Appropriate pattern formation following regulative regeneration in the hindbrain neural tube

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SUMMARY

Patterns of neuronal differentiation and axon outgrowth were examined in regenerated neural tissue of the hindbrain of the chicken embryo. Specific regions of the hindbrain neural tube corresponding to identified rhombomeres were selectively removed unilaterally by microsurgery. Of the embryos that survived, about 35% exhibited regulative regeneration, wherein the missing hemi-rhombomere was reconstituted to normal size and morphology through compensatory proliferation and migration from adjacent tissue. The capacity for regeneration depended on the embryonic stage at which the ablation was performed and on whether the midline floor plate was included in the ablation. Ablations performed prior to the formation of morphologically overt rhombomere boundaries led to significantly lower frequencies of regulative regeneration than ablations performed after the formation of boundaries. Ablations that included the floor plate led to significantly lower frequencies of regulative regeneration than ablations sparing the floor plate. Regulative regeneration was also observed at low frequency following excision of an entire rhombomere.

Within the regenerated hemi-rhombomere, identified neuron groups appeared in their normal positions and differentiated normal patterns of migration and axon outgrowth. The fidelity of this patterning, however, depended on the mediolateral position of the neuron group, being more reliable for neuron groups in the basal plate than in the alar plate. These results confirm the extensive regulative potential of the neural tube and demonstrate the capacity for appropriately patterned cellular differentiation within the regenerated tissue.

Key words: pattern formation, regulation, regeneration, hindbrain, rhombomere, floor plate, chicken, neural tube

INTRODUCTION

During roughly the first half of this century, and especially in the 1940s and 1950s, a great deal of interest was focused on the capacity of the central nervous system to regulate following lesions of the neural tube (reviewed in Cowan and Finger, 1982). Although there were instances of controversy (Wenger, 1950), it eventually became clear that most, if not all, regions of the neural tube of anamniotes and amniotes have the capacity to repair restricted lesions through a compensatory proliferation in neighboring neural tissue. The techniques available at the time, however, provided limited information regarding patterning and cellular differentiation within the regenerated tissue. After a lull of several decades, the problem was reexamined by Cowan and Finger (1982), who specifically addressed the capacity for regulative regeneration in the region of the neural tube that gives rise to the mesencephalic optic tectum in the chicken embryo. Their technical repertoire included radioactive birthdating and anterograde axonal tracing, allowing them to ascertain the cytoarchitecture, the spatiotemporal pattern of neuronal generation and deposition, and the pattern of retinal axon ingrowth in the regenerated tectal tissue. On the basis of their findings and those of earlier studies, the following general features emerged: (1) no regeneration occurs after complete bilateral ablations; (2) regeneration after unilateral ablations occurs exclusively from the contralateral, unoperated side, with no contribution from tissue rostral or caudal to the lesion; (3) the regenerated tissue derives predominantly or exclusively from the alar plate of the contralateral, unoperated side; (4) the regenerated side can exhibit normal or nearly normal morphology and cytoarchitecture and can receive a normally patterned afferent input. In one early study, intrinsic axonal growth within and from regenerated tissue in the urodele spinal cord was observed using a silver stain (Holtzer, 1951).

On this backdrop, a great many interesting questions remain partially or completely unanswered. Where and by which progenitors are the cells of the regenerated tissue produced, and how is their number regulated? How do they move to fill in the lesion and generate a structure of normal morphology? Are there any spatial and temporal limitations that constrain the regenerative process? To what extent does the regenerated tissue exhibit normal patterns of neuronal differentiation and connectivity?

Since the studies of Cowan and Finger (1982), this field has lain relatively fallow, aside from recent studies by...
Guthrie and Lumsden (1991), who have demonstrated the capacity for regeneration of hindbrain neuromere (rhombomere) boundaries, and by Scherson et al. (1993) and Hunt et al. (1995), who have confirmed the capacity for regeneration of the dorsal region of the hindbrain neural tube and demonstrated the reconstitution of neural crest and the establishment of normal patterns of Hox gene expression in the regenerated tissue.

During the past ten years, new information has been collected about the regionalization of the neural tube, both at a descriptive and at a mechanistic level, providing a much richer context for the understanding of regenerative events. Having first observed the phenomenon of regulation several years ago in the hindbrain of the chicken embryo (Jansen, Petursdottir and Glover, unpublished results), we now wish to undertake a more systematic and comprehensive analysis of its phenomenology and mechanism. This manuscript presents our first attempts in this endeavor, where we characterize regulative regeneration following hemi-ablation of the hindbrain neural tube. We focus specifically on the stage-dependence of regeneration, the origin of the regenerated tissue, the effect of ablating the midline floor plate and the spatial pattern of neuronal differentiation within the regenerated tissue.

The advantage of studying regulative regeneration in the hindbrain is twofold. First, there is a rapidly growing description of the patterning of its constituent neural populations (Glover and Petursdottir, 1991; Glover, 1993; Fritzsch et al., 1993; Simon and Lumsden, 1993; Clarke and Lumsden, 1993), and of potentially underlying patterns of regulatory gene expression (see, for example, Keynes and Krumlauf, 1994; Tsuchida et al., 1994). Second, the overt segmentation of the hindbrain into rhombomeres provides visual landmarks that facilitate the precise ablation of well-defined regions of the neural tube (Källén, 1955; Vaage, 1969; Lumsden, 1990). Here, we take advantage of these features to show that specific axonal pathways and neuron populations that are functionally identifiable by their characteristic axonal trajectories differentiate in the appropriate spatial pattern following regulative regeneration of individual hemi-rhombomeres. We show further that the completeness of regeneration and the fidelity of neuronal patterning in the regenerate are contingent on several factors, including whether the ablation is performed before or after the formation of rhombomere boundaries, whether the ablation includes the midline floor plate, and where the regenerated neuronal groups are located along the floor plate-roof plate axis. We provide direct evidence that intact neuroepithelial tissue not only contralateral to, but also rostral and caudal to, the ablation can contribute to the regenerate. Lastly, we show that the compensatory reaction in neighboring intact tissue can support the regulative regeneration of whole rhombomeres following bilateral ablations.

**MATERIALS AND METHODS**

**Surgery**

White Italian chicken eggs were obtained from local suppliers and incubated in a forced draft incubator until the desired stage. A window was cut in the shell over the embryo and a 0.1% solution of Fast Green dye (Sigma) or India fountain pen ink (Pelikan) in chicken Ringer (Glover et al., 1986) was sterilized by filtration and injected beneath the embryo to aid in visualization. The embryo was staged according to the criteria of Hamburger and Hamilton (1951). Tungsten needles, sharpened by flame-etching, were used to cut and deflect the overlying vitelline membrane and then to excise a chunk of tissue, containing a defined piece of the hindbrain neuroepithelium along with overlying surface ectoderm and immediately adjacent paraxial mesoderm, which was then removed from the embryo.

A total of 551 embryos were operated: in 411 half of rhombomere 4 (r4) was excised, in 91 half of r5 was excised, and in 49 the entire r4 was excised. Most of the ablations were made after the relevant boundaries defining r4 and r5 had formed and could be discriminated in the dissecting microscope (by stage 10+). Surgical incisions were then visually guided by the rhombomere boundaries and the underlying notochord, which, by virtue of lying immediately subjacent to the floor plate, provided an indication of the lateral edges of the floor plate. The notochord was left intact in all cases except for one set of bilateral ablations (n=12) (Fig. 1). Prior to the formation of the boundaries, the territory corresponding to r4 and r5 is contained within prorhombomere B, whose rostral boundary becomes the rostral boundary of r4 and whose caudal boundary becomes the caudal boundary of r5 (Vaage, 1969). Thus, at these stages, one incision was placed at the appropriate boundary and the other was placed midway along prorhombomere B. Since the r4/r5 boundary was not visible, the precision of the mid-prorhombomere B incisions was correspondingly compromised, with the possibility that tissue destined to form the r4/r5 boundary and a very small portion of the adjacent rhombomere was excised along with the tissue destined to form the target rhombomere. To check the effect of ablations known to violate a rhombomere boundary, in 12 of the r4 or r5 hemiablations performed after the formation of boundaries, both boundaries were excised along with the hemi-rhombomere.

At stage 10 and earlier, when the hindbrain neuroepithelium has not yet fused at the dorsal midline, excision of a hemi-rhombomere did not require a midline dorsal incision. At later stages, after dorsal fusion, the dorsal incision was made just lateral to the midline, contralateral to the hemi-rhombomere that was excised, to ensure that none of the dorsal part of the excised hemi-rhombomere remained (Fig. 1). In different experiments the floor plate was either spared (n=451) or excised along with the hemi-rhombomere (n=51) (Fig. 1).

About 65% of the operated embryos survived the ablation and continued to develop (Table 1), but development was sometimes delayed, presumably due to the stress of the operation. Most of the embryos were allowed to develop for a few days, after which the hindbrain was dissected from the embryo and examined for regulative regeneration, defined operationally as any instance of a hindbrain boundary becoming the caudal boundary of r5 (Vaage, 1969). Thus, at these stages, one incision was placed at the appropriate boundary and the other was placed midway along prorhombomere B. Since the r4/r5 boundary was not visible, the precision of the mid-prorhombomere B incisions was correspondingly compromised, with the possibility that tissue destined to form the r4/r5 boundary and a very small portion of the adjacent rhombomere was excised along with the tissue destined to form the target rhombomere. To check the effect of ablations known to violate a rhombomere boundary, in 12 of the r4 or r5 hemiablations performed after the formation of boundaries, both boundaries were excised along with the hemi-rhombomere.

**Axonal tracing**

To reveal patterns of axon outgrowth and the location of specific neuron groups, small crystals of 3×10^3 M Llysine xfixable rhodamine dextran-amine, or of the lipophilic tracer Dl (both from Molecular Probes, Eugene, Oregon) were applied to nerves or axon tracts following transection with tungsten needles. The preparations were then incubated in vitro for several hours, fixed in paraformaldehyde, mounted whole on glass slides and photographed under a fluorescence microscope. These procedures have been described in more detail elsewhere (Glover et al., 1986; Glover 1995).

**Tracing the origin of the regenerate with Dl**

To determine the origin of regenerated tissue, neural tube tissue adjacent to the ablation was labeled with Dl using a method very similar to that described by Scherson et al. (1993). A stock solution
of 0.5% Dil in 100% EtOH was diluted either 1:10 or 2:11 in 0.3 M sucrose and then applied using a micropipette to the rostral or caudal edges of the excision or to the contralateral hemi-rhombomere immediately after the ablation in 62 embryos (unilateral ablation, n=42; bilateral ablation, n=20). The micropipette was pulled from borosilicate glass capillaries (0.78 mm outer diameter, 0.45 mm inner diameter; model GD-1, Narashige), and the tip was broken to a diameter of about 10 μm. The micropipette was then backfilled with freshly made Dil solution and pressure pulses were applied to force the solution into the tip. Application to the neural tube was made by 5 to 25 msec pressure pulses (20 psi) using a Picospritzer (General Valve). Each embryo was examined in ovo under the fluorescent microscope immediately after Dil application to assess the localization of tracer. Any embryo in which the ablation or Dil injection were not clean and precise was discarded. The embryos were then allowed to develop for 2 days before dissection, fixation and examination under the fluorescence microscope.

**Histology**

To follow the morphogenetic course of regeneration, 64 embryos were fixed at intervals from 1 to 48 hours after the unilateral excision of r4 (sparing the floor plate) and embedded in Historesin plastic (Leica). 1-7 μm thick transverse sections were cut through the regenerating tissue on a microtome and stained with 0.5% Toluidine Blue dye in 0.5% borax.

To determine whether compensatory proliferation occurred during the regeneration, cell profiles were counted on both the regenerating and control sides of r4, and in r4 of unoperated controls. Five experimental embryos, in which ablations were performed at stages 10^ or 11, and 5 unoperated control embryos were included in this analysis. The embryos were killed at stages 13 to 16 (matched for operated and unoperated categories), corresponding roughly to the time period at which regulative regeneration nears completion following ablations performed at stages 10^ or 11. In each embryo, all cell profiles containing a clearly defined nucleus were counted in a series of 10 transverse sections evenly distributed along the length of r4. These data were then segregated into the categories ‘unoperated’, ‘regenerating’ and ‘control’, the latter two representing cell profile counts made from the ablated and control sides of the operated embryos.

**Statistics**

Statistical analyses were performed manually and subsequently checked using the Statview 4.0 program on a Power Macintosh computer.

**RESULTS**

Since we were most familiar with patterns of neuronal differentiation in rhombomere 4 (r4), we focused our efforts on that rhombomere. In the first series of experiments, we excised r4 unilaterally at stages 9 to 12^ (about 30 to 50 hours of development), sparing the floor plate (Fig. 1A,B). During the operation, the floor plate itself was not distinctly visible. The location of the ventral incision was instead guided by cutting along one or the other edge of the notochord, which is immediately subjacent to the floor plate and easily visible through the neural tube in ovo (Fig. 1, 2A). Between stages 9 and 12^ only a small fraction of the neurons in r4 are born and begin to differentiate; these are reticular neurons whose axon growth is first detected at about stage 12 (Sechrist and Bronner-Fraser, 1991). Thus, at the time that we performed the ablation, few if any of the neurons that we examined following the completion of regeneration had yet been generated.

**Regulative regeneration of r4**

About 35% of the embryos that survived the ablation exhibited regulative regeneration, defined as the reformation of a complete r4 with normal or nearly normal morphological appearance (Fig. 2B). In the other embryos, regeneration was either absent or partial. In embryos with no regeneration (about 50%), there was no sign of a regenerated hemi-rhombomere, merely a cleft lying between the adjacent intact rhomboemers (Fig. 2D). In embryos with partial regeneration (about 15%), regenerated tissue was clearly present, but either did not fill the space normally occupied by the relevant hemi-rhombomere, or contained areas of low tissue thickness or integrity (Fig. 2C).

Regulative regeneration occurred at all stages, but the frequencies of regulative, partial, and no regeneration depended on the stage at which the ablation was performed (Table 1). Prior to formation of both rhombomere boundaries, the ablation of the territory corresponding to a future hemi-rhombomere led to a frequency of regulative regeneration about one third that obtained following ablation after the formation of both boundaries. The frequency of partial regeneration was higher after the earlier ablations, however, such that the
combined frequency of regulative and partial regeneration was similar for ablations before and after boundary formation (Table 1).

To determine whether the regulative regeneration as defined by external morphology was indeed a consequence of compensatory cellular proliferation instead of merely cell movement and growth, we counted cell profiles in the regenerated tissue, in the contralateral control tissue and in unoperated stage-matched controls. The number of cell profiles on the regenerated side was typically about 20% lower than on the contralateral side, but on neither side was the average number of cell profiles significantly different from that in stage-matched normal embryos (Fig. 3). We observed, moreover, no appreciable differences between experimental and normal embryos in the number of cell profiles in r3 or r5 (data not shown). Unless one invokes a substantial immigration of cells from outside the neural tube, these data cannot be reconciled with anything but a compensatory proliferation in r4. This accords with Källén’s (1955) report of increased mitotic activity in the contralateral tissue during the first few hours after unilateral ablation in the hindbrain.

Table 1. Frequency of survival and regulative and partial regeneration following unilateral ablation of r4, sparing the floor plate

<table>
<thead>
<tr>
<th>Stages of operation</th>
<th>Survived</th>
<th>Regulative regeneration</th>
<th>Partial regeneration</th>
<th>No regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10+ – 12+ (after)</td>
<td>105/139 (75%)</td>
<td>47/105 (45%)</td>
<td>10/105 (10%)</td>
<td>48/105 (45%)</td>
</tr>
<tr>
<td>9 – 10 (before)</td>
<td>64/121 (53%)</td>
<td>10/64 (16%)</td>
<td>19/64 (30%)</td>
<td>35/64 (54%)</td>
</tr>
<tr>
<td>Total</td>
<td>169/260 (68%)</td>
<td>57/169 (34%)</td>
<td>29/169 (17%)</td>
<td>83/169 (49%)</td>
</tr>
</tbody>
</table>

Embryos that received DiI injections are not included in this data set. The data are compared for regeneration following ablations performed before and after the formation of both the rostral and the caudal boundaries of r4. A χ² analysis rejected the null hypothesis that whether the ablation is performed before or after formation of the rhombomere boundaries has no bearing on the frequencies of regeneration (χ²=20.1, d.f.=2, P<0.005).

Fig. 2. Regulative regeneration following unilateral ablation of r4. (A) A dorsal view of a stage 10+ embryo immediately after an ablation sparing the floor plate. The notochord (arrows) can be seen through the neural tube. The embryo has been explanted from the egg and non-neural tissue deep to the notochord has been removed to improve visibility of the ablation. (B) A case of regulative regeneration, seen in a ventral view of the hindbrain of an embryo subjected to ablation of r4 and allowed to develop to stage 24+. The hindbrain has been flattened under a coverslip to improve visibility. Rhombomeres 2 through 6 are indicated on the unoperated side. The ablated side of r4 has regenerated completely, and there are no morphological discontinuities or malformations at the boundaries of the regenerated tissue. There is a slight misalignment of the rhombomeres. We deliberately show this hindbrain because it is among the least normal of the cases of regulative regeneration; most of the cases were indistinguishable from controls. The modest distortion is probably due to a cervical scoliosis, a condition that also occurs occasionally in unoperated controls where it may be associated with misalignment of the rhombomeres along the midline (unpublished observations). C and D show, respectively, cases of partial and no regeneration, seen in dorsal views of hindbrains from embryos subjected to ablation of r4 and allowed to develop to stage 23. Rhombomeres 2 through 6 are indicated on the unoperated side. Scale bars: 100 μm (A), 200 μm (B-D).
Regulative regeneration in the hindbrain

Fig. 3. Cell profile counts demonstrate that regulative regeneration occurs through compensatory proliferation. Each point represents the total number of cell profiles on one side of r4 counted in a single section from 1-5 embryos. Each circle represents the average number of cell profiles per section for a given embryo. Lines connect the circles corresponding to opposite sides of the same experimental embryo. The Mann-Whitney U test showed that the average values for the regenerating or control sides of the operated embryos were not statistically different from the average values for the unoperated embryos (P>0.3).

ering tissue. Regeneration occurred over the course of several hours (Fig. 4). Within 1 to 3 hours after the ablation, neural tube tissue had appeared on the ablated side both ventrally and dorsally. There was also marked encroachment of mesenchymal tissue into the vacant space. As regeneration proceeded, both the ventral and dorsal fronts of neural tube tissue advanced. A loose network of cells lay between the two fronts; it is uncertain whether these originated from the mesenchyme or the neural tube or both. In a few cases, the gap between ventral and dorsal fronts was bridged by more structured cellular aggregates of varying forms (Fig. 5). Typically, by 9 to 12 hours, the regenerating hemi-rhombomere was continuous dorsal to ventral and, by 13 to 24 hours, had attained a form and thickness similar to the unablated side.

To determine the origin of the cells making up the regenerate, we applied DiI focally to either the rostral or caudal edges of the excision or to the intact, contralateral side immediately after the ablation, and examined the regenerate for the presence of DiI-positive cells 2-3 days later. Most of the embryos survived this procedure (37/42=88%), and about half of the surviving embryos exhibited regulative or partial regeneration (n=19; Table 2). To avoid confounding the statistical treatment of the data in Table 1, none of the DiI-labelled embryos are included in that data set. Of the embryos exhibiting regeneration, 58% (11/19) had DiI-positive cells in the regenerated tissue (Fig. 6A,B, Table 2). That at least some of these cells were neurons was apparent from their possession of growing axons (not visible in Fig. 6). In 3 embryos, DiI-positive cells were not present in the regenerate, but rather rostral, caudal or contralateral to the regenerate, in accordance with the site of DiI application. In 5 embryos, however, there were no DiI-positive cells in the neural tube at all (Table 2), although DiI-positive cells could be seen in peripheral tissues. Since, in all of these cases, DiI had been applied to the most dorsal aspect of the intact tissue, it is likely that this particular application site preferentially labels neural crest cells with a high probability of departing the confines of the neuroepithelium.

Pattern formation within the regenerate

To determine whether specific neuron groups had differentiated normally in the regenerated hemi-rhombomere, we applied axonal tracers to either the medial longitudinal fascicule (mlf), the common facial/vestibulo-cochlear nerve root or the lateral longitudinal fascicule (llf) at stages 23 to 32 (4 to 7 days of development). The retrogradely labeled neuron groups that we examined included (1) a medial group of commissural ascending interneurons that lies in the basal plate of r4 and projects in the contralateral mlf (Díaz and Glover, unpublished observations), (2) the facial motoneurons and vestibulo-cochlear efferent neurons, which are located in the basal plate of r4 and r5 (Fritzsch et al., 1993; Simon and Lumsden, 1993) and (3) the lateral vestibulospinal tract (LVST) group, which is located in the alar plate of r4 and projects in the llf (Glover and Petursdottir, 1991; Glover, 1993). To assess the pattern of differentiation, we compared the spatial domain and approximate number of neurons of a given group in the regenerated hemi-rhombomere to the counterpart in the unablated hemi-rhombomere or in a normal embryo (Fig. 7, Table 3).

The labelling of longitudinal axons obtained following tracer application showed that the mlf and llf lay in the normal positions within the regenerated r4 and contained the number and type of axons expected from the same labelling procedure in normal embryos. The labelling of neuron groups showed that normal patterning of differentiation could be obtained for both basal and alar plate populations in the regenerated hemi-rhombomere (Fig. 7). However, the fidelity of patterning varied with mediolateral location. The two basal plate groups were always found in the appropriate spatial domains, but one of them, the cranial nerve efferent neurons, showed substantial numerical variation (Table 3). The LVST group in the alar plate was also found in the appropriate spatial domain, but showed even more numerical variation and was sometimes absent (Table 3).

Table 2. Source of regenerated tissue determined by DiI labeling

<table>
<thead>
<tr>
<th>Type of r4 ablation</th>
<th>Site of DiI application</th>
<th>Regulative partial regeneration</th>
<th>DiI+ cells in regenerate</th>
<th>No DiI+ cells in neural tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>unilateral</td>
<td>contralateral</td>
<td>13</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>(ventral or lateral)</td>
<td>(8)</td>
<td>(7)</td>
<td>(0)</td>
</tr>
<tr>
<td></td>
<td>(dorsal only)</td>
<td>(5)</td>
<td>(1)</td>
<td>(4)</td>
</tr>
<tr>
<td>bilateral</td>
<td>rostral</td>
<td>6</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>caudal</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Each value represents the number of embryos in each category; values in parentheses represent subsets.
To determine whether appropriate patterning is also exhibited following the regulative regeneration of other rhombomeres, we ablated r5 unilaterally in the same fashion in 85 embryos at stages 10+ to 12+ and examined the patterning of the cranial nerve efferent neurons specific to that rhombomere. Regulative regeneration and partial regeneration were obtained for all stages of ablation with frequencies of, respectively, 30% (22/74 surviving embryos) and 24% (18/74), not significantly different from those obtained for r4 ($\chi^2=3.2$, d.f.=2, $P \geq 0.2$). Normal patterning following regulative regeneration showed a quantitative profile similar to that seen for the cranial nerve efferent neurons of r4 (Table 3).

In addition to having the appropriate axonal trajectory, the cranial nerve efferent neurons in both r4 and r5 exhibited a normal pattern of migration. A subset of these neurons, which has been shown to give rise specifically to the vestibulocochlear nerve efferents in normal embryos (Fritzsch et al., 1993), translocated across the midline and into the intact hemi-rhombomere (not shown).

### The effect of ablating the floor plate

In a separate series of experiments, we ablated r4 or r5 unilaterally along with the midline floor plate, but without damaging the notochord (Fig. 1C,D), in 51 embryos ranging from stage 10+ to stage 12+, that is, after the formation of both rhombomere boundaries. In these experiments, the survival rate was identical to that obtained following ablations sparing the floor plate (38/51=75%, c.f. Table 1). The frequencies of regulative and partial regeneration were, respectively, 13% (5/38) and 16% (6/38). By contrast, the frequencies of regulative and partial regeneration at the same stages when the floor plate was spared were 39% and 16% (data for r4 and r5 combined). A Chi-square analysis on the combined material for r4 and r5 rejected the null hypothesis that ablation of the floor plate has no effect on the frequencies of regeneration ($\chi^2=10.0$, d.f.=2, $P<0.01$). In each of the 5 cases of regulative regeneration, the regenerated tissue included a floor plate of normal dimensions that continued smoothly into the floor plate in adjacent rhombomeres (not shown). In cases of partial regeneration, the floor plate either could not be distinguished morphologically at all (that is, there was no sign of a boundary demarcating a floor plate structure from adjacent neural tissue), or was clearly deficient. Axonal tracing in the 5 cases of regulative regeneration showed that the efferent neurons in the basal plate were absent in one case, and were present in the appropriate spatial domain but were numerically deficient, falling in the ‘+’ or ‘++’ categories of Table 3, in 4 cases.

### Regulative regeneration following more extensive ablations

The pronounced capacity for regulative regeneration demonstrated by the hindbrain neural tube following the ablation of a hemi-rhombomere prompted us to test the capacity for regeneration following more extensive ablations. In 12 of the 179 embryos that survived the excision of half of r4 or r5 following the formation of both the rostral and caudal boundaries, we had excised both boundaries as well. Regulative regeneration, including the reformation of the boundaries, occurred in 4 of these embryos, partial regeneration in 4, and no regeneration in 4. The equivalent proportions were not significantly different.

![Fig. 4. The extent of regeneration is shown in photographs (A, 1 hour; B, 5 hours; C, 14 hours) and camera-lucida drawings of transverse sections taken at different time points following unilateral ablation of r4 (floor plate spared). The camera lucida drawings are of the same sections as photographed but are all shown at the same scale. Vertical hatching represents surface ectoderm, triangles represent loosely organized mesenchyme, and horizontal hatching represents a more densely packed mesenchyme that probably includes neural crest. Scale bars: 50 µm (A,B), 100 µm (C, drawings).](image-url)
Regulative regeneration in the hindbrain

...different from those observed in the experimental population as a whole ($\chi^2=3.0$, d.f.=2, $P@0.25$).

In a separate series of experiments, we ablated r4 bilaterally in 37 embryos sparing the underlying notochord, and in 12 embryos including the underlying notochord, at stages 10+ to 12. Of these, 29 embryos in the first group (notochord spared) and 9 embryos in the second group (notochord ablated) survived until examined 2-3 days later (78% total survival). In many of the surviving embryos, tissue bridges, containing axons and in some cases neuronal cell bodies, had formed across the ablation, but the deficits were extensive and the hindbrains were malformed. None of the embryos from which both r4 and the underlying notochord were ablated showed signs of regeneration. On the contrary, in 5 cases wherein the notochord was spared, r4 had regenerated (17%) and exhibited a normal or nearly normal morphology including the presence of boundaries to the neighboring r3 and r5, and with appropriately positioned common facial/vestibulo-cochlear nerve roots and associated ganglia.

Table 3. Differentiation of identified neuron groups following regulative regeneration of r4 and r5

<table>
<thead>
<tr>
<th>Neuron group</th>
<th>Number of embryos</th>
<th>+++</th>
<th>++</th>
<th>+</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVST (alar plate r4)</td>
<td>16</td>
<td>1</td>
<td>2</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>efferent neurons (basal plate r4)</td>
<td>15</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>commissural interneurons (basal plate r4)</td>
<td>3</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>efferent neurons (basal plate r5)</td>
<td>11</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>–</td>
</tr>
</tbody>
</table>

The estimated number of neurons in each group relative to normal is coded from +++ (within the range exhibited by normal embryos at the same stage) to 0 (no neurons observed).

...different from those observed in the experimental population as a whole ($\chi^2=3.0$, d.f.=2, $P@0.25$).

In a separate series of experiments, we ablated r4 bilaterally in 37 embryos sparing the underlying notochord, and in 12 embryos including the underlying notochord, at stages 10+ to 12. Of these, 29 embryos in the first group (notochord spared) and 9 embryos in the second group (notochord ablated) survived until examined 2-3 days later (78% total survival). In many of the surviving embryos, tissue bridges, containing axons and in some cases neuronal cell bodies, had formed across the ablation, but the deficits were extensive and the hindbrains were malformed. None of the embryos from which both r4 and the underlying notochord were ablated showed signs of regeneration. On the contrary, in 5 cases wherein the notochord was spared, r4 had regenerated (17%) and exhibited a normal or nearly normal morphology including the presence of boundaries to the neighboring r3 and r5, and with appropriately positioned common facial/vestibulo-cochlear nerve roots and associated ganglia.

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<th>Neuron group</th>
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<th>+++</th>
<th>++</th>
<th>+</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVST (alar plate r4)</td>
<td>16</td>
<td>1</td>
<td>2</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>efferent neurons (basal plate r4)</td>
<td>15</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>commissural interneurons (basal plate r4)</td>
<td>3</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>efferent neurons (basal plate r5)</td>
<td>11</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>–</td>
</tr>
</tbody>
</table>

The estimated number of neurons in each group relative to normal is coded from +++ (within the range exhibited by normal embryos at the same stage) to 0 (no neurons observed).

Fig. 5. Morphological variants observed during the regeneration process. (A) A transverse section through a regenerating hemi-rhombomere about 6 hrs after the ablation. The gap appears to be bridged by a squamous epithelium (arrows) that might be similar to the initial bridging structure reported by Cowan and Finger (1982). (B) A transverse section through a regenerating hemi-rhombomere about 9 hours after the ablation. Here there is a large mass of tissue suspended between the dorsal and ventral fronts by a thin epithelium (arrows). Scale bar: 150 μm.

Fig. 6. The origin of the regenerated tissue demonstrated by Dil labeling in ovo. Flat-mount preparations at stages 20-22 show the location of Dil-positive cells in the hindbrain after ablation of r4 on one side (A,B) or bilaterally (C), immediately followed by focal application of Dil to neighboring intact tissue, and with subsequent development for 2-3 days during which regeneration occurred. Rhombomeres are indicated by numbers on the side of the ablation. (A) Labeling obtained after Dil application to the ventral portion of r4 on the side contralateral to the ablation. Dil-positive cells are spread throughout most of the basal plate and much of the alar plate in the regenerated hemi-r4. (B) Labeling obtained after Dil application to the ventral portion of r5 on the ablated side. Dil-positive cells are spread throughout most of the regenerated hemi-r4. (C) Labeling obtained after Dil application bilaterally to the ventral portion of r3. Dil-positive cells are spread throughout the regenerated r4 bilaterally. It was not uncommon to see Dil-positive cells within the floor plate distributed over several rhombomere lengths as seen here. Scale bar: 200 μm.
embryo showed that LVST neurons and facial motoneurons were present on both sides, demonstrating r4-specific patterning in the regenerate (not shown). Both neuronal populations, however, were numerically deficient, falling in the ‘+’ category of Table 3.

DISCUSSION
Regulative regeneration of the neural tube has been well-documented in amphibians and avians and has been observed previously in the hindbrain of the chicken embryo (Källén, 1955; Guthrie and Lumsden 1991; Scherson et al. 1993; Hunt et al. 1995). The primary motivation for our study was the dearth of information regarding the factors that support or constrain the regenerative process and of the degree to which the regenerated neural tissue becomes normally patterned and its constituent cells differentiate. This information has a significance beyond the province of developmental mechanisms: with the use of embryonic implants being considered as a possible therapy for brain and spinal cord injury, a more comprehensive knowledge of the potential (and limits to potential) of specific regions of the neural tube to proliferate and to generate appropriate neuronal populations will be a necessary parameter in the formulation of both clinical strategy and medical policy.

This is the first study that has examined regeneration of the neural tube (1) at the resolution of single, defined rhombomeres, (2) quantitatively, over a range of stages before and after the overt formation of rhombomere boundaries, and (3) through selective labeling of identified neuronal populations, providing evidence of the definitive differentiated characters of the regenerated rhombomeres. We show that the hindbrain neural tube is capable of regenerative regeneration after the loss of a hemi-rhom-

![Diagram](https://via.placeholder.com/150)

Fig. 7. The appearance of identified neuron groups in the normal and regenerated r4. a1 – c1 show the site at which axonal tracers were applied and the axonal trajectory characteristic for the relevant group. a2 – c2 illustrate the normal appearance of the LVST, efferent neuron, and commissural interneuron groups, respectively. a3 – c3 and a4 illustrate the appearance of these groups in regenerated hemi-rhombomeres. a3 – c3 are examples of groups graded numerically as (+++) in Table 3, while panel a4 is an example of a group graded as (+). Scale bars: 100 μm (a2-4, b2-3), 200 μm (c2-3).
Limitations to regulative regeneration

On the basis of our results, we conclude that regulative regeneration of a hemi-rhombomere: (1) occurs at lower frequency if ablations are performed prior to formation of both of the rhombomere’s boundaries, (2) is not prevented by coablation of the boundaries after they have formed, and (3) is not prevented, but is significantly decreased in frequency, by coablation of the midline floor plate. In addition, we conclude that regulative regeneration can occur after ablation of an entire rhombomere.

Why isn’t reconstitution of the ablated tissue always successful? In the experimental paradigm with the highest success rate, namely unilateral ablation sparing the floor plate, the histological snapshots at timed intervals suggest a gradual advance of neural tissue on both a ventral and a dorsal front, with encroachment of mesenchymal tissue into the diminishing gap. The validity of this scenario is not guaranteed, of course, considering the variable appearance of the regenerating tissue (Fig. 5) and especially considering the impossibility of predicting which of the timepoint-sacrificed cases would eventually be among the 35% with regulative regeneration (see below). It was the most typical observation nonetheless, and its relevance here is that it appears as if both neural tube and mesenchymal tissue respond to the ablation by proliferating and competing for the vacated space. In a dynamic competition of this sort, domination by the mesenchymal tissue might underly the many instances in which partial or no regeneration occurred. Direct evidence that conditions at the advancing front are very dynamic, indeed fluid, comes from the observations of Scherson et al. (1993), who show that cells can dissociate from the ventral front of the regenerating neural tube and emigrate as ectomesenchyme to populate normal neural crest derivatives in the periphery.

Prior to the formation of the r4/r5 boundary, ablation of the territory corresponding to a future hemi-rhombomere leads to a significantly lower frequency of regulative regulation. This is associated with a higher frequency of partial regeneration but, even when regulative and partial regeneration are combined, the regenerative response is lower than after both rhombomere boundaries have formed. It might be argued that ablations performed before both boundaries are formed risk removing a few cells destined to lie outside of the target hemi-rhombomere and that the removal of the extra few cells could compromise the regenerative response. This does not seem likely. When we intentionally excised a hemi-rhombomere together with both boundaries at stages 10+ or 11 (shortly after boundary formation), regulative regeneration occurred at a frequency similar to that obtained when boundaries were spared. In previous studies, we have observed regulative regeneration following the ablation of a region corresponding to roughly 2 to 3 hemi-rhombomeres after boundary formation (Glover, Petursdottir and Jansen, unpublished observations). Scherson et al. (1993) have observed regulative regeneration following unilateral or bilateral ablation of the alar plate and much of the basal plate of several presumptive rhombomeres prior to boundary formation. Hunt et al. (1995) have observed regulative regeneration at frequencies as high as 75% following bilateral ablation of much of the alar plate of several presumptive rhombomeres prior to boundary formation. Moreover, the regulative regeneration that we observed following complete bilateral ablation attests to the vigor of the regenerative response. Thus, we feel that the lower frequency of regulative regeneration following ablations performed before boundary formation is not due to accidental removal of too much tissue, but rather represents some other limitation on the regenerative response. This might be related to the number of progenitor cells available for compensatory proliferation. Since the progenitor cell population is known to increase during early stages of neural tube development (see for example Gray et al., 1988; Frederiksen and McKay, 1988), perhaps the number available in the hindbrain before boundary formation is still insufficient to mount a regenerative response that can prevail in the face of mesenchymal encroachment. In the future, it will be important to perform similar ablations at still earlier stages to determine whether regenerative capacity is a progressive phenomenon or is subject to specific critical periods.

When the floor plate is excised along with the hemi-rhombomere, the frequency of regulative regeneration also falls dramatically. As argued above, it is unlikely that this merely represents a general effect of ablating a larger percentage of the neural tube. Ablation of adjacent boundaries, for example, represents at least as large an extra deficit as ablation of the floor plate. Rather, it seems most likely that the specific loss of the floor plate compromises in some way the capacity for regulative regeneration. One possibility is that the floor plate might provide a necessary but purely mechanical bridge that directs cells in the intact basal plate across the midline and into the gap. Secondly, it might participate in actively signalling the need for proliferation pursuant to the loss of adjacent tissue, or be required in some other way to initiate or maintain the proliferative response in the intact hemi-rhombomere. Thirdly, the floor plate might provide a secondary trophic support for the regenerating tissue that is necessary for its survival. Finally, the progenitor cells within the floor plate might themselves be responsible for much of the proliferation that occurs. Whatever the case, a hemi-rhombomere lacking the adjoining floor plate appears incapable of fully reconstituting its missing mate unless it also successfully regenerates the floor plate. Our results are therefore consistent with the following interpretation: although the presence of a normal floor plate does not guarantee regulative regeneration, the absence of a normal floor plate precludes it.

The Dil-labