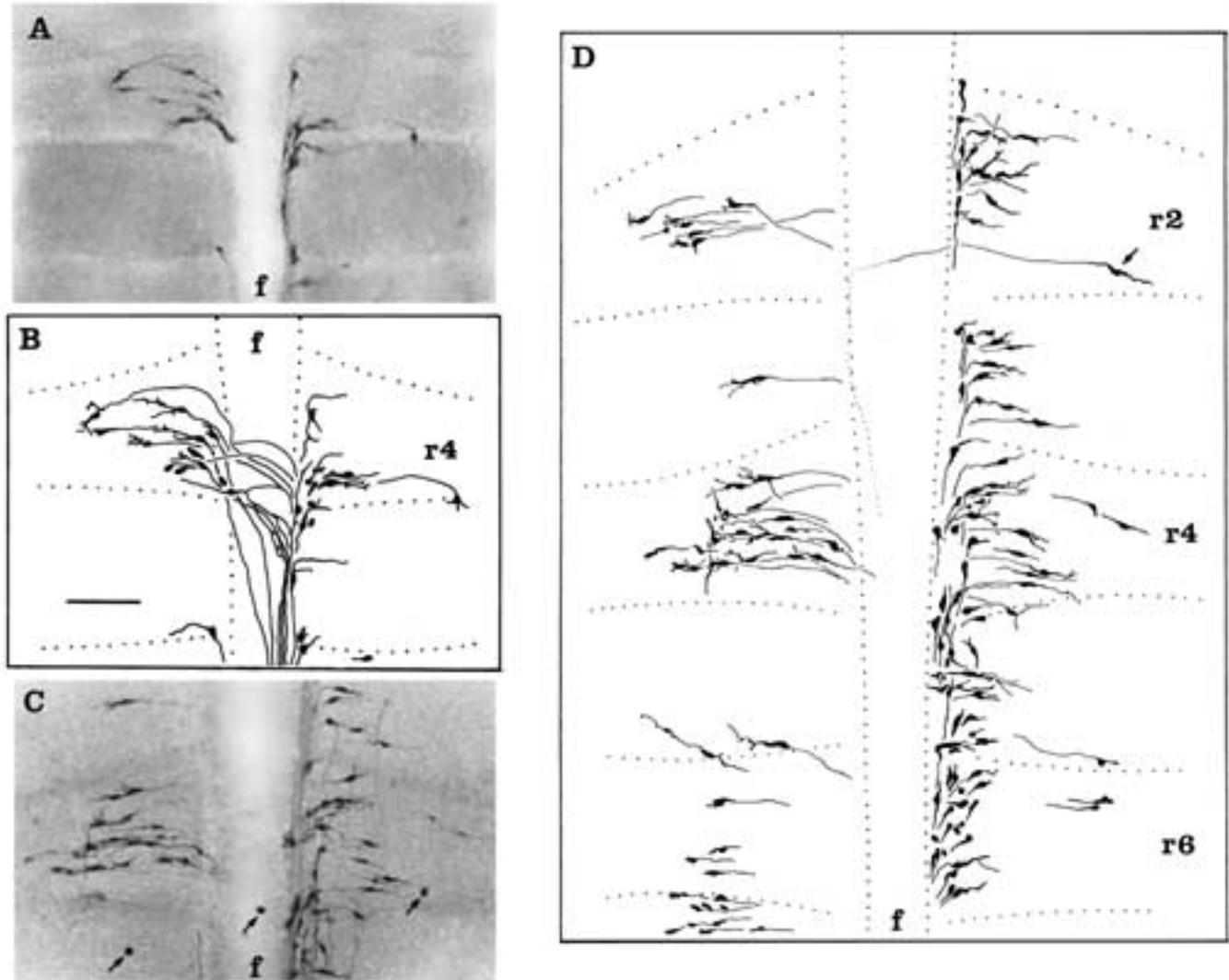
**Table 1. Maximum number of each cell type labelled with HRP in each rhombomere at stage 20**

	r1	r2	r3	r4	r5	r6	r7	r8
<b>idm</b>	15	34	12	23	19	27	13	12
<b>cdm</b>	14	54	12	28	11	11	22	15
<b>iam</b>	4	5	5	13	17	14	9	6
<b>cam</b>	10	6	3	13	23	16	22	22
<b>idl</b>	36	28	5	18	4	7	0	0
<b>cdl</b>	2	2	0	1	0	0	0	0
<b>ial</b>	0	1	1	7	5	8L	1L	0L
						1M	7M	5M
<b>cal</b>	0	1	0	0	1	0	0L	0L
							1M	5M

Data compiled from 37 preparations, including at least one HRP application to every segmental level from caudal midbrain to rostral spinal cord. The **ial** and **cal** cells are subdivided into lateral (L) and medial (M) types.



