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The Relationship between Rhombomeres and Vestibular Neuron Populations as Assessed in Quail–Chicken Chimeras

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The aim of this study was to evaluate the role segmentation plays in the determination of neuronal identity in the hindbrain. We focused on two specific sets of hindbrain neurons, namely, the vestibulospinal and vestibulo-ocular neurons, which comprise distinct groups that can be identified and distinguished by virtue of their axonal projection pathways. The relationship between rhombomeres and the vestibular neuron groups was assessed by a combination of quail–chicken chimeric grafting and selective retrograde axonal tracing. Individual quail hemirhombomeres were transplanted homotopically and isochronically into a chicken embryo host. Subsequently, vestibulospinal and vestibulo-ocular neurons with specific axon trajectories were labeled retrogradely with biotin-conjugated dextran-amines. The relationship between the spatial domains of the vestibular neuron groups and rhombomere-derived domains had the following features: (1) some groups were derived from single rhombomeres; (2) some groups were derived from multiple contiguous rhombomeres; (3) two groups occupied domains that could not be defined in terms of whole rhombomere lengths; (4) some groups spanning multiple rhombomeres exhibited an internal cytoarchitectonic organization that related to individual rhombomeres; and (5) some groups exhibited limited boundary violation. These results support the notion that positional information within defined domains of the neural tube provides a groundplan for the regional determination of neuronal identity and axon pathfinding, and that hindbrain segmentation contributes to this process. But they also indicate that segmentation is not the only mechanism that defines the rostrocaudal domains of neuron types. Moreover, they emphasize that the relationship between rhombomeres and neuronal determination cannot be couched simply in terms of segmental iteration or of bimeric (paired rule) specification. © 1998 Academic Press

INTRODUCTION

The generation of diverse yet regionally specific neuronal phenotypes in the central nervous system implies the existence of positional determinants with stereotyped distributions within the neural tube. In the hindbrain region, the neural tube is partitioned into a series of neuromeres, called rhombomeres (Lumsden, 1990; Vaage, 1969). Each rhombomere expresses a unique combination of transcription factors, thus endowing the rhombomeric domains with distinct molecular cues that could in principle establish rhombomere-specific patterns of neuronal differentiation (Keynes and Krumlauf, 1994). Indeed, several functionally identified neuronal populations have been found to correlate to rhombomeric domains at the early stages when rhombomere boundaries are visible. These include the motoneurons and parasympathetic neurons of the cranial nerve nuclei (Auclair *et al.,* 1996; Gilland and Baker, 1993; Lumsden, 1990), the cochlear efferent neurons (Fritzsch *et al.,* 1993; Simon and Lumsden, 1993), and certain populations of vestibular neurons (Glover, 1989, 1994, 1996).

In the classical anatomical description of the vestibular system, the vestibular neurons in the hindbrain are grouped into nuclei on the basis of cytoarchitectonic features (cell size, shape, and distribution). More recently, the vestibular neurons have been shown in the chicken embryo to be organized in coherent clusters with characteristic patterns of axon trajectory and termination among motoneuron targets (Fig. 1; see Glover, 1994; Glover and Petursdottir,

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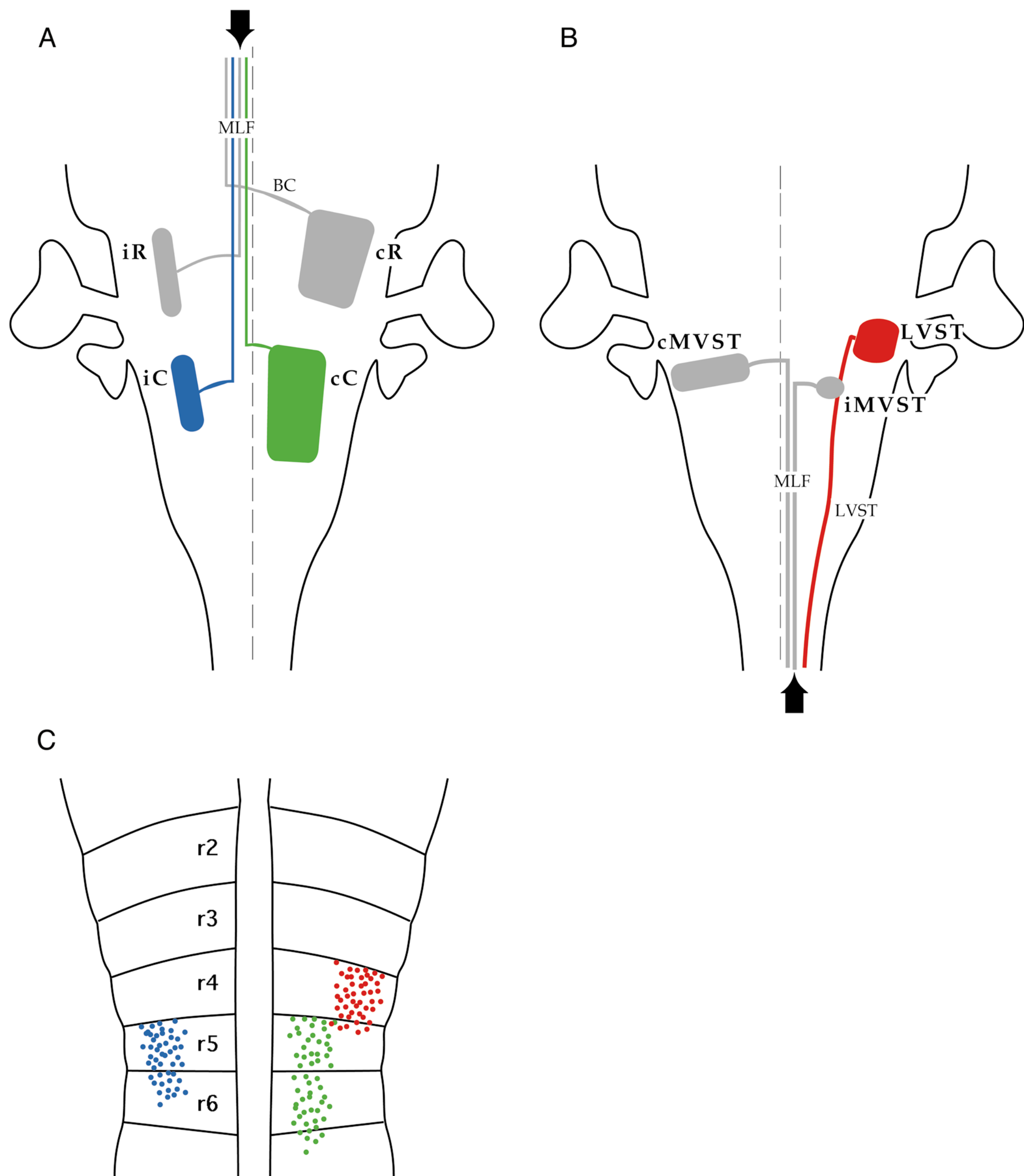

FIG. 1. Vestibular projection neuron groups can be defined on the basis of hodology and occupy specific rhombomeric domains at early stages. (A) The rough locations and axon trajectories of the four hodologically defined groups of vestibulo-ocular neurons in horizontal projection at d11. The groups are named according to the side on which their axons project and their rostrocaudal location (iR, ipsilateral rostral; cR, contralateral rostral; iC, ipsilateral caudal; cC, contralateral caudal). The major axon tracts of projection are the medial longitudinal fascicle (MLF) and the brachium conjuctivum (BC). The arrow indicates the approximate site of tracer application that gives retrograde labeling of these groups. (B) The rough locations and axon trajectories of the three hodologically defined groups of vestibulospinal neurons in horizontal projection at d11. The groups are named according to a nomenclature used in vestibular physiology wherein the vestibulospinal projections are separated into a medial and a lateral vestibulospinal tract (MVST and LVST). The MVST projection originates from two groups, one projecting ipsilaterally (iMVST), the other contralaterally (cMVST). The major axon tracts of projection are the MLF and the LVST. The arrow indicates the approximate site of tracer application that gives retrograde labeling of these groups. (C) The locations of the iC, cC, and LVST groups at earlier stages (about stages 22–25) when rhombomeres (r) are still visible. The individual groups are color-coded as in A and B. The iR, cR, iMVST, and cMVST groups are not shown because they cannot be identified unambiguously at these stages.

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TABLE 1
Summary of Preparations by Labeled Vestibular Group and Grafted Rhombomere

Projection	Side	Labeled groups	r3	r4	r5
v-spinal	Ipsi	LVST, iMVST	—	3 (2)	5 (1)
	Contra	cMVST	—	1 (1)	—
v-ocular	Ipsi	iR, iC	(1)	2 (2)	—
	Contra	cR, cC	(2)	3 (1)	2

Note. The number of completely normal preparations is followed in parentheses by the number of preparations with minimal defects in the basal or floor plates.

1998). The relationship between neurons and their axon trajectories is called "hodology" (from "hodos", the Greek word for "path"). On the basis of this concept, the organization of the vestibular system has been described as a "hodological mosaic," wherein vestibular neurons with specific axonal projection phenotypes are localized to specific, segregated domains of the hindbrain neural tube (Fig. 1; see Glover, 1989, 1994; Glover and Petursdottir, 1998). Recent studies in the zebrafish indicate that hodology as an organizational feature of the vestibular system is likely to be conserved phylogenetically (Baker *et al.,* 1996). Since hodological patterns are the end result of complex molecular interactions between growing axons and potential pathways and targets, a stereotyped hodological organization is likely to be related to regionally specific patterns of gene expression.

At early stages of neural tube development, when rhombomeres are visible, some of the vestibular neuron groups are strikingly correlated with rhombomeric domains (Fig. 1C; see Glover, 1989, 1996). Because of the link between rhombomeric domains and transcription factor expression (Keynes and Krumlauf, 1994), the vestibular hodological mosaic has been proposed as a genetically predetermined pattern of connections from which species-specific variants might be sculpted (Glover, 1994; Glover and Petursdottir, 1998). To test this notion, it is necessary to elucidate the relationship between vestibular neuron clusters and the spatiotemporal pattern of gene expression within the hindbrain. As a first step, we are trying to assess completely the relationship between vestibular neuron groups and rhombomeric domains.

Unfortunately, the mature pattern of vestibular neurons is not fully evident at the stages when rhombomeres are visible (compare Figs. 1A and 1B to Fig. 1C). Some groups are insuffiently populated to allow for reliable demarcation at early stages, and certain groups cannot be identified unambiguously until later stages because of delayed differentiation (Glover and Petursdottir, 1991). Indeed, the overt morphological boundaries between rhombomeres disappear well before all hindbrain neurons have been born and migrated to their definitive positions (Hemond and Glover, 1993). Thus, fate-mapping techniques that indelibly mark individual rhombomeres are required to assess the relationship between neuronal populations and rhombomeric domains in the mature hindbrain. The main goal of the present report was to use the quail–chicken chimera technique to render specific hemirhombomeres visible at late stages of development when all the vestibular groups can be labeled, thus permitting a direct assessment of this relationship.

In a general survey of rhombomeric domains using the quail–chicken chimera technique, Marín and Puelles (1995) found that many mature hindbrain nuclei occupied either single or multiple rhombomere-derived domains. For those stretching over multiple rhombomeres, cytoarchitectonic subdivisions were often correlated to single rhombomeres. In particular, the set of classically defined vestibular nuclei spanned all the rhombomeres, and some of the nuclear subdivisions were roughly in register with rhombomere boundaries. Here, we combine the quail–chicken chimera technique with retrograde labeling of the vestibular neuron groups identified by axon trajectory. This has allowed us to identify how rhombomere boundaries relate to the vestibular hodological mosaic. Our study also provides new information relevant to the question of what kind of "segmentation rules," if any, are at play in the regional determination of neuronal identity in the hindbrain.

MATERIALS AND METHODS

Fertilized chicken and quail eggs were obtained from the animal facility at the University of Murcia or from local suppliers and incubated at 38°C. Homotopic, isochronic grafting of single hemirhombomeres (r3, r4, or r5) was performed as previously described (Marín and Puelles, 1995) on the second day of incubation. The developmental timepoint of the operation was assessed by stage (Hamburger and Hamilton, 1951) and the number of fully formed somites and ranged from stages 10^+ to 12, corresponding to 11–14 somites. The chimeras were then reincubated for 9 to 10 days, reaching stages 36–38, at which time they were removed from the egg into ice-cold chicken physiological saline (137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na phosphate, 5 mM Hepes, 11 mM glucose, pH 7.4) and decapitated. The brain was dissected free and maintained *in vitro* for retrograde axonal labeling with biotin dextran amine (BDA), as previously described (Glover *et al.,* 1986; Glover, 1995). Following labeling, the tissue was fixed in buffered 4% paraformaldehyde or a periodic acid–lysine–paraformaldehyde mixture (McLean and Nakane, 1974) for 6 h and cryoprotected overnight in 20% sucrose in phosphate buffer (pH 7.4, 4°C) containing 0.1–0.2% hydrogen peroxide to quench endogenous peroxidases. Sections were cut on a cryostat at 20–30 μm in the sagittal or horizontal plane and collected onto glass slides. Quail cells were detected by immunohistochemistry. The sections were incubated overnight at room temperature with the QCPN antibody (Developmental Studies Hybridoma Bank, University of Iowa), diluted 1:5 in 0.1% sodium azide, 0.05% Triton X-100 in 0.1M PBS, or for 2 days at 4°C in the same antibody diluted 1:10. Biotinylated secondary antibody treatment and visualization with a Vectastain ABC kit (Burlingame, CA) were standard, leading to nuclear labeling with the antibody and cytoplasmic labeling with

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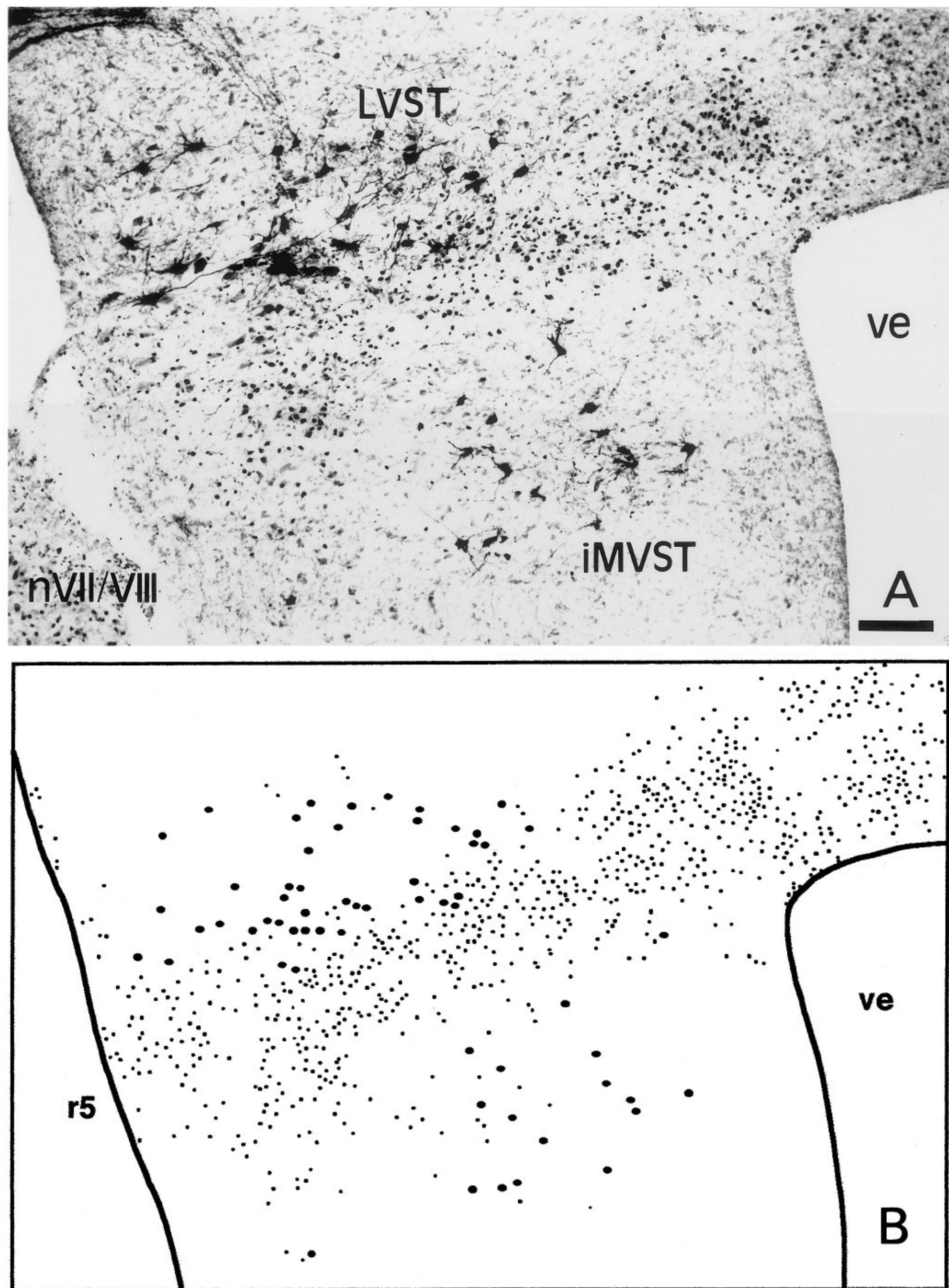

