

Pathfinding by cranial nerve VII (facial) motorneurons in the chick hindbrain

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Summary

Cranial nerve VII (facial) motorneurons begin extending axons through rhombomeres 4 and 5 (R4 and R5) in the chick hindbrain on the second day of incubation. Without crossing the midline, facial motorneuron axons extend laterally from a ventromedial cell body location. All facial motorneuron axons leave the hindbrain through a discrete exit site in R4. To examine the importance of the exit site in R4 on motorneuron pathfinding, we ablated R4 before motorneuron axonogenesis. We find that mechanisms intrinsic to R5 direct the initial lateral orientation of R5 motorneuron axons.

Upon reaching a particular lateral position, all R5 motorneuron axons must turn. In normal embryos the axons all turn rostrally to reach the nerve exit in R4. In embryos with R4 ablated, sometimes the axons turn rostrally and sometimes they turn caudally. A model combining permissive fields and chemotropic cues is presented to account for our observations.

Key words: axonal guidance, hindbrain, facial nerve development.

Introduction

The development of a functional nervous system depends upon correct matching of neurons and their targets, a process that begins as neurons extend axons through the embryonic environment. The mechanisms which ensure that neuronal axons consistently and precisely find their way to their target are not completely understood. In order to study axonal pathfinding within the vertebrate central nervous system, we have turned to the chick hindbrain. The chick hindbrain can be easily and conveniently prepared as a wholemount preparation. It is readily accessible to experimental manipulations both *in vitro* and *in ovo*. Using an antibody that labels cranial motorneurons (Burns et al., 1991), we have begun to study axonal pathfinding by cranial nerve VII (facial) motorneurons in the chick hindbrain.

The vertebrate hindbrain is transiently segmented. In zebrafish embryos nine neuromeres have been described (Hanneman et al., 1988), while in chicks there are eight rhombomeres, with rhombomere 1 (R1) the most rostral (reviewed by Lumsden and Keynes, 1989). Rhombomeres first begin to be visible between Hamburger and Hamilton (HH) stages 9-12 early on the second day of incubation (21 days to hatching). Shortly afterwards, beginning at HH stage 13, cranial motorneurons arise bilaterally along the midline. The motorneurons that we have studied are those that belong to

the visceral sensorimotor nerves V, VII, IX and X. A schematic diagram showing the pathways these motorneurons take in the hindbrain is shown in Fig. 1. Motorneurons of cranial nerve V (trigeminal) are located in R2 and R3 and project laterally within the hindbrain to exit to the periphery at a defined point in R2. Motorneurons of cranial nerve VII (facial) are located in R4 and R5 and project laterally within the hindbrain to exit to the periphery at a defined point in R4. Motorneurons of cranial nerve IX (glossopharyngeal) and X (vagal) are located in R6-8 and project laterally within the hindbrain to exit to the periphery, not at a defined point, but distributed along most of the length of R6-8.

Facial motorneurons are the first motorneurons in the hindbrain to extend axonal processes. During the second day of incubation, facial motorneuron axons extend through rhombomeres 4 and 5 when the terrain is relatively simple and the number of cell types relatively small. Facial motorneuron axons in R4 extend directly towards their exit site, which is also in R4. Facial motorneuron axons in R5 take a more circuitous path, extending first laterally and then turning anteriorly to reach the exit site in R4. The predictability with which facial motorneurons follow these simple rules prompted us to examine in greater detail their axonal extension, and to test possible sources of guidance cues.

Guidance cues that help pattern axonal extension

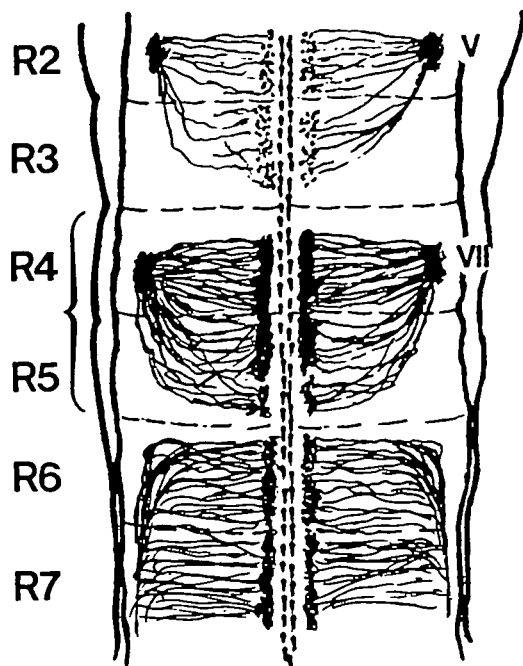


Fig. 1. Schematic representation of chick hindbrain wholemount preparation. The hindbrain is shown filleted open, after the dorsal roofplate was cut. The ventral midline is in the middle, the dorsal edges are to each side, and rostral is at the top. Rhombomeres and cranial nerve roots are numbered. In all subsequent figures R4 and R5 are bracketed as shown here.

could theoretically exist as cell surface or matrix bound molecules that are fixed in place, or they could exist as diffusible molecules. In order to function as a guidance cue, immobilized molecules would be expected to be distributed in a restricted spatial pattern, and diffusible molecules in a gradient. Evidence that both these types of guidance cues may exist in developing nervous systems has been reviewed by Dodd and Jessell (1988).