**Fig. 3.** Typical CL drawn distributions of ascending and descending mlf neurons when filled from middle (A, stage 18), caudal (B, stage 20) and rostral (C, stage 20) HRP applications (hatched areas). In general contralaterally projecting mlf cells are more lateral than the ipsilaterally projecting cells, but as shown in A this relationship does not hold for the more caudal ascending mlf cells as here **iam** cells can occupy both medial (curved arrows) and more lateral positions (straight arrows). r4 is rhombomere 4. Rostral is up. Bar is 500  $\mu$ m.



**Fig. 4.** Development of **idm** and **cdm** cells in r4 from stage 17 (A,B) to stage 20 (C,D). HRP was applied to the right-hand side mlf at the r7/8 boundary for A and B and the r6/7 boundary for C and D. Note that the **cdm** cells (left-hand side) are shifted laterally in the older specimen. The specimen in A is reproduced as a CL drawing in B to illustrate the variability in the angle of crossing of the commissural axons. The specimen in C is drawn in D and illustrates the continuous column of **idm** cells (right side) in the basal plates of r2 to r6, and the more lateral **cdm** cells, most of which are filled in r2 and r4. Arrowed cell in r2 has an unusual axon that crosses the floor plate (f) twice to join the ipsilateral mlf. In D many of the **idm** cells adjacent to the floor plate are aligned along the rostrocaudal axis while the more lateral cells tend to be aligned circumferentially. Arrows in C are red blood cells. Rostral is up. Bar is 110  $\mu\text{m}$  for A and C, 100  $\mu\text{m}$  for B and D.

less elaborate than those of the circumferentially aligned cells. Some cells have a morphology that is suggestive of a transitional form from a rostrocaudal to a circumferential alignment, thus the two morphologies may reflect differing stages of maturity rather than two distinct cell types.

#### Neurons with contralateral descending axons in the mlf (**cdm** cells)

Like the **idm** neurons, these cells differentiate early within the hindbrain. Present in approximately similar numbers to the **idm** cells, **cdm** neurons are also more numerous in even-numbered rhombomeres than in odd-numbered rhombomeres. Their cell bodies are largely confined to the basal

plate (Figs 3A, 4D), but a few labelled cells are found in the medial half of the alar plate. At stages 16 and 17, most of these cells lie close to the floor plate (Fig. 4A,B) and like **idm** cells some are circumferentially and some are rostrocaudally aligned. Axons from rostrocaudally aligned cells tend to cross the floor plate at more oblique angles than axons from circumferentially aligned cells. By stage 19, rostrocaudally aligned cells close to the floor plate are rare; most **cdm** cells are now found in positions that are characteristically more lateral than those of the ipsilaterally projecting **idm** cells (Figs 3B, 4D). By stage 20, their dendrites ramify in the marginal zone at the level of the llt, i.e. more lateral than **idm** cell dendrites.

### Neurons with ipsilateral ascending axons in the mlf (**iam** cells)

Neurons with ipsilateral ascending axons in the mlf are broadly similar to those described with ipsilateral descending mlf axons (**idm** cells) but are fewer in number, especially in the rostral rhombomeres (Table 1; Fig. 3C). Like the **idm** neurons, **iam** cells are medial and may be subdivided into rostrocaudally and circumferentially aligned cells. These cells have some dendritic processes, but in general appear less mature than **idm** and **cdm** cells.

In r4 and caudally, a second class of **iam** neuron may be present. These cells lie in the medial half of the alar plate and appear as a longitudinal column separated from their more medial counterparts (Fig. 3A). Although especially prevalent in the more caudal rhombomeres, a few examples of these more lateral **iam** cells have been found in the rostral rhombomeres, they are thus not a diagnostic feature of the caudal rhombomeres.

### Neurons with contralateral ascending axons in the mlf (**cam** cells)

These usually bipolar cells are present in similar numbers and have a similar rostrocaudal distribution to the **iam** cells. In the rostral rhombomeres, they lie lateral to the **iam** cells, but in r5 and caudally this positional segregation is less apparent because here some **cam** cells are more medial, and (as described above) some **iam** cells are more lateral than their rostral counterparts (Fig. 3A).

### Summary of mlf neurons

Basal plate neurons with axons in the mlf fall into in four categories, according to whether they ascend or descend and whether or not they cross the midline before doing so. Those whose axons descend in the ipsilateral or contralateral mlf are the first to differentiate in the hindbrain. By stages 17-21, they already have elaborate dendritic processes. They are more numerous in even-numbered rhombomeres than in odd. Neurons with ascending axons occupy the same region of the basal plate but they are less numerous and their dendritic processes are less mature than those with descending axons.

### Neurons with ipsilateral descending axons in the llt (**idl** cells)

The earliest and most numerous neurons with ipsilateral descending llt axons are in r1 and r2 (Fig. 5A; Table 1). Their cell bodies lie at the level of, or just medial to, the tectobulbar axons (Fig. 5A). They are usually bipolar cells with a short dorsal process and are essentially oriented circumferentially but with a slight caudal slant. Axons are initially directed medially but quickly curve caudally to join the medial edge of the tectobulbar axon tract.

Similar cells can also be found in rhombomeres 3, 4, 5 and 6 (Fig. 5B). Here the cells occasionally lie more lateral to the llt axons. This class of cell has not been filled caudal to r6.

### Neurons with contralateral descending axons in the llt (**cdl** cells)

These cells (Fig. 5A',B',C') are first seen in r2 at stage 17

and at later stages have also been filled in r1 and more rarely in r4. A maximum of only 2 cells per rhombomere were labelled. The cell bodies are bipolar, circumferentially oriented and generally positioned midlaterally.

### Neurons with ipsilateral ascending axons in the llt (**ial** cells)

This class of neuron is found most often caudal to r3 and can be divided into two cell types. In the medial alar plate of r4, 5 and 6 typical bipolar neurons are present with axons that run directly into the llt (Fig. 5C). Caudal to r5 a second class of **ial** neuron appears. These cells lie close to the floor plate and have laterally directed dendritic processes on their cell bodies and proximal axon (Fig. 5D). Their rostrally directed axons run for up to 100  $\mu$ m in the mlf before curving laterally to join the llt. A few of these cells are present in r6 but they are more numerous caudally. They appear to form a column of regularly spaced cells extending down into the cervical spinal cord (Fig. 5E).

### Neurons with contralateral ascending axons in the llt (**cal** cells)

Only two examples of these cells, one in r2 and one in r5, have been found rostral to r7. In r7 and caudally a few medial cells with contralateral llt axons are present. The contralateral portions of their axons usually run for a short distance in the mlf before following a curved path to the llt in a similar manner to the medial **ial** cells described above (Fig. 5D,E). The **cal** cells are less numerous than the medial **ial** cells.

### Summary of llt neurons

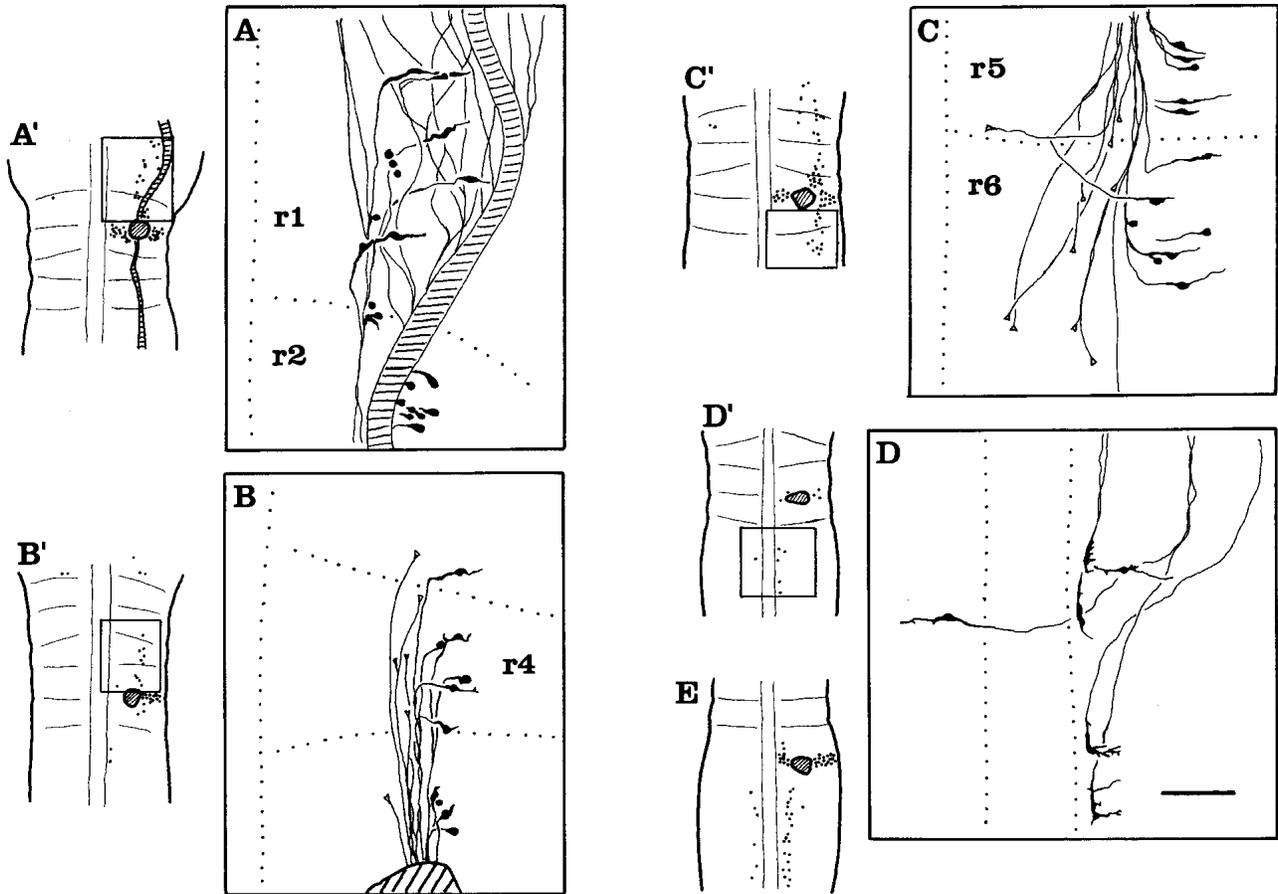
In general, neurons with axons in the llt develop later than those with axons in the mlf. Examples of cells with elaborate dendritic processes are rare. The majority of llt neurons lie in the alar plate. Like the mlf cells, llt neurons can be divided into four basic types depending on their ipsilateral or contralateral and ascending or descending projection. Only the ipsilateral descending llt cells are present in similar numbers to the mlf cells (Table 1). Neurons of the other three types, especially the contralateral llt cells, are rare.