FIG. 2. Combined quail–chick chimeric grafting and retrograde axonal tracing with BDA allows for comparison of projection neuron groups and rhombomeric domains. (A) Horizontal section from a chicken embryo with a graft of a quail hemirhombomere 5 and with BDA injected into the upper cervical spinal cord ipsilateral to the graft. BDA-labeled vestibulospinal interneurons are recognizable by their dark cytoplasmic staining that reveals dendrites and axons. Two identified vestibulospinal groups can be seen: the LVST and the iMVST. Quail cells are recognizable by their immunostained nuclei. (B) Drawing of the same photograph with large dots indicating vestibulospinal neurons and small dots indicating quail nuclei. The quail graft occupies a wedge of the hindbrain, but with some intermingling with adjacent chicken tissue. Scale bar, 250 μm.

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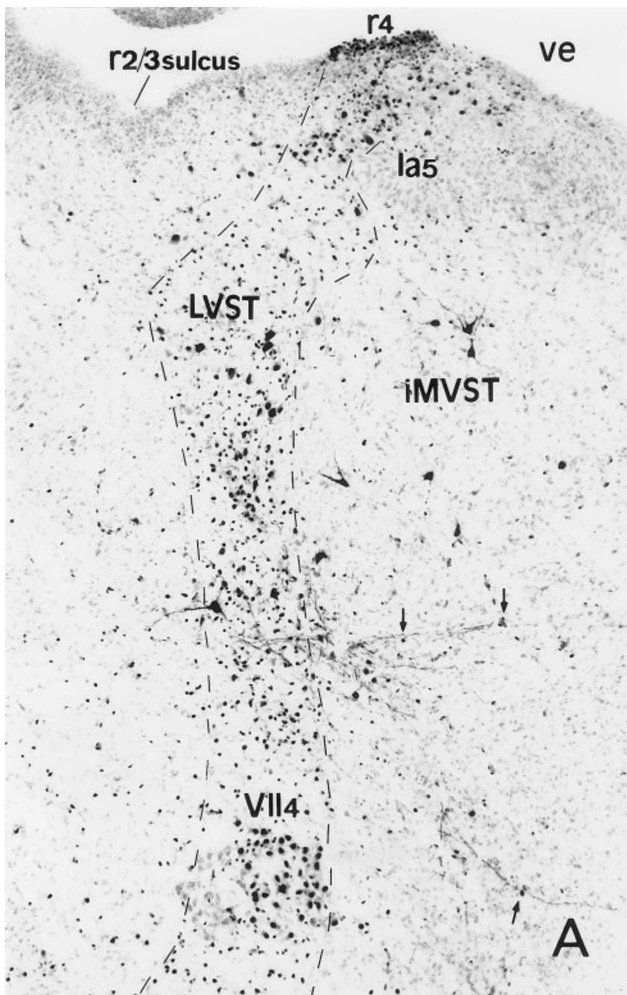

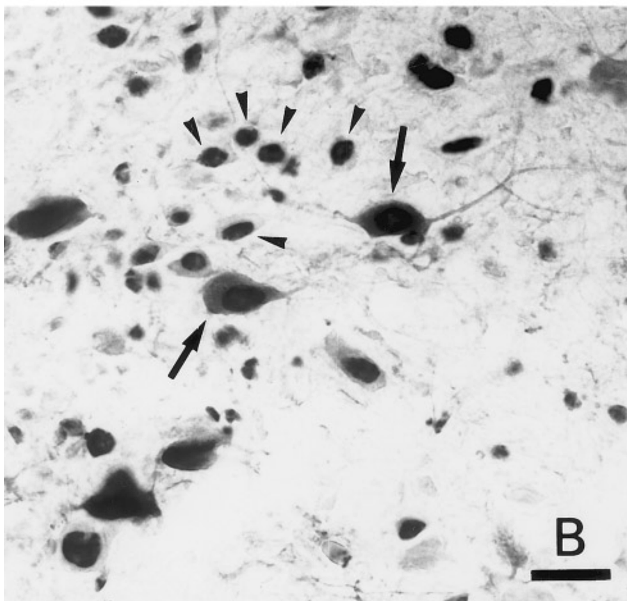

BDA. Some sections did not receive antibody to show BDA-labeled cells only.

Vestibulospinal and vestibulo-ocular neuron groups were identified using criteria defined by Glover and Petursdottir (1991, 1998). Vestibulospinal groups were labeled retrogradely and unilaterally from approximately the first cervical spinal segment, whereas vestibulo-ocular groups were labeled retrogradely and unilaterally from the medial longitudinal fascicle just caudal to the trochlear nucleus (see Figs. 1A and 1B). Based on their axon trajectories and positions, the retrogradely labeled neurons could be assigned to one of three vestibulospinal or four vestibulo-ocular groups.

The overall survival of embryos following the grafting procedure was 55%. In 34 of 38 surviving embryos that developed to stages 36–38, the graft had visibly integrated into the host neural tube, and 26 of these embryos were judged to be either completely normal ($n = 16$) or had only minimal defects in the basal plate or floor plate at the site of the graft ($n = 10$). As the vestibular groups of interest lie predominantly in the alar plate, we analyzed all of these preparations but based our interpretations primarily on those that were completely normal (Table 1).

Digital images of sections containing BDA-labeled interneurons and donor quail cells were imported into Photoshop (Microsoft) format using a Leaf Lumina camera. Plots of cell positions were generated in a digital overlay constructed in the Canvas (Deneba) drawing program (Fig. 2). Horizontal or sagittal projections of individual preparations were constructed by aligning the rostral and caudal limits of the grafted rhombomere. Since these limits are skewed with respect to the horizontal and sagittal planes of the head, the projections do not provide a true representation of the position of the groups with respect to the projection outline, but do indicate reliably the topological relationship between group position and rhombomeric domain. To obtain quantitative estimates of the proportions of neurons lying in neighboring rhombomeres, retrogradely labeled neuron profiles were counted directly from the sections in the microscope. Only BDA-labeled neurons lying clearly to either side of the graft–host interface received definitive rhombomere assignments in this analysis; those lying within the boundary zone (see below) were deliberately left ambiguous.

FIG. 3. The boundary between graft and host rhombomeres is defined as the zone of intermingled graft and host cells. (A) Sagittal section of a hindbrain with r4 quail graft and BDA-labeled vestibulospinal neurons ipsilateral to the graft. Rostral is to the left. Quail cells are tightly contiguous at the ventricular surface, but are more spread toward the ventral brain surface. The dashed lines indicate the approximate location of the boundary zone of intermingling between graft and host tissue. Arrows indicate a few of the quail cells (probably oligodendrocytes) that have penetrated further into the host tissue along fiber tracts. At such sites of more extensive graft cell dispersal, the boundary is assumed to follow the generally straight ventricular-to-pial trajectory, thus cutting across the area of dispersal. (B) Most BDA-labeled neurons can be identified as graft- versus host-derived, irrespective of location, by virtue of the quail nucleus-specific marker. Higher magnification view showing the appearance of BDA+/quail+ cells (LVST neurons, arrows) and BDA−/quail+ cells (arrowheads). Scale bar, 260 μm (A) and 50 μm (B).

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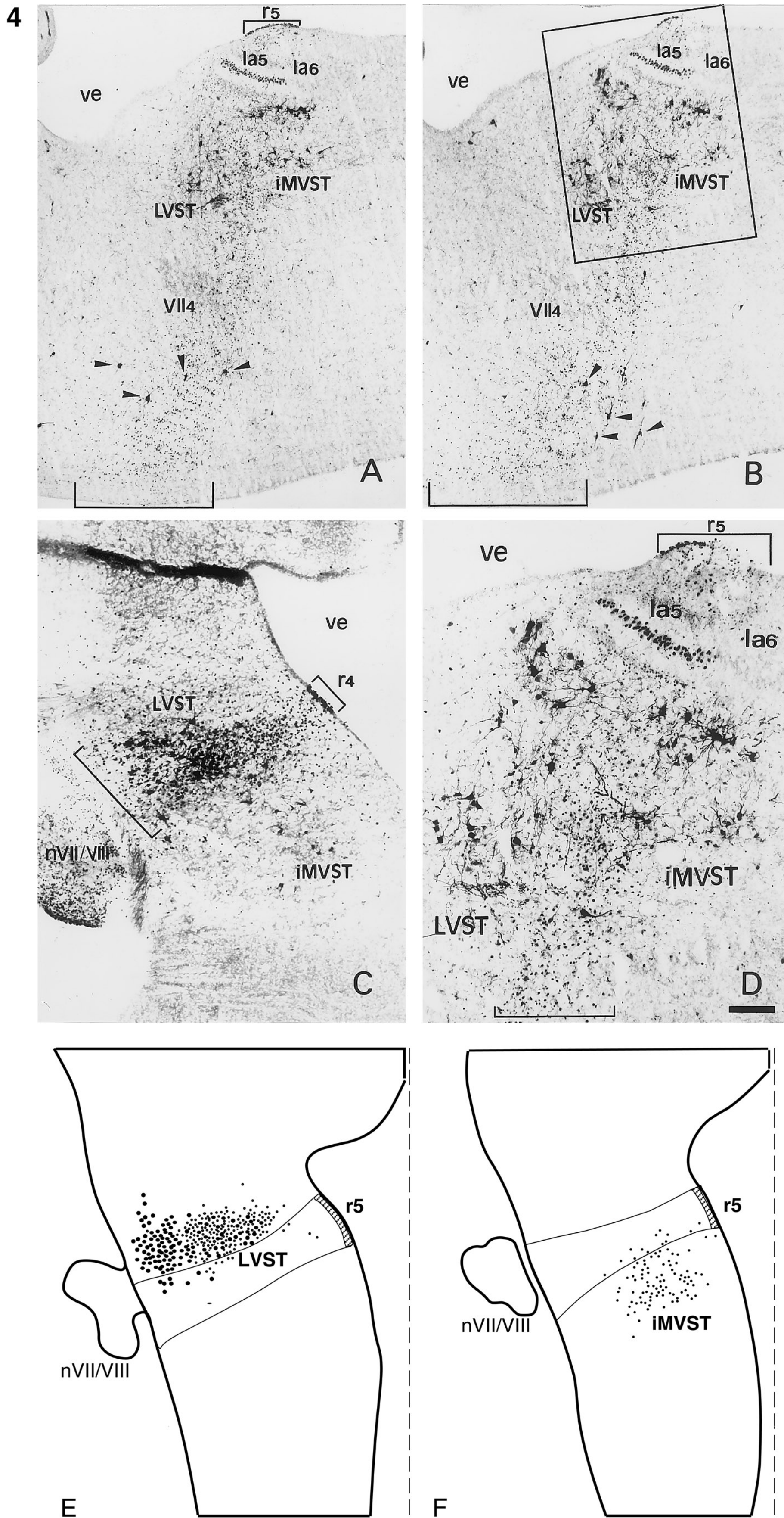
4
r5
la5
la6
ve
iMVST
LVST
VII4
A
la5
la6
ve
iMVST
LVST
VII4
B
ve
r4
LVST
nVII/VIII
iMVST
C
r5
ve
la5
la6
iMVST
LVST
D
r5
LVST
nVII/VIII
E
r5
nVII/VIII
iMVST
F

RESULTS

There are three hodologically defined vestibulospinal groups: the LVST, iMVST, and cMVST groups, each of which projects along a specific pathway to the spinal cord (Fig. 1B; see also Glover and Petursdottir, 1991). There are four hodologically defined vestibulo-ocular groups: the iR, cR, iC, and cC groups, each of which projects along a specific pathway to their target motoneurons in the oculomotor nucleus (Fig. 1A; see also Glover, 1994; Glover and Petursdottir, 1998). To assess the relationships of these groups to individual rhombomeres, we grafted a quail r3, r4, or r5 into a chicken host, and subsequently labeled the vestibulospinal or vestibulo-ocular neurons with BDA.

As previously observed (Marín and Puelles, 1995; Wingate and Lumsden, 1996), the domain derived from a single rhombomere occupied at stages 36–38 a wedge of the hindbrain, narrowest at the ventricular surface and widening toward the pial surface (Figs. 2 and 3A). At the ventricular surface, graft cells were packed tightly, providing a sharp transition from graft to host tissue (Fig. 3A). The transition was less sharp deep to the ventricular surface, however, with intermingling of graft and host cells up to several cell diameters. At specific sites, especially near fiber tracts, graft cells dispersed much more extensively into host territory, up to 1 or 2 rhombomeres distant (Figs. 2 and 3A; see also Marín and Puelles, 1995). Preliminary results using cell type-specific antibodies suggest that many of these cells are oligodendrocytes (S. Martínez, personal communication). Irrespective of location, most BDA-labeled neurons could be distinguished as graft- versus host-derived (Fig. 3B). The definition of rhombomere boundaries at the graft–host interfaces is described in the legend to Fig. 3.

The Vestibulospinal Groups Relate Principally to Single Rhombomeres

The LVST group was contained systematically within r4 grafts (Fig. 4C) and roughly bounded caudally by r5 grafts (Figs. 4A, 4B, 4D, and 4E). A few LVST neurons (about 5%) lay in some preparations just within r3 ($n = 1$) or r5 ($n = 8$). On the basis of the quail-specific nuclear marker, at least half of these were derived from r4, evidently having migrated or dispersed to the proximate regions of r3 and r5.

The iMVST group lay almost entirely caudal to r5 grafts in two well-labeled cases (Figs. 4A, 4B, 4D, and 4E), with only 2 and 7% of the labeled cells lying in r5. At least some of the iMVST cells in r5 were quail-negative and thus not derived from r5 (presumably derived from r6). In four other cases the iMVST group was not labeled completely and it was difficult to delineate its domain, but the majority of cells lay clearly within r6.

The cMVST group lay largely caudal to r4 grafts (Fig. 5), but about 15–20% of the cMVST neurons were located well within the lateral part of r4 (Figs. 5A, 5B, and 5E). Similarly, most of the commissural axons of the cMVST neurons, easily identified at the midline, lay clearly within r5, but a smaller proportion could be seen in r4 (Fig. 5C). Some of the cMVST neurons in r4 were quail-positive and thus r4-derived. By comparison with r5- and r6-specific landmarks identified by Marín and Puelles (1995), the caudal boundary lay at about the r5/6 border. For example, the cMVST group lay beneath only the r5 portion of the auditory nucleus laminaris, and its commissural axons traversed the r5 portion, but not the r6 portion, of the abducens motor nucleus (Fig. 5D). Similar comparison with established segmental landmarks allowed us to place the caudal boundary of the iMVST group at about the r6/7 border.

In summary (see Figs. 9 and 10), the LVST group is localized to r4 (r4-derived), with a minor spillover into r3 or r5 (at least partly r4-derived), the cMVST group is localized largely to r5 (r5-derived) but with a substantial component in the lateral portion of r4 (at least partly r4-derived), and the iMVST is localized to r6 (presumably r6-derived), with a minor spillover into r5 (also presumably r6-derived). Thus, each of these groups relates principally to a single rhombomere, and r4, r5, and r6 each contain a unique set of vestibulospinal axon projection phenotypes.