Motorneurons in the chick hindbrain are labeled by DM-GRASP antibodies. DM-GRASP is a new member of the immunoglobulin superfamily of adhesion molecules, and has been shown to promote the extension of neurites *in vitro* (Burns et al., 1991). Other antibodies which are likely to crossreact with DM-GRASP are SC1 (Tanaka and Obata, 1984), BEN (Pourquie et al., 1990), and JC7 (El-Deeb et al., 1992). Using anti-GRASP labeled hindbrain wholemounts, we describe here the early development and trajectory within the hindbrain of facial motorneurons. Preparations were also labeled with antibodies against acetylated tubulin and G4, in an attempt to look at all the early neurons and axons in the hindbrain. Since facial motorneuron axons seem to "home in" on their exit site in R4, it is possible that cells at the R4 exit site secrete a chemoattractant that lures facial motorneurons towards it. Alternatively, local guidance cues within each rhombomere could dictate the paths that facial motorneurons take. To test these two possibilities, we tried to remove the R4 exit site by ablating R4 early in embryonic day 2. The pathfinding of R5 motorneurons

in the absence of its exit site is in many ways quite normal, and the defects occur principally in the turn that R5 motorneurons make to reach the exit site. These results are discussed in a model for R5 motorneuron pathfinding that combines permissive fields for axon extension with chemotropic guidance cues.

Materials and methods

Wholemount antibody labeling

Fertile White Leghorn chicken eggs were obtained commercially (Truslow Farms, MD) and incubated at 37°C for the desired length of time. Embryos were taken out of the egg and the head removed. After opening the roofplate along the dorsal aspect of the hindbrain, heads were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Hindbrains were then dissected free from the rest of the brain and the branchial arches, and washed in PBS. Primary antibody incubations with antibodies against DM-GRASP, acetylated tubulin, and G4 were for 3 days at 4°C, and all solutions contained 0.5% Triton X-100. Acetylated tubulin antibody TuJ1 was the kind gift of A. Frankfurter (University of Virginia). After washing in PBS, hindbrains were then incubated in horseradish peroxidase (HRP)-coupled secondary antibody (Jackson Immunoresearch, PA) for 2 days at 4°C. After thorough washing, the HRP was reacted with diaminobenzidine (DAB) intensified with Co^{2+} (Adams, 1981). Hindbrains were cleared in 90% glycerol, carefully stripped of pial membranes, and viewed on a Zeiss Axiovert microscope equipped with Nomarski optics.

Rhombomere 4 ablations

Eggs were swabbed with a dilute solution of Wescodyne followed by 70% ethanol, and an effort was made to perform the ablations aseptically. A 1 cm² window in the egg shell was carefully prepared by cutting the shell with a scalpel knife. In order to visualize the embryo easily, a small amount of India ink (Pelikan Fount India Ink, diluted 1:10 with sterile PBS) was injected under the vitelline membrane. R4 ablations were performed on embryos between HH stages 10-12. On one side of the hindbrain, two transverse incisions at the R3/R4 and R4/R5 boundaries were made with a disposable ophthalmic knife (Alcon Surgical, TX). A longitudinal incision was made along the ventromedial edge of R4. The isolated R4 was then removed by gentle suction through a micropipette. The window in the shell was sealed with a piece of Blendern surgical tape (3M Medical-Surgical Division, MN), and the egg replaced in the incubator. After a further 24-36 hours of incubation, the embryos generally had developed to HH stages 15-17. The hindbrain was dissected out, fixed, stained with DM-GRASP antibody and visualized as described above.

Results

Development of motorneurons visualized by DM-GRASP immunoreactivity

At early stages in the chick hindbrain, antibodies to DM-GRASP label floorplate cells and cranial motorneurons. Floorplate cells become immunoreactive first. Antibody labeling was observed beginning at HH stage 11, during the second day of incubation. Not all floorplate cells label with the antibody at the earliest

stages. Fig. 2A shows that at HH stage 13 floorplate cells caudal to rhombomere 5 are labeled with GRASP antibody, while those in rhombomere 5 and rostral are not labeled. Immunoreactivity of floorplate cells gradually extends rostrally as development proceeds.

The first cranial motoneurons are labeled by GRASP antibody at HH stage 13. These motoneurons are cranial nerve VII (facial) motoneurons, located in rhombomeres 4 and 5 (Fig. 2A). The motoneuron cell bodies are located bilaterally along the midline, and they rapidly extend axons. A few hours later, at HH stage 14⁻, facial motoneuron axons have projected laterally in both R4 and R5 (Fig. 2B), and another few hours later, at HH stage 14⁺, R4 motoneuron axons have reached the nerve exit site, 200 μm away, in R4 (Fig. 2C). About a half day of incubation (21 days to hatching) covers the time from when the motoneurons first became immunoreactive to the time that R4 facial motoneurons begin exiting the hindbrain. Other motoneurons in R2 (trigeminal) and R6-8 (glossopharyngeal and vagal) also begin to be immunoreactive to GRASP antibody by HH stage 14.

R5 motoneuron axons are a little behind those in R4 in getting to the nerve exit site, and they begin to exit at HH stage 15 (Fig. 2D). While most R4 motoneuron axons tend to project straight towards the exit site without much wandering, R5 motoneuron axons wander quite a bit in their initial extension within R5. Their trajectories are not identical even on two sides of the same embryo (see Fig. 2D). Frequently their initial extension is in a caudal orientation, in what is ultimately the "incorrect" direction. However, R5 motoneuron axons remain in R5 and are not observed to cross into R6. The general axonal orientation is lateral, and the axons always reach a certain point, about 200 μm lateral from the ventral midline, at which they all turn rostrally to attain the nerve exit. We refer to this position as the lateral motor boundary.

On the third day of incubation, at HH stage 20, the number of axons has increased substantially, and the facial motoneuron projection appears more orderly (Fig. 2E). R4 motoneuron axons head straight for the nerve exit, and R5 motoneuron axons project directly laterally and make a sharp right angle turn at the lateral motor boundary to reach the nerve exit. Aside from the ventro-lateral motoneuron projections at all levels of the hindbrain, three distinct lateral longitudinal pathways are also labeled. The most medial of these pathways descends from the mesencephalon, and is thus a tectospinal tract. The middle of these three lateral longitudinal tracts descends from the trigeminal root, and is the descending sensory tract of V. The most dorsolateral of the tracts ascends from the area of the facial root. Windle and Austin (1936) observed that the sensory root of the facial and vestibular nerves are very closely associated, and this lateral ascending tract is probably the vestibulo-cerebellar tract.

Development of neurons visualized by tubulin immunoreactivity

In the early hindbrain, DM-GRASP antibodies label

only floorplate cells and cranial motoneurons. A number of different classes of neurons are found in the hindbrain, and interactions of facial motoneuron axons with other axons could be an important part of facial motoneuron pathfinding. In order to visualize all the neurons in the hindbrain, wholemount preparations were labeled with antibodies to acetylated tubulin (TUJ1). Acetylated tubulin is specifically expressed in neural tissues (Moody et al., 1989). Furthermore, tubulin is probably expressed by all neurons (Yaginuma et al., 1990; Kuwada et al., 1990).

TUJ1 antibody labels neurons in R2, R4, and R6-8 at HH stage 13 (Fig. 3A). Most of these neurons have cell bodies located laterally and axons projecting ventrally (Fig. 3B), and are probably reticular neurons. Upon reaching the midline, their axons turn to extend caudally upon floorplate cells. It is clear at higher magnification that while some of the cells labeled by tubulin antibodies are motoneurons along the midline, the majority are reticular neurons that project towards the floorplate (Fig. 3B). At HH stage 14, the lack of tubulin-labeled neurons and axons in R3 and R5 is striking in comparison to the labeling in the even numbered rhombomeres (Fig. 3C). While the majority of neurons are still reticular, at higher magnifications motoneuron axons projecting laterally can be seen (Fig. 3D). At HH stage 16, tubulin antibody labeling of reticular neurons has decreased, and the pattern of tubulin immunoreactivity is very similar to the pattern observed with DM-GRASP (Fig. 3E). The difference between the two antibodies at stage 16 is that tubulin antibodies label the ventral longitudinal fascicle and do not label floorplate cells. Tubulin antibody labeling of motoneurons, like that of reticular neurons, also decreases; and by HH stage 21 almost all the tubulin immunoreactivity is expressed only by longitudinal fibers traversing the hindbrain (Fig. 3F). This pattern of immunoreactivity at HH stage 21 is in contrast to that observed with DM-GRASP antibodies, which label uncrossed circumferential pathways mainly, and only a few (lateral) longitudinal pathways (compare Figs 2E and 3F).

Rhombomere 4 ablations

In order to test the significance of the nerve exit site in R4 for facial motoneuron pathfinding, we ablated R4 between HH stages 10-12, at a time prior to motoneuron axonogenesis. Several possible outcomes on facial motoneuron pathfinding in R5 were anticipated. (1) The exit site in R4 might secrete a chemotropic cue that directs facial motoneurons towards it. In the absence of R4, the cue would not be produced. Consequently, perhaps R5 facial motoneurons would now turn caudally, or they might become completely disorganised in their pattern of extension. (2) Local cues within R5 might dictate the pattern of R5 motoneuron extension, and R4 is not essential for R5 motoneuron pathfinding. Thus in the absence of R4, R5 motoneuron extension might be quite normal, with the exception that axons would accumulate at the rostral R5 boundary because they cannot exit the

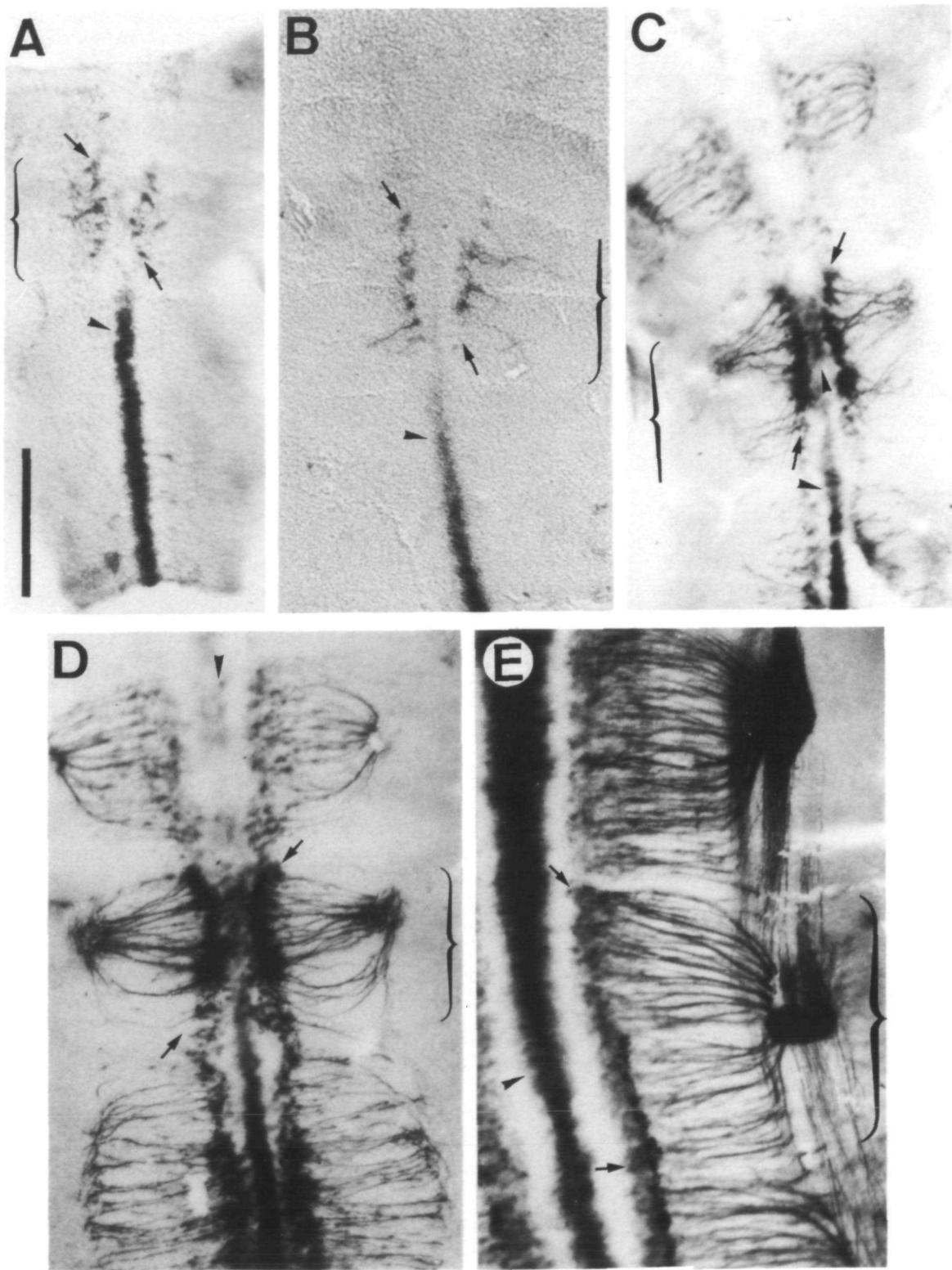


Fig. 2. DM-GRASP-labeled wholemount chick hindbrains. Wholemount chick hindbrains viewed from the pial surface at (A) HH stage 13, (B) HH stage 14⁻, (C) HH stage 14⁺, (D) HH stage 16, (E) HH stage 20. Rostral is towards the top of each panel, and the midline is at the center except in (E), where only half the hindbrain is shown and the midline is on the left. Brackets indicate R4 and R5 as schematized in Fig. 1. Arrowheads indicate floorplate cells, arrows indicate facial motoneurons. Bar: 240 μ m (A,B), 300 μ m (C, D, E).

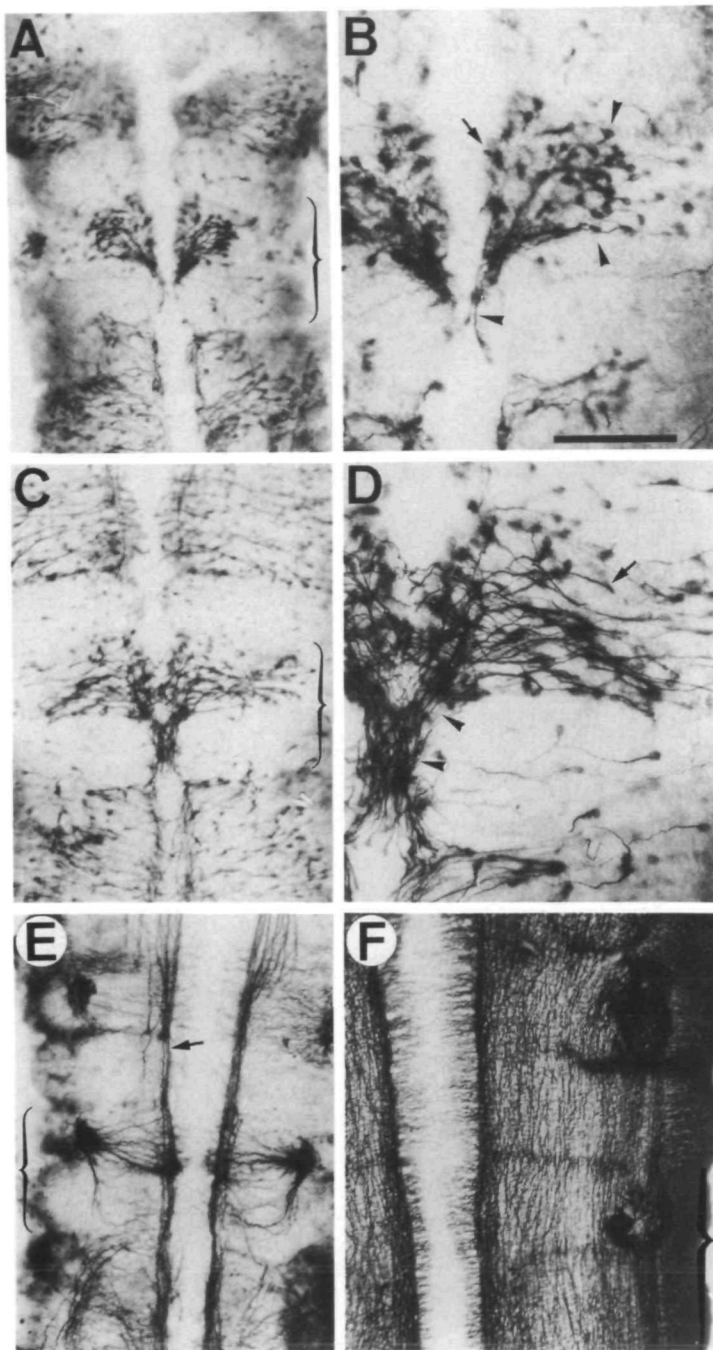


Fig. 3. Acetylated-tubulin-labeled wholemount chick hindbrains. Wholemount chick hindbrains viewed from the pial surface at (A, B) HH stage 13, (C, D) HH stage 14, (E) HH stage 16, (F) HH stage 21. Rostral is towards the top of each panel, and the midline is at the center (left column of panels) or towards the left edge (right column of panels). Arrowheads in B, D indicate reticular neurons and axons, arrows in B, D indicate facial motoneurons and facial motoneuron axons. Arrow in E indicates ventral longitudinal tract. Brackets show rhombomeres 4 and 5 as schematized in Fig. 1. Bar: 240 μm (A, C), 50 μm (B, D), 300 μm (E).

Table 1. *Rhombomere 4 ablated embryos*

A. Summary of R4 ablations

Result of operation	No. of embryos	% of total
Dead	6	8
Unanalyzable	22	28
Normal	25	32
Analyzable	26	33
Total	79	—

B. Direction of R5 motoneuron axon turn at the lateral motor boundary

Direction of turn	No. of embryos	No. of embryos with residual R4	% of total
Rostral	13	7	50
Caudal	4	2	15
Both	3	2	12
Unclear	6	5	23
Total	26	—	—

A shows the outcome of all operated embryos, B shows the analysis of R5 motoneuron pathfinding among the 26 analyzable embryos. It lists the direction of the turn made by R5 motoneuron axons upon reaching the lateral motor boundary, and the number of embryos in each category with residual R4 tissue.

hindbrain. (3) R5 might not make its own nerve exit site because an exit is already available in R4 by the time a significant number of R5 motoneuron axons need to exit. Thus in the absence of R4, R5 might now make its own exit site.

Among the 79 operated embryos, 22 were unanalyzable (Table 1A) due to abnormalities in the control side, or poor immunohistological staining. 25 embryos resulted in a morphologically normal hindbrain and normal GRASP labeling on the operated side. It was critical to remove all of the rhombomere, without taking some of the floorplate (which resulted in grossly abnormal embryos) or leaving a small bit of R4 behind (which then tended to regenerate into an intact and full sized R4).

Finally, 26 out of 79 operated embryos had normal rhombomere structure and motoneuron staining on the control side, but abnormal rhombomere structure and motoneuron staining on the operated side (Table 1B). The operated side of the hindbrain is either missing R4 completely (10 embryos) or contains remnants of R4 (16 embryos). In addition, removal of R4 quite often resulted in some scar tissue upon healing.

In all 26 embryos, the initial motoneuron projection in R5 was normal. R5 motoneuron axons in operated embryos projected laterally until they reached the lateral motor boundary (Figs 4 and 5). Once they reached the lateral motor boundary, R5 motoneuron axons ceased their lateral projection and did one of several things (Table 1B). In some embryos R5 motoneuron axons projected normally, that is they turned rostrally. In other embryos, however, either all

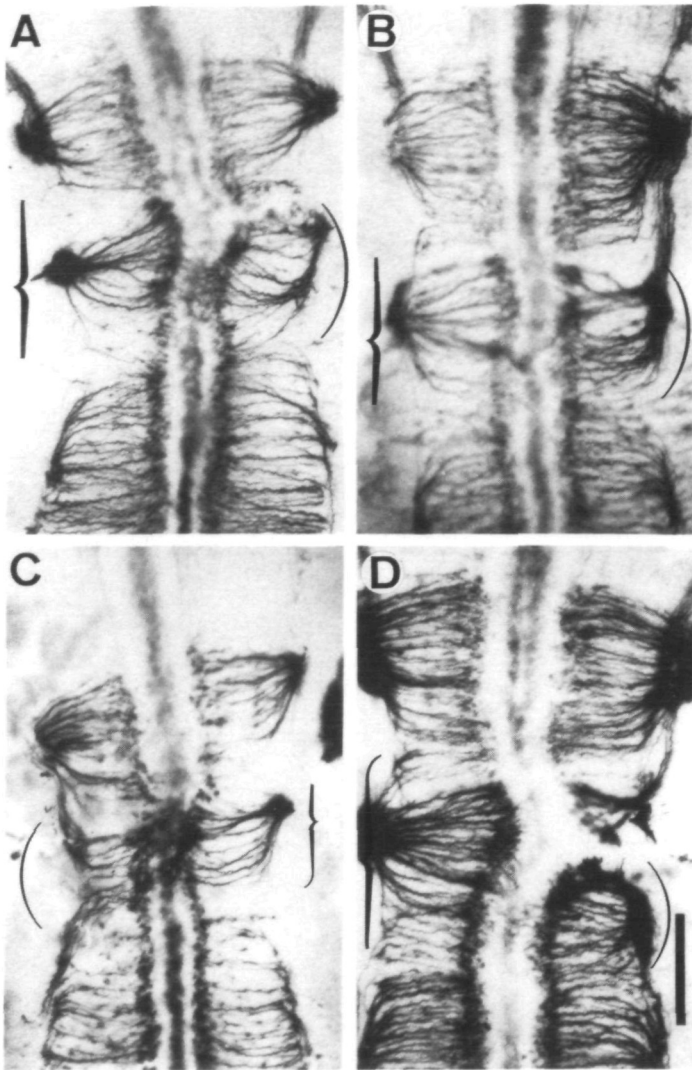


Fig. 4. Rhombomere 4 ablations. Wholemount operated hindbrains, viewed from the pial surface. Rostral is towards the top and the midline is in the center of each panel. (A) R4 on the right side ablated at HH stage 11, embryo fixed at stage 15. (B) R4 on the right side ablated at stage 12, embryo fixed at stage 17. (C) R4 on the left side ablated at stage 12, embryo fixed at stage 15. (D) R4 on the right side ablated at stage 11, embryo fixed at stage 17. Bracket shows R4 and R5 on unoperated side; arc shows R5 on operated side. Bar: 250 μm for all panels.

the axons turned caudally, or some turned caudally and some turned rostrally. In still other embryos the axons did not clearly turn one way or the other at the lateral motor boundary. However, in no case did R5 motorneuron axons keep growing laterally and cross the lateral motor boundary.

In 13 of the 26 embryos, the axons of R5 motorneurons turned rostrally upon reaching the lateral motor boundary. Fig. 4A and 4B show two examples from this group of embryos. In both cases R4 was ablated on the right side of the hindbrain. In the embryo shown in Fig. 4A, a small, neat scar was visible but no residual R4 tissue was detected. This is one of our most normal

looking embryos. R5 motorneuron axons extend from the midline laterally as they normally would, and turn rostrally once they reach the lateral motor boundary. They do not extend beyond R5. In the embryo shown in Fig. 4B, remnants of R4 were probably present. In this embryo, R5 motorneuron axons extend laterally and then turn rostrally, and continue to extend rostrally through R3 to the exit in R2. This embryo was fixed at a slightly older stage than the one in Fig. 4A.

In 3 embryos, R5 motorneuron axons diverged with some turning rostrally and some turning caudally. An example is shown in Fig. 4C. R4 was ablated on the left side of the hindbrain. Axons in the rostral part of R5 turned rostrally, and those in the caudal part turned caudally. No residual R4 tissue was detected.

In 4 embryos, R5 motorneuron axons turned caudally. One example is shown in Fig. 4D. R5 motorneuron axons grew laterally first and then turned caudally when they reached the lateral motor boundary. A small rostral portion of R4 remained. Axons from this remaining R4 grew laterally, then turned rostrally and at least part of the axons reached R2. Since the scar in Fig. 4D is relatively large, it is possible that R5 motorneuron axons turned caudally because the scar tissue forms a physical barrier and hinders R5 motorneuron axons from turning rostrally. Nevertheless, upon reaching the lateral motor boundary motorneuron axons can extend caudally when forced to do so, even though normally they never would.

In 6 cases, R5 motorneuron axons on the ablated side did not extend either caudally or rostrally upon reaching the lateral motor boundary. Fig. 5 shows one example of such an embryo. On both the control side (Fig. 5A) and the operated side (Fig. 5B), R5 motorneuron axons extend from the midline laterally until they reach the lateral motor boundary. Even though there is still plenty of room for further lateral extension, in neither the control nor operated side do R5 motorneuron axons continue to extend beyond the lateral motor boundary. On the control side, the axons smoothly turned rostrally. However, on the operated side, a dark mass of immunoreactivity is located at the lateral motor boundary. It appears that the axons are extensively branched, and piled up on themselves.

Discussion

We describe the early development and pathfinding of facial motorneurons within the chick hindbrain. These motorneurons are part of cranial nerve VII, a mixed sensorimotor nerve. The motorneurons were visualized with antibodies against DM-GRASP, a recently discovered member of the immunoglobulin superfamily of cell adhesion molecules (Burns et al., 1991).

Model for R5 motorneuron pathfinding

Facial motorneurons are distributed in R4 and R5 in the chick hindbrain. We have studied in greater detail the pathfinding of facial motorneurons in R5. Based on observations of both normal and operated embryos, we

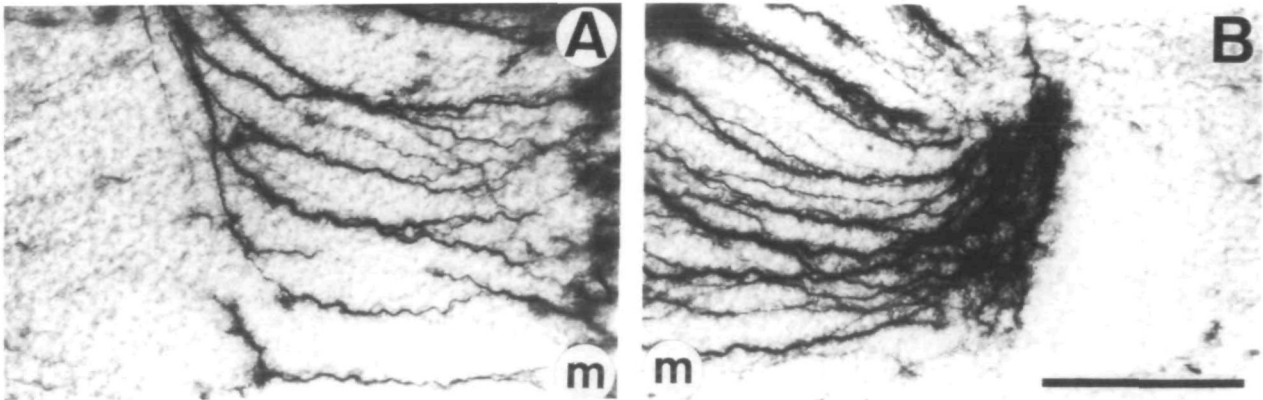


Fig. 5. Rhombomere 5 motorneuron axons following R4 ablation. Wholemount hindbrain preparation, viewed from the pial surface, showing rhombomere 5 only. R4 was ablated at HH stage 12, the embryo was fixed at stage 15. (A) is the control side, (B) is the operated side, with rostral towards the top and the midline (m) labeled. Bar: 100 μ m for both panels.

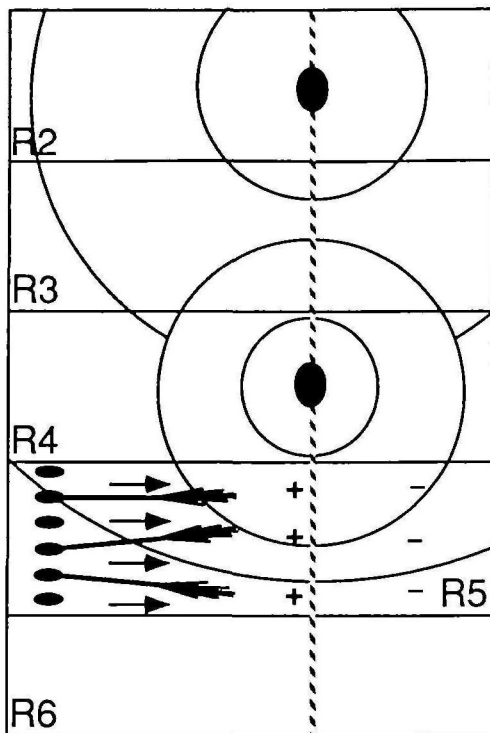


Fig. 6. Model for R5 motorneuron pathfinding. Exit sites in R2 and R4 are shown as dark circles, the lateral motor boundary is the dashed line running rostral-caudally. For details see text.

propose that facial motorneuron axons in R5 extend initially in a permissive field. A generally lateral orientation of growth is observed, but the axons do not appear to be following discrete pathways. Upon reaching the lateral motor boundary, R5 motorneuron axons are forced to make a turn. We hypothesize that the direction of the turn is influenced by a chemotropic signal from R4. This model of our results is schematized in Fig. 6, and discussed in detail in the following paragraphs.

Permissive fields

In both normal embryos and embryos with R4 ablated, R5 motorneuron axons all extend from medial to lateral, and remain within R5. Given these constraints, the axons otherwise appear able to project freely (Fig. 6). There do not appear to be true pioneer axons that the later axons follow, nor are individual preparations exact replicas of each other. We propose that the initial phase of R5 motorneuron extension takes place within a permissive field, rather than along a distinct pathway. The idea of permissive fields suggests that it is as important to look for boundary conditions as for particular pathway labels. We suggest that for R5 motorneurons the important boundaries that confine the initial phase of axon extension are the floorplate medially, the segment boundaries rostrally and caudally, and the lateral motor boundary laterally.

Floorplate might be inhibitory

Since facial motorneuron somas are located adjacent to floorplate cells yet do not cross the midline, it is possible that motorneuron axons extend laterally because they are inhibited from extending medially across the floorplate. Floorplate cells have many important properties. They have been implicated in inducing motorneuron differentiation through the release of a diffusible factor (Yamada et al., 1991). Floorplate cells also seem to be an important source of guidance cues for extending axons. In the zebrafish spinal cord, floorplate cells are thought to attract certain identified axons and to facilitate crossing, while inhibiting other identified axons from doing so (Kuwada et al., 1990). In the rat spinal cord, floorplate cells may attract commissural axons by secreting a chemoattractant (Placzek et al., 1990).

Segment boundaries restrict cell movement

Cell lineage tracing studies have shown that rhombomere segment boundaries restrict the movement of clonally related cells (Fraser et al., 1990). We propose that motorneurons also respect segment boundaries,

and do not usually cross them. R5 motorneuron axons are not observed to cross from R5 into R6, and cross into R4 only via the lateral motor boundary. Other axons may respond differently to segment boundaries, for example some axons have been observed to extend within the boundary (Lumsden and Keynes, 1989).

Lateral motor pathway

The lateral motor boundary itself could be a very attractive substratum that positively holds on to R5 motorneuron axons, or it could be the edge of an inhibitory wall that the axons can not penetrate (Fig. 6). Other axons are found in positions dorsal to the lateral motor boundary, so dorsal hindbrain regions are not inhospitable to all axons. We feel that the lateral motor boundary specifies a turn, but not a direction. In R4-ablated embryos, R5 motorneuron axons turn at the right place, but in either direction. Although in half of the ablated embryos R5 motorneuron axons still turn rostrally, the invariability of the rostral turn is lost. Thus it is plausible that R4 is the source of a positive signal to turn rostrally (Fig. 6).

Chemotropic cues

If R4 is influencing the rostral turn made by R5 motorneurons, it is likely to be a chemotropic cue, given the distances involved. The width of rhombomere 5 at HH stage 15 is about 175 μm , so a signal from R4 would have to diffuse at least that far and remain active if it were to influence R5 motorneuron orientation. These distances are not unrealistic, given the chemotropic cues that have already been demonstrated in the nervous system. These include cues involved in the pathfinding of trigeminal neurons to the maxillary pad, spinal commissural neurons to the floorplate at the ventral midline, and the sprouting of corticospinal fibers into the pons (Lumsden and Davies, 1986; Tessier-Lavigne et al., 1988; Heffner et al., 1990).

We hypothesize that the nerve exit in R2 for the trigeminal nerve might also be secreting a chemo-attractant (Fig. 6), to which R5 motorneuron axons in embryos missing R4 could respond. Trigeminal motorneurons are located in R2 and R3, and always exit in R2. In embryos with R4 ablated, R5 motorneuron axons do not make their own exit and in fact do not exit without finding another exit site, namely the one in R2. In terms of segmental identity, R2 and R4 are thought to share similar properties, as well as R3 and R5 (Guthrie and Lumsden, 1991). Rhombomere grafting experiments have demonstrated that R5 motorneuron axons, besides exiting through R4, can exit through the R2 exit site (Guthrie and Lumsden, 1991). R3 trigeminal motorneurons can also exit through both R2 and R4 exit points (Guthrie and Lumsden, 1992). Thus R2 and R4 exert the same influence over R3 and R5 motorneurons. In the absence of R4, R2 may be an effective but more distant substitute source of the chemotropic cue to turn rostrally.

Sensory-motor interactions

The facial nerve is a mixed sensory and motor nerve. A

factor which we have not addressed is the possible interactions between motor and sensory neurons within the nerve. We have observed a specialised morphology at the exit site in 1 μm plastic sections, as well as highly adherent cells just peripheral to the exit site (Nayak and Chang, unpublished observations). Facial motorneurons can be seen exiting the hindbrain and projecting straight into a mass of HNK-1⁺ cells (Chang, unpublished observations). Cranial neural crest cells begin migrating around HH stage 10, and are in position at the future ganglion site adjacent to the nerve exit by the time that R4 facial motorneurons are exiting the hindbrain at HH stage 14 (D'Amico-Martel and Noden, 1983). Given these considerations, it is possible that sensory neurons of the VII root ganglion induce the formation of a nerve exit. That sensory neurons are important for determining the site of cranial nerve exits has already been suggested for amphibian trigeminal and facial nerves (Jacobson, 1976), and for the chick trigeminal nerve (Moody and Heaton, 1983).

Since sensory axons of both the VIIth and VIIIth nerves enter the hindbrain in close proximity to where facial motorneurons exit (Windle and Austin, 1936), it could be that axon-axon interactions between sensory and motor axons determine where motorneuron axons exit. This is probably not the case, as we have seen no evidence that sensory axons enter the hindbrain before the motorneurons exit.

Conclusion

We propose that pathfinding by facial motorneurons in R5 of the chick hindbrain takes place first in a permissive field, followed by a chemotropic signal to turn rostrally upon reaching the boundary of the field. These hypotheses regarding pathfinding in part of the vertebrate central nervous system leave many questions to be answered. For example, what processes set up the boundaries of a permissive field? What determines the general orientation of growth within the field? Do R2 and R4 secrete a chemotropic cue that R5 motorneurons respond to? What are the molecules that subserve these functions? We hope that a combination of cell biological and molecular techniques will help to answer some of these questions.

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