### Lateral neurons with medial axons

The most lateral region of the alar plate contains many neurons with simple, near spherical cell bodies and medially directed axons. HRP stabs into this region label the axons of these cells and their growth cones, which are often found in the floor plate but have seldom grown as far as the contralateral mlf. No axons from these cells are found in the ipsilateral longitudinal tracts but their contralateral destination is not yet apparent. Their cell bodies lie close to the central axons of the primary sensory neurons, suggesting they may be destined to become second order sensory neurons.

### Rare neurons of uncertain classification

The axons of a few cells were seen to run from one of the major longitudinal tracts to the other at some point in their trajectory. These neurons therefore do not easily fall into the simple categories described above. In all cases (17 cells



**Fig. 5.** Neurons from the lateral longitudinal tract. A and B are CL drawings of **idl** cells within boxed regions in A' and B' respectively. Axons from the r1 cells in A typically join onto the medial edge of the descending tectal axons (hatched axon tract). Ascending axons ending in growth cones (shown diagrammatically as triangles) are positioned just medial to the **idl** cells of r3, 4 and 5 in B. C and D are CL drawings of **ial** cells within boxed areas in C' and D' respectively. C shows some laterally positioned **ial** cells in r5 and r6 and some descending lateral axons with growth cones just medial to these cells. One axon has made a sharp turn to grow down the r5/6 boundary. D shows five medial **ial** and one medial **cal** cell each with axons that curve laterally from the mlf to run in the llt. E is a low power view showing a column of medial **ial** and **cal** cells extending into the rostral spinal cord. Bar is 100  $\mu\text{m}$  for A-D.

in 6 specimens), these are basal cells whose axons first run for a variable distance in the mlf before curving laterally into the llt. This axonal behaviour may represent aberrant growth which is later eliminated, rather than genuine but rare neuronal phenotypes. As described in an earlier section, many mlf axons are in fact displaced laterally from the mlf towards the llt. The large majority of these intermediate axons do not however reach the llt, but rather run parallel to it within the relatively axon sparse region between llt and mlf.