The Vestibulo-Ocular Groups Relate to Single or Multiple Rhombomeres

The cR group lay rostral to r3 grafts with virtually no constituent neurons located within r3 in either of two preparations (Figs. 6A, 6C, and 6E).

The cC group as a whole stretched from the caudal part of r4 to the rostral part of r7. At its medial aspect it lay almost entirely caudal to the r4/5 boundary (Figs. 6B and 6D). Laterally, however, a substantial number of cC neurons lay within the caudal part of r4 (Figs. 6B, 6C, and 6F). Due to

FIG. 4. The relationship of the LVST and iMVST vestibulospinal groups to r4 and r5. Sagittal sections of a hindbrain with an r5 graft (A, B, and D; rostral to left) and horizontal section of a hindbrain with r4 graft (C; rostral at top). D is a higher magnification view of the outlined field in B. Note that the iMVST group lies below the r6 portion of nucleus laminaris. Domains of the grafted hemirhombomeres are indicated by brackets at the pial and ventricular surfaces. Arrowheads in A and B indicate isolated BDA-labeled reticulospinal neurons. E and F show dot plots of the LVST and iMVST groups in horizontal projection (which can be compared directly to C and to Fig. 2A), generated from photographs as described under Materials and Methods. The domains of the grafted rhombomeres are marked by hatching in the ventricular zone and by boundary lines. The sizes of the dots roughly represent the relative sizes of the neurons. Abbreviations: ve, ventricle; la5 and la6, r5 and r6 portions of nucleus laminaris, respectively; VII4, r4 portion of facial motor nucleus; nVII/VIII, facial and vestibuloacoustic nerves. Scale bar, 525 μm (A and B), 400 μm (C), and 260 μm (D).

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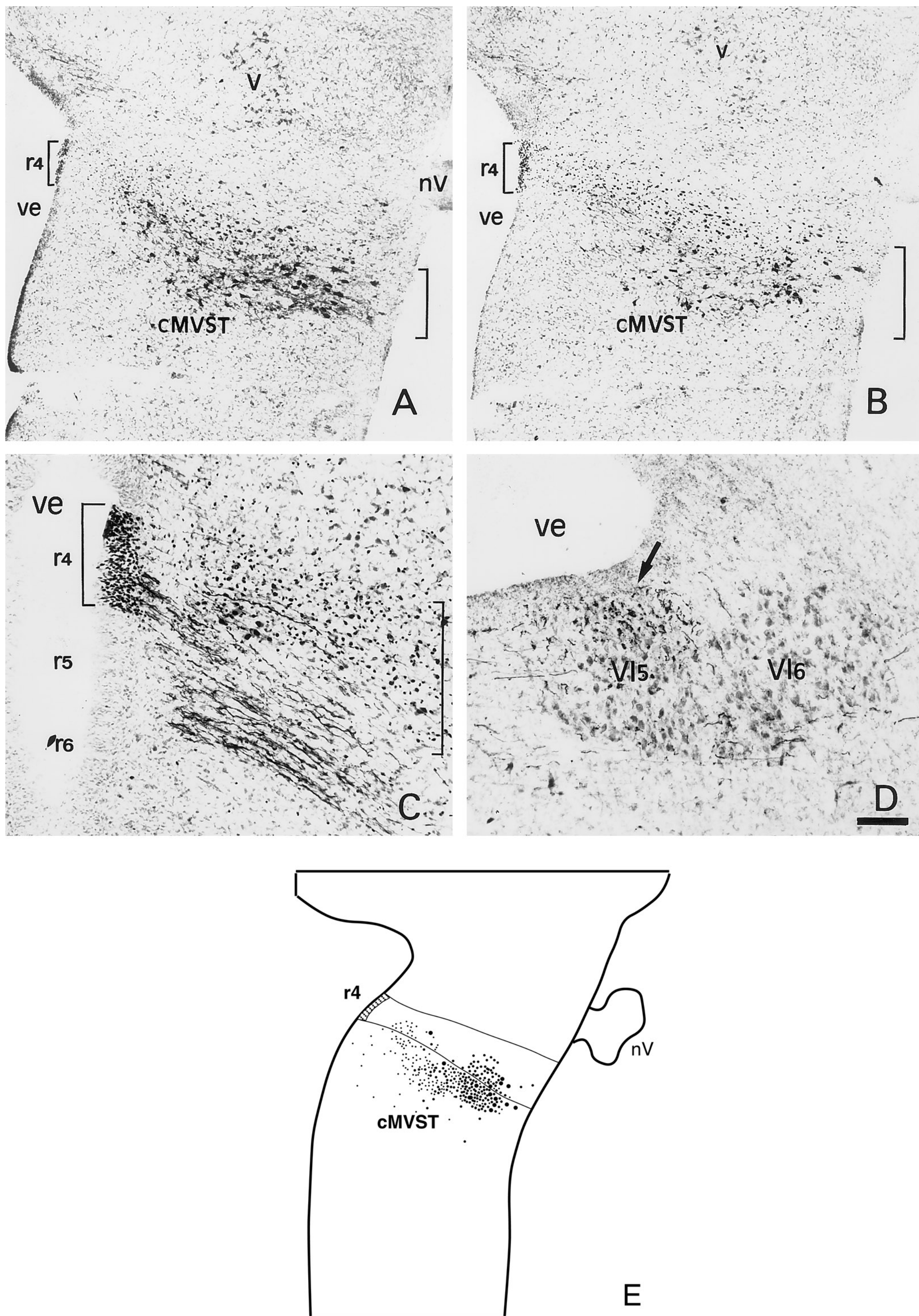

FIG. 5. The relationship of the cMVST vestibulospinal group to r4. Horizontal (A, B, C; rostral at top) and sagittal (D; rostral to left) sections of hindbrains with r4 graft. Domains of the grafted hemirhombomeres are indicated by brackets at the pial and ventricular surfaces. C and D show the location of cMVST commissural axons as they course toward the midline. Note that cMVST axons coursing through the abducens nucleus relate only to its rostral, r5-derived part (arrow in D). E shows a dot plot of the cMVST group in horizontal projection (which can be compared directly to A and B), generated as described in the legend to Fig. 3. Abbreviations: ve, ventricle; V, trigeminal motor nucleus; nV, trigeminal nerve; VI5 and VI6, r5 and r6 portions of abducens motor nucleus, respectively. Scale bar, 400 μm (A and B) and 200 μm (C and D).

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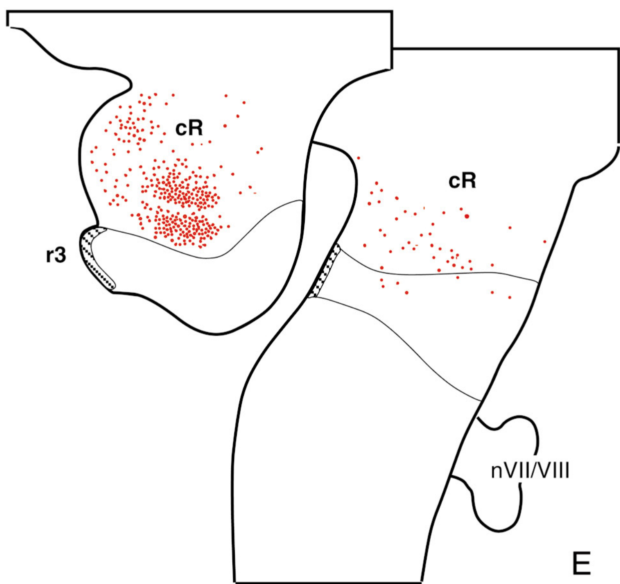
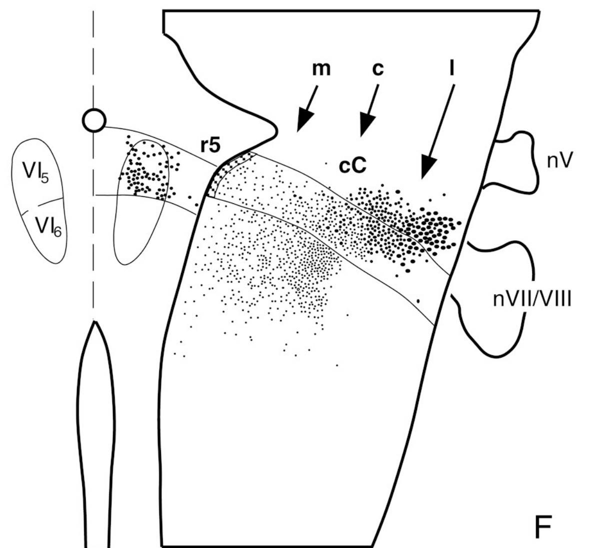

cR
r3
cR
nVII/VIII
E


m
c
l
r5
cC
nV
VI5
VI6
nVII/VIII
F

heavy BDA labeling that obscured the quail-specific nuclear marker, it was unclear whether these were derived from r4 or r5.

Though spanning several rhombomeres, the cC group had subdivisions that were partly in register with the individual rhombomeric domains. In horizontal projection, four components could be distinguished on the basis of position and cytoarchitecture (Fig. 6F). The first, lying most medially and ventrally, was composed of projection neurons within the r5 portion of the abducens motor nucleus (Figs. 6F and 7). The second (medial) and third (central) components were both related to the alar plate of r5 and r6 and the rostral part of r7 (Figs. 6B, 6D, and 6F). The fourth (lateral) component lay in the rostral region of r5 and the caudal region of r4 (Figs. 6B, 6C, and 6F).

The bulk of the iC group was clearly localized to r5, and the bulk of the iR group was clearly located in r2 and r3 (Fig. 8). There were, however, a few labeled neurons (<10%) in r4 that could be assigned only arbitrarily to either iC or iR (Figs. 8A, 8E, and 8F).

Comparison with established segmental landmarks (Marín and Puelles, 1995) allowed us to estimate the locations of the rostral boundaries of the iR and cR groups and the caudal boundary of the cC group (Figs. 6 and 8).

To summarize (see Figs. 9 and 10), the cR group lies in r2 and extends into r1. The cC group spans r4 through r7, with four characteristic subcomponents localized to specific rhombomeric and mediolateral domains. The iR group lies largely within r2 and r3, and the iC group lies largely within r5. Thus, these groups relate to one, two, or more rhombomeres, and each rhombomere contains a unique set of vestibulo-ocular axon projection phenotypes.

DISCUSSION

Hindbrain segmentation certainly appears to make an important contribution to demarcating axon projection phenotypes within the vestibular complex. Our principal finding, however, is that the axon projection phenotypes of the vestibular neurons have a complex relationship to the rhombomeres. Some of the hodologically defined groups of vestibular projection neurons relate to single rhombomeres, whereas others relate to multiple contiguous rhombomeres. Different rhombomeres thus contain different sets of projection phenotypes.

A secondary finding is that the correlation to rhombomeric domains is not absolutely strict. All the groups exhibit minor boundary transgressions by a few percent of the total number of constituent neurons. Two groups, though, have substantial portions that extend well into adjacent rhombomeres, such that their domains cannot be defined in terms of whole rhombomere lengths. Before discussing the significance of the rhombomeric pattern, it is instructive to examine the possible causes of boundary transgression.

Potential Causes of Boundary Transgression

In principle, technical artifacts such as imprecision in resecting the host and donor rhombomeres, damage to the rhombomere boundaries during surgery or implantation, or aberrant intermingling of the heterospecific quail and chicken cells following graft integration could lead to a contamination of one rhombomeric domain by cells from an adjacent rhombomere. On the other hand, a natural source of boundary violation by active cell migration has also been reported in the chicken embryo, with as many as 15% of cells in single labeled neuroepithelial cell clones penetrating from their rhombomere of origin into a neighboring rhombomere while boundaries are still visible (Birgbauer and Fraser, 1994). The LVST group epitomizes this phenomenon: a few of its constituent cells derive from r4 but wind up in r3 and r5 (see Fig. 10). In this case, the transgression is clearly not a technical artifact, as a similar degree of spillover is seen at earlier stages in the absence of any experimental disruption of the rhombomere boundaries (Fig. 1C; J. C. Glover, unpublished results). In the case of the

FIG. 6. Relationship of the cR and cC vestibulo-ocular groups to r3 (A) and r5 (B–D). (A) Horizontal section (rostral at top) showing the cR group, which lies rostral to r3. (B) Horizontal section showing the cC group with its distinct medial, central, and lateral components (m, c, l); the medial and central components lie in r5, r6 and the rostral part of r7, and the lateral component straddles the r4/5 boundary. Note that dispersed reticular neurons (re) in r2 are also labeled with BDA. (C) Sagittal section (rostral to left) showing the cR neurons at the base of the cerebellum and also the lateral component of cC. (D) Detail of a sagittal section (rostral to left) across the central component of cC, illustrating its r5 and r6 portions as they relate to nucleus laminaris. Boundaries of the grafted hemirhombomeres are indicated by brackets at the pial and ventricular surfaces. The boundary between r2 and r3 is also visible as a marked sulcus at the ventricular surface in C (see Marín and Puelles, 1995). E and F show dot plots of the cR and cC groups, respectively, generated as described in the legend to Fig. 3. These can be compared directly to A and B. In each of E and F, two partial projections are shown for clarity. In E, they show differentially the dorsal (left) and ventral (right) cells of the cR group. In F, they show differentially the ventral cC neurons within the abducens motor nucleus (left) and the more dorsal cC neurons (medial, central, and lateral components, right). Abbreviations: ve, ventricle; cc, cerebellar cortex; a, nucleus angularis; p, principal trigeminal sensory nucleus; V2 and V3, r2 and r3 portions of principal trigeminal motor nucleus; Os, nucleus olivaris superior; Dd4, r4 portion of dorsal Deiter's nucleus; la5 and la6, r5 and r6 portions of nucleus laminaris; mc, nucleus magnocellularis; nV, trigeminal nerve; nVII/VIII, facial and vestibuloacoustic nerve complex; VI5 and VI6, r5 and r6 portions of abducens motor nucleus; m, c, and l, medial, central, and lateral subdivisions, respectively. Scale bar, 200 μm (A), 525 μm (B), 400 μm (C), and 260 μm (D).

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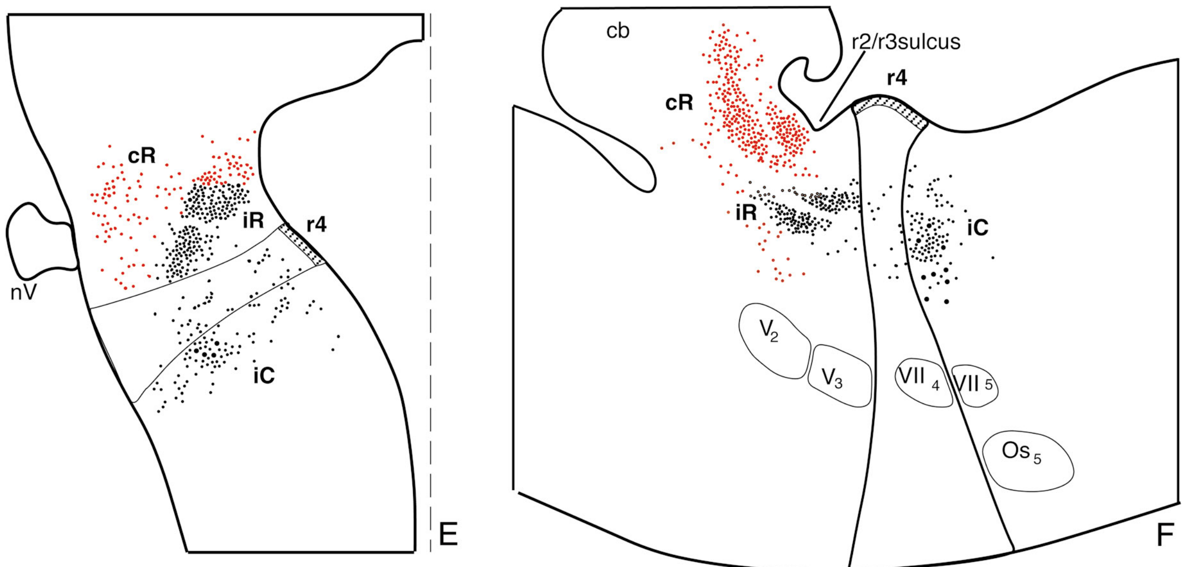

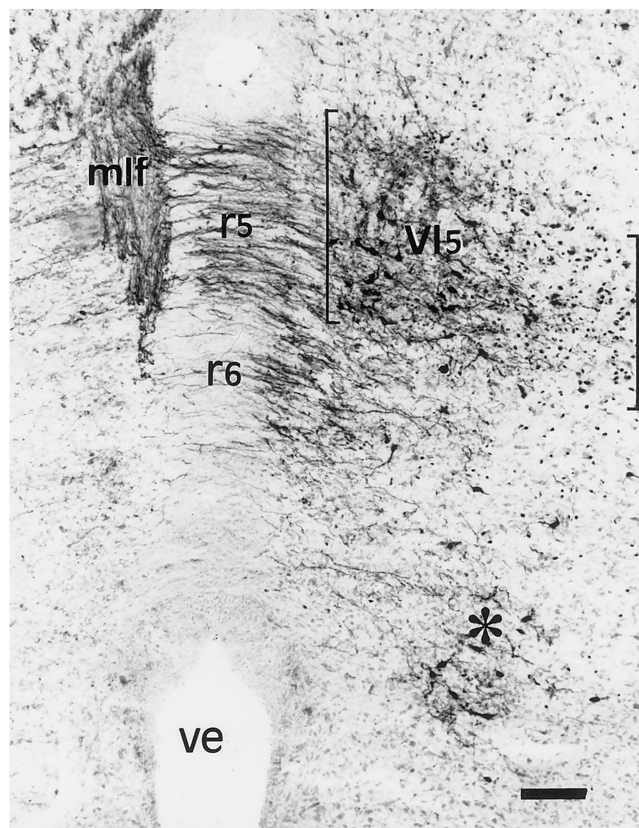

FIG. 7. Horizontal section (rostral at top) showing the ventral cC neurons and their commissural axons in r5 and r6 in an embryo with an r5 graft (boundaries indicated by brackets). Note that some neurons in the more caudal nucleus prepositus hypoglossi are also labeled with BDA (asterisk). Abbreviations: ve, ventricle; VI5, r5 portion of abducens motor nucleus. Scale bar, 200 μm.

other groups, it is not yet clear whether the boundary transgressions occur as a result of technical artifact or normal cell movement.

Alternatively, some examples of noncoincidence we have observed might not represent transgressions at all, but rather arise because the regional specification of vestibular neuron identity is not slavishly in register with the rhombomeres. Perhaps some gene products responsible for specifying neuronal identity have a "sloppy" distribution with respect to the rhombomere boundaries. This could result, for example, from a gradual spatial restriction of gene expression from larger domains down to single rhombomeric domains (as is the case for HoxB1 and r4; see Sundin and Eichele, 1990). If determination occurred before the gene expression domain had retreated to its definitive rhombomeric territory, then the resultant neuron type would not be neatly delimited by a rhombomere boundary.

Another important feature is that, aside from the minor boundary transgressions, whole rhombomere lengths are not obligatory units of specific neuron types. The cMVST and cC groups provide clear examples, where a substantial portion of each group is localized to a fraction of a rhombomere length (r4 in both cases). In the case of the cMVST group, at least some of the r4 component indeed derives from r4 instead of having migrated from r5 (see Fig. 10). In the case of the cC group the origin of the r4 component is not clear. Importantly, the relevant component of the cC group (the lateral component) straddles the r4/5 boundary but does not extend into the caudal half of r5 and is thus almost exactly anti-correlated to the rhombomeric periodicity (see Fig. 6F). Marín and Puelles (1995) describe additional examples of this phenomenon. For example, the r3 portion of the trigeminal motor nucleus lies only in the rostral part of r3, the r4 and r5 portions of the central motor nucleus of the facial nerve are aggregated at the r4/5 boundary, and the r6 portion of the glossopharyngeal motor nucleus lies only in the caudal part of r6. Further research is required to determine how these examples of noncoincidence between rhombomeric domains and neuronal phenotypes arise.

How Do Specific Axon Trajectory Features Relate to the Rhombomeres?

Vestibulospinal neurons differentiate in three contiguous rhombomeres (r4–r6), whereas vestibulo-ocular neurons differentiate in seven rhombomeres (r1–r7, but evidently sparsely in r4). Thus, whether the axon of a vestibular neuron ascends or descends in the hindbrain is not patterned at single rhombomere resolution. What about whether the axon of a vestibular neuron crosses the midline before ascending or descending? Ipsilaterally projecting ves-

FIG. 8. Relationship of the iR and iC vestibulo-ocular groups to r4. (A and B) Sagittal sections (rostral to left) showing the iR, iC, and cR groups. The cR group (also labeled here because the cR commissural axons passed through the injection site) lies dorsal to and extends further rostral than the iR group. (C) Horizontal section (rostral at top) showing the iR group. (D) Detail of B. Boundaries of the r4 graft are indicated by brackets at the pial and ventricular surfaces. The boundary between r2 and r3 is also visible as a marked sulcus at the ventricular surface in A and B. E and F show dot plots of the cR (red dots), iR, and iC groups in horizontal and sagittal projections, respectively, generated as described in the legend to Fig. 3. E and F can be compared directly to C and B, respectively. Abbreviations: cb, cerebellum; a3, r3 portion of nucleus angularis; re, reticular neurons; 1a, nucleus laminaris; nV, trigeminal nerve; V2 and V3, r2 and r3 portions of principal trigeminal motor nucleus; VII4 and VII5, r4 and r5 portions of facial motor nucleus; Os5, r5 portion of superior olivary nucleus. Scale bar, 525 μm (A and B), 400 μm (C), and 260 μm (D).

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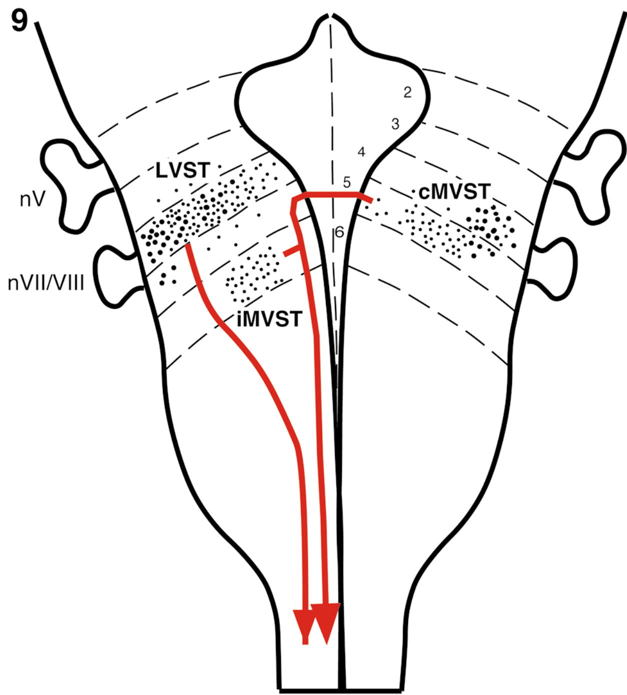

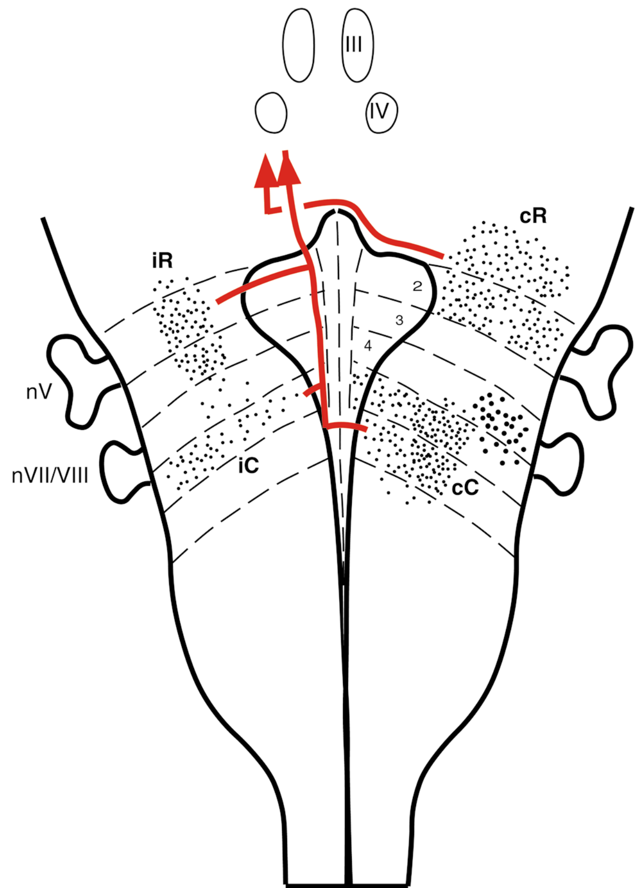

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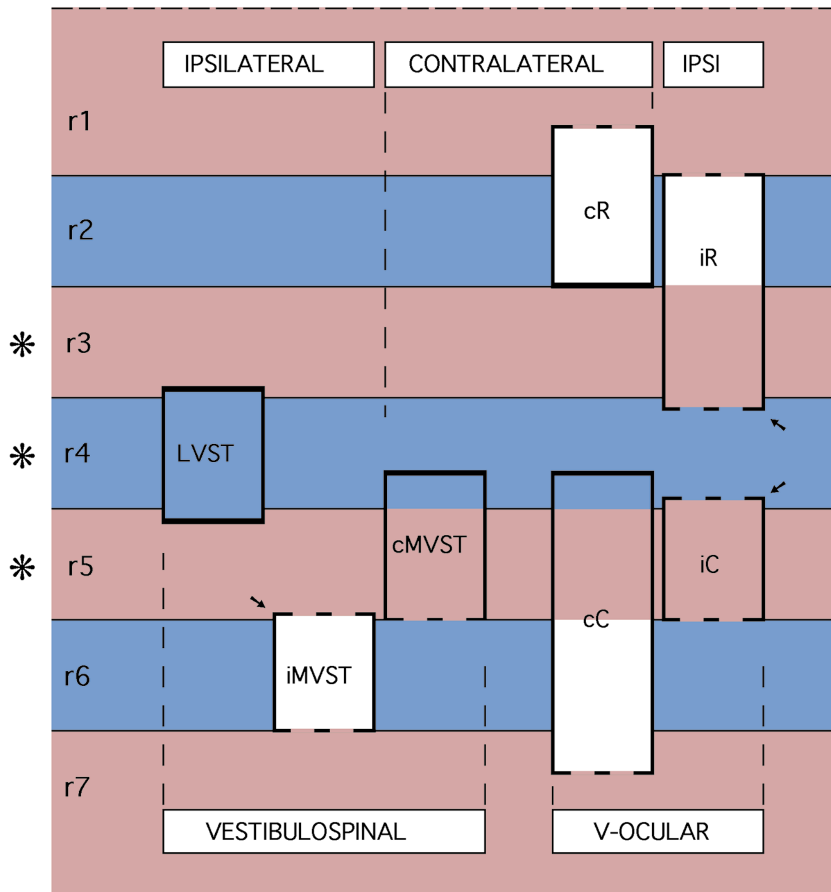

tibulospinal and vestibulo-ocular neurons differentiate in r2–r6, whereas their commissural counterparts differentiate in r1, r2, and r4–r7 (evidently rarely in most of r4). Again, single rhombomeres are not the spatial units of determination. If these two trajectory features (direction and laterality) are combined, in only three cases is there a strong correlation between a single rhombomere and a specific neuron type: (1) the LVST group, which is the only group whose axons descend ipsilaterally in the lateral vestibulospinal tract, is largely restricted to r4; (2) the iMVST group, which is the only group whose axons descend ipsilaterally in the MLF, is largely restricted to r6; and (3) the iC group, whose axons ascend ipsilaterally in the MLF, is largely restricted to r5. All other combinations of axon trajectory features arise in multimeric units (Fig. 10).

Among the multimeric groups, there are some indications that specific combinations of axon trajectory features may relate to pairs of adjacent rhombomeres, although such rhombomere pairs are not mutually exclusive. For example, the cR group (contralateral, ascending in brachium conjunctivum and MLF) lies largely in r1 and r2, and the iR group (ipsilateral, ascending in MLF) lies largely in r2 and r3. The cMVST group (contralateral, descending in MLF) lies largely in r5 but also has a substantial number of cells in the caudal part of r4. The cC group (contralateral, ascending in MLF) is more extensive from this point of view (r4–r7), but its medial and central components lie largely in r5 and r6, and its lateral component straddles the boundary between r4 and r5 and is, therefore, related to both of these rhombomeres.

Multimeric organization may have functional significance. It has been argued previously, on the basis of termination patterns on the different oculomotor neuron pools, that the cC group may be expected to comprise functional subdivisions (Glover, 1996). This brings up the possibility that the individual rhombomeric compartments of a multimeric group might specify functional differences within a population of neurons of uniform axon trajectory. This notion is supported by the correlation of specific cytoarchitectonic subdivisions of the cC group to specific rhombomeres. Elucidation of the relationship between rhombomeres and functional features such as termination pattern, afferent input, neurotransmitter content, and electrophysiological properties will be required to resolve this issue.

Relationship to Proposed Models of Segmental Organization

Is the rhombomeric distribution of the hodologically defined vestibular neuron groups consistent with proposed models of the segmental organization of hindbrain neuron populations? One model proposes that neurons of all axon projection phenotypes are segmentally iterated (Clarke and Lumsden, 1993). This is clearly not the case for the mature pattern of the vestibular neurons (although these are only a fraction of the neurons in any given rhombomere and the differences we report here may have gone undetected in the general population analyzed by Clarke and Lumsden (1993)). To account for reported discrepancies with their model (see Glover and Petursdottir, 1991), Clarke and Lumsden (1993) proposed that an initial segmental iteration is modulated by regressive events such as cell death or axon retraction. However, studies of the early patterning of vestibular and reticular projection neurons (Glover, unpublished) rather support the view that rhombomeric domains exhibit striking differences in their content of projection phenotypes from the same early stages analyzed by Clarke and Lumsden (1993).

Another model, based on the distribution of cranial nerve motoneurons and the expression patterns of certain Hox genes, proposes that the rhombomeres imbue the hindbrain with a paired rule system for neuronal determination. That is, neurons of a given phenotype arise in two-rhombomere units (Lumsden, 1990; McKay *et al.,* 1994). This clearly does not hold for all the vestibular groups studied here (see Fig. 10).

The "segmental plan" of the vestibular hodological mosaic thus does not fit systematically either of these two models. There is no clear evidence of a general segmental iteration nor of a consistent bimeric organization. These models, which are derived from studies of the segmental organization of invertebrates and anamniote vertebrates, are insufficient to account for the specification of axon pathfinding by hindbrain projection neurons in an amniote vertebrate.

FIG. 9. Overview of the different vestibular projection neuron groups and their relationships to the rhombomeres in horizontal projection. Red arrows indicate axon trajectories and tracts.

FIG. 10. A schematic semiquantitative representation of the rhombomeric domains of the different vestibular projection neuron groups. The groups are arranged by hodological classification (vestibulospinal (descending axons) versus vestibulo-ocular (ascending axons) and ipsilaterally versus contralaterally projecting). The rhombomeres that were subjected to transplantation are indicated by asterisks. The rostral and caudal limits of each group are shown in relation to rhombomere boundaries. Limits denoted by solid lines were determined directly by transplantation. Most of the limits denoted by broken lines were estimated through comparison with rhombomere-specific landmarks. Those indicated by arrows were tested directly by transplantation, but remain uncertain because of ambiguity in defining the identity of neurons (see text). The color of each group indicates which rhombomere it was derived from; the origin of white groups or regions was either not tested or remains unclear.

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