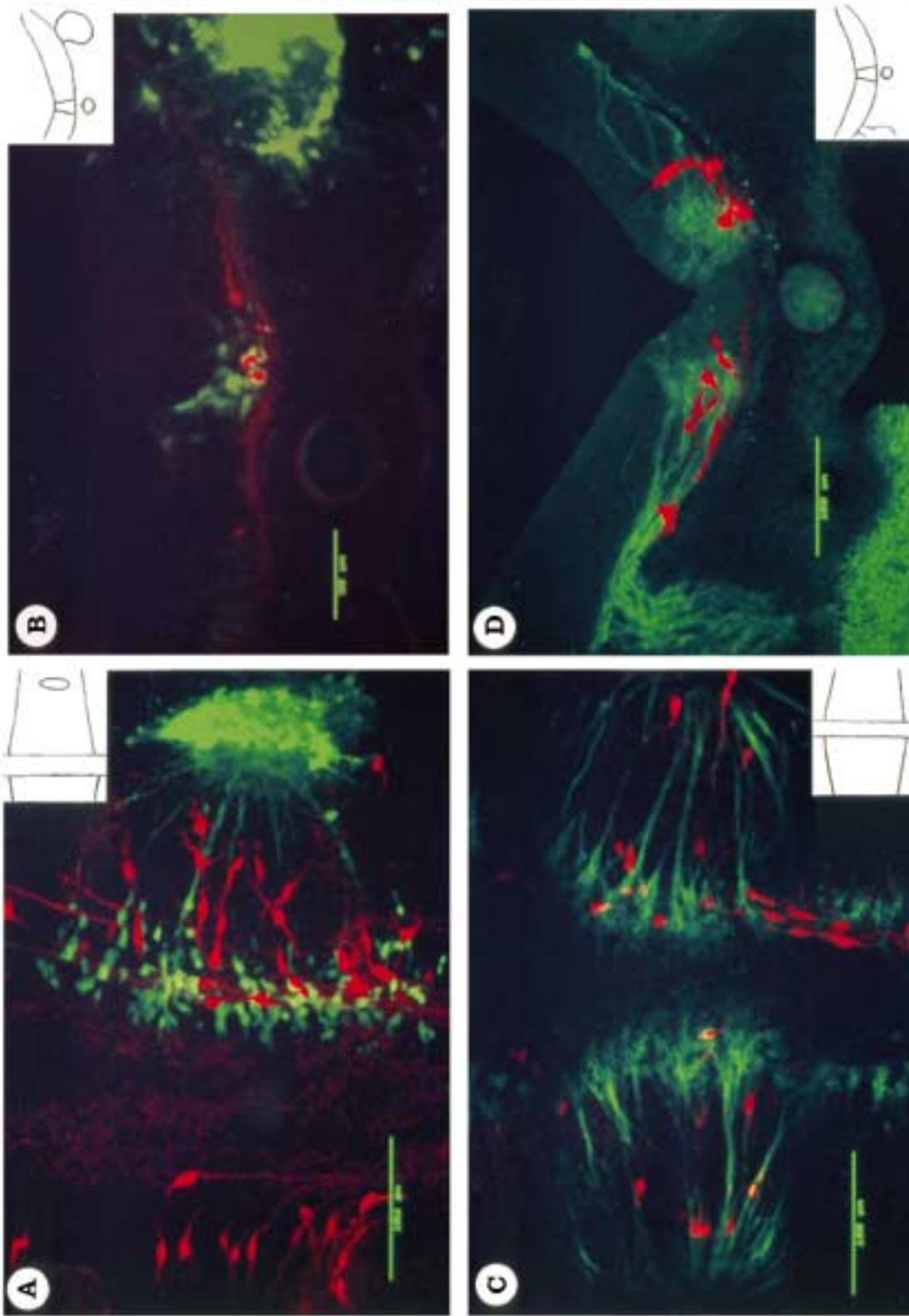
#### Spatial overlap of broad classes of neurons

On the basis of retrograde tracing experiments, certain regions of the hindbrain neuroepithelium (such as the basal plate of r4) would appear to produce a variety of neuronal phenotypes. To discover the extent to which diverse neuronal types are either mixed together in such regions or else are segregated from each other within discrete subregions, we performed a number of double tracing experiments using fluorescent dextrans and/or specific antibodies (Fig. 6). By applying LFD to both the ipsilateral and contralat-

eral mlf at the r5/6 border and LRD to the nerve root of r4 the distributions of mlf-projecting neurons and motor neurons has been compared (Fig. 6A,B). Extensive overlap of motor neurons, **idm** neurons and **cdm** neurons was noted in the most medial region of the basal plate at stage 16/17. An identical distribution (Fig. 6C,D) was seen when the motor neurons were stained with DM2, a monoclonal antibody that recognises a surface glycoprotein (Burns et al., 1991) that is expressed on branchiomotor neurons (Guthrie and Lumsden, 1992) before and during initial axon outgrowth (H. Simon, S. Guthrie and AL, unpublished data). Fig. 6D shows that **idm** neurons (right side) and **cdm** neurons (left side), both labelled from the right mlf, lie within the motor column.

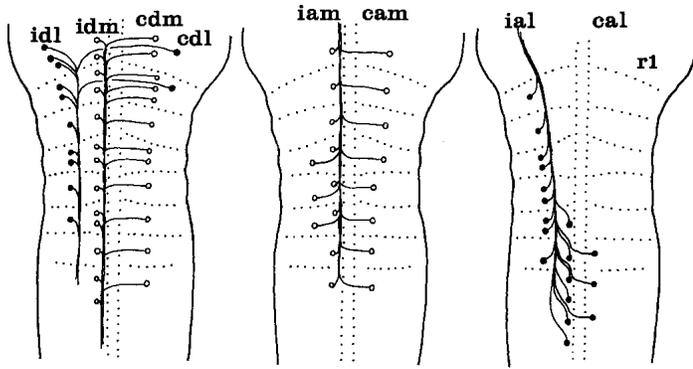
#### DISCUSSION

During development the hindbrain is transiently subdivided into a segmental series of compartments called rhombomeres. Once delineated by interfaces with its neighbours,



**Fig. 6.** Double labelled preparations of r4 to show overlapping domains of motor neurons, **idm** and **cdm** cells. (A,B) A flat-mount (A) and a 75  $\mu$ m vibratome section (B) through preparations that have been double labelled with (i) LFD from the vii/viii nerve exit point (motor neurons, green) and (ii) LRD from both the ipsilateral and contralateral mlf (**cdm** and **idm** neurons, red). (C,D) A flat-mount (C) and a 75  $\mu$ m vibratome section (D) through preparations that have been double labelled with (i) indirect immunofluorescence

using DM2 primary antibody (motor neurons, green) and (ii) LRD retrograde tracing from the right side mlf (**cdm** and **idm** neurons, red). Insets in A and C show positions of floor plate and rhombomere boundaries, and insets in B and D show outlines of neuroepithelium with floor plate and underlying notochord, and vii/viii nerve exit point. Rostral is up for A and C, dorsal is up for B and D.



**Fig. 7.** Summary diagrams to show the distribution and relative positions of the eight classes of neuron described here at stage 20. A guide to relative abundance is given by the number of cells illustrated in each rhombomere, actual numbers are given in Table 1. Unfilled cells have axons in the mlf and filled cells have axons in the llf. Note that cells within a given class are loosely arranged into longitudinal columns and that most rhombomeres contain some cells from most of the classes.

each rhombomere behaves as a unit of lineage restriction (Fraser et al., 1990). Genetic analyses suggest that each rhombomere's positional identity could be encoded by a combination of *Hox* and related genes (Graham, 1992). In the chick, cranial nerve roots are associated with specific rhombomeres and the motor neurons of particular cranial nerves derive from specific pairs of rhombomeres (Lumsden and Keynes, 1989). Neuronal differentiation proceeds in a rhombomere-specific pattern (Lumsden and Keynes, 1989). The rhombomeres thus appear to be programmed to develop along particular, position-specific pathways and may provide an important mechanism that allows for regional diversification in the CNS. In this paper, we have investigated the emerging neuroanatomy of chick rhombomeres in detail and have found that the basic neuronal composition of each rhombomere (with the possible exception of r7 and r8) is remarkably consistent from one rhombomere to the next (summarised in Table 1 and Fig. 7); thus each may develop according to an initially highly conserved plan, generating similar axonal pathways and a set of similar basic neuronal types. Regional differences that are apparent at these early stages appear to arise from variations on a basic theme rather than by abrupt phenotypic changes.

We have based these conclusions on the systematic analysis of neuronal position and axonal projection patterns of all ascending and descending neurons at stages of development when rhombomeres are most prominent. Our data support the view, proposed from the analysis of reticulospinal neurons in the zebrafish larva, that each rhombomere may have evolved by the duplication of and subsequent divergence from a primitive segmental unit (Metcalf et al., 1986). In the zebrafish, the identification of individually distinct neurons has led to the proposal that segmental homologues are present within clusters of hindbrain neurons and that intersegmental variation of neuronal organisation could have arisen through changes in the ipsilateral or contralateral projection of axons as well as the

deletion and addition of neuronal phenotypes. Metcalfe et al. (1986) argue that such changes have led to each rhombomere containing a unique set of the different neuronal classes. Individually identifiable neurons are probably not present in the chick hindbrain but the similarities of neuronal position, axonal projection pathways and simple dendritic morphologies suggest that groups of similar neurons in different rhombomeres are also segmental homologues. Diversification of chick rhombomeres becomes apparent when the relative numbers and balance of cells of each neuronal type is considered. For example, although the hindbrain contains a continuous column of basal *idm* cells at stage 20 (Fig. 4D), more *idm* cells are present in r2, r4 and r6 than in the intervening rhombomeres (Table 1; Figs 3B, 4D). While in r2 there are more *cdm* cells than *idm* cells, in r6 it is the *idm* cells that predominate. Also, ipsilateral descending *llt* cells (*idl* cells) are numerous in r1, r2 and r4, but are scarce in r3, r5 and r6, and apparently absent from r7 and r8. The relative abundance of some neurons in the even-numbered rhombomeres may be simply a reflection of their advanced neuronal differentiation (Lumsden and Keynes, 1989). If true then neuronal numbers in odd-numbered rhombomeres may catch-up at later stages. This is probably not the case, however, as reticulospinal neurons have been shown to be clustered into three groups in older chick embryos (Glover and Petursdottir, 1991) as well as in other species (lamprey, Rovainen, 1967; zebrafish, Kimmel et al., 1982; *Xenopus*, van Mier and ten Donkelaar, 1984).

A number of features of neuronal organisation suggest that the developmental plan of rhombomeres 7 and 8 may be significantly different to that of the rostral rhombomeres. First, caudal to r6 two new cell types appear that are also present in the rostral spinal cord. These are the medial neurons with axons that ascend in either the ipsi- or contralateral *llt* (Fig. 5D,E). Their axons usually run for a short distance in the mlf and then take a characteristically curved trajectory from mlf to llf; a pathway not taken by any more rostral cells. Secondly, *idl* cells have not been found in r7 and r8 but are present in reasonable numbers in other rhombomeres (Table 1). Third, rhombomere boundaries caudal to r6 are less well-defined morphologically and whole-mount neurofilament stains reveal a near continuous, non-segmented overall neuronal and axonal organisation that is not obviously different to that of the rostral spinal cord. This region may thus have a transitional organisation with some features of the hindbrain (e.g. dorsal branchiomotor exit points) and some features of the non-segmented spinal cord. A transitional region is also apparent in the caudal hindbrain of the zebrafish (Hannemann et al., 1988; Trewarow et al., 1990).

### Regional diversification increases later in development

As the chick hindbrain develops beyond the stages studied here its complexity increases and a number of more distinct regional specialisations become apparent (Glover and Petursdottir, 1988, 1991; Petursdottir, 1990). For example, even at stage 20.5 HRP injections into the cervical spinal cord show that reticulospinal neurons are clustered into midbrain, isthmic (or pontine), bulbar and vagal groups

(Glover and Petursdottir, 1991). It is likely that the **idm** and **cdm** cells of successive even-numbered rhombomeres are the forerunners of these three clusters of reticulospinal neurons; this therefore suggests that the **idm** and **cdm** cells described here in r3 and r5 are destined to have shorter axons than those in r2, r4 and r6. Clustering of reticulospinal cells is thus initiated early by the precocious development of **idm** and **cdm** cells in r2, r4 and r6 and may be sharpened subsequently by axon retraction or cell death in the adjacent regions.

By stage 24, the nucleus of the lateral vestibulospinal tract can be identified at the level of the eighth nerve as a collection of lateral cells with ipsilateral descending axons in the llt (Glover and Petursdottir, 1991). This nucleus probably includes the r4 **idl** cells described in this paper. However, because we also find similar **idl** neurons in r3, r5 and r6 the eventual restriction of this nucleus may result from a later specialisation of r4, possibly from a local influence of the vestibular afferents.

Two further mechanisms to generate regional diversity should also be considered. The most obvious is that later born cells are generated in region-specific patterns and the second is differential cell migration. For example, up to stage 21, in the region of the vestibular nerve, nearly all cells with axons in the mlf are located in the basal plate, yet by embryonic day 11 two neuronal groups with mlf axons are present in the alar plate (the nuclei of contralateral medial vestibulospinal tract and ipsilateral medial vestibulospinal tract of Glover and Petursdottir, 1991). These could arise from a lateral migration of previously basal cells, or from the later generation of a new alar cell type. As discussed above for the lateral vestibulospinal nucleus, this regional specialisation may result from local interactions with the incoming vestibular afferents.

### Motor neurons and vestibulo-acoustic efferent neurons

In addition to the neurons described here the early hindbrain also contains motor neurons and vestibulo-acoustic efferent neurons. The motor neurons can be divided into two classes. First, the somatic motor neurons that innervate the eye muscles (trochlear motor neurons in r1, abducens motor neurons in r5 and 6) and the tongue muscles (hypoglossal motor neurons in r8) form a discontinuous medial column of cells. Their axons (except the unusual dorsally looping trochlear axons) exit the hindbrain via ventral roots that lie medial, alongside the floor plate. Secondly, with the exception of r1, the hindbrain contains a continuous column of branchial and visceral motor neurons whose axons exit via the dorsolateral cranial nerve roots (trigeminal in r2 and 3, facial in r4 and 5, glossopharyngeal in r6 and 7, and vagal and accessory in r7 and 8). Vestibulo-acoustic efferent neurons differentiate in the basal plate of r4, like the facial motor neurons, and have axons that exit in the dorsal cranial nerve root of r4 (Simon and Lumsden, unpublished data). The early organisation of these three classes of efferent neuron demonstrates a greater degree of rhombomere diversity than does the organisation of the longitudinally projecting neurons. For instance, the vestibulo-acoustic efferent cells are particular to r4 (later migrating into the contralateral r4 and r5, Simon and Lumsden,

unpublished data), somatic motor neurons are particular to r1, 5, 6 and 8, and, although the dorsally exiting motor neurons are present in each rhombomere except r1, they are differentially specified such that motor neurons in adjacent pairs of rhombomeres exit into particular nerve roots in r2, r4, r6 and r7 (Lumsden and Keynes, 1989; Guthrie and Lumsden, 1992).

Our analysis of hindbrain projection neurons is based solely on the early phenotype of these cells, as revealed by cell shape, position and axonal pathway choice. As development proceeds, cells with initially similar phenotypes may be destined to follow more divergent paths of maturation, perhaps according to (or allowing for) different presynaptic and postsynaptic target specificities. An analysis of the distribution of cell surface molecules and of neurotransmitter content may reveal whether any such diversification is apparent at early stages when morphology alone reveals none.

### Positional overlap and segregation of cell types

In this study, we describe eight basic neuronal types within the chick embryo hindbrain. Motor neurons and vestibulo-acoustic efferent neurons are also developing in the basal plate at this time and the most lateral region of the alar plate contains neurons whose medially directed axons have not yet extended beyond the floor plate, but whose position is suggestive that they may later become second order sensory neurons. The hindbrain thus contains at least 11 different types of neuron at stages 17-21. Many of these neuronal classes are loosely clustered in segmental groups that appear to be aligned into columns that extend through the length of the hindbrain. Some of the columns occupy distinct mediolateral positions but for others there is pronounced overlap. The most obviously segregated columns are those in the alar plate where the most lateral are the circumferential neurons that we tentatively suggest are second order sensory cells, and just medial to these are the neurons whose axons project into the llt. In the basal plate, the situation is more complicated. By stage 20 most **cdm** cells lie more lateral than the **idm** cells, but they reach this position by migrating from an earlier more medial location (see Figs 4, 6D). The spatial domains of **idm** and **cdm** cells are thus initially overlapping. There is also considerable spatial overlap between both the **idm** and **cdm** cells and the hindbrain motor neurons (Fig. 6) which differentiate close to the floor plate (Moody and Heaton, 1981; Guthrie and Lumsden, 1992).

Lineage analyses (Wetts and Fraser, 1988; Holt et al., 1988; Walsh and Cepko, 1992) and heterochronic grafting experiments (McConnell and Kaznowski, 1991) suggest that in many vertebrate systems neuronal phenotype is determined not by lineage, but by local environmental interactions within the neuroepithelium during or after the last cell division. However, the situation may be somewhat different in the early chick hindbrain where lineage analysis demonstrates that the majority of neuronal precursors generate, after three to four successive multiplicative divisions, neurons of only a single phenotype (Lumsden, Clarke, Keynes and Fraser, unpublished data). Together with the present demonstration that multiple neuronal phenotypes are interspersed with each other in the basal plate of the

hindbrain, this suggests that here phenotype decisions might be made in early precursors and are remembered through several rounds of mitotic division and clonal dispersal. To evaluate this hypothesis, more precise data are required on the position and movements of neuronal precursors and their lineal descendants. In the insect CNS, neuronal phenotype is determined in part by position and cell interactions and in part by lineage (Doe and Goodman, 1985; Kuwada and Goodman, 1985), perhaps similar mechanisms operate in the chick hindbrain.

## Conclusions

We have found that early in its development, the hindbrain of the chick embryo displays a uniformity of neuronal composition between successive rhombomeres that is remarkable considering the regional specialisations that later emerge in this part of the brain (Williams et al., 1989). The iteration of a comparatively uniform set of early cell types suggests that segmentation of the hindbrain has consequences that reach beyond the mere allocation of subregions. Rather than the rhombomeres each being distinctly and uniquely specialised, the repeated units represent, at the cellular level, a theme on which local variations are subsequently built. This is consistent both with absence of single rhombomere-specific expression of known genes that could control segment identity (*Hoxb-1* expression in r4 is exceptional) and with the capability of retinoic acid to transform the phenotype of one rhombomere into that of another (Marshall et al., 1992), presumably by spatial alteration of elements of the *Hox* code.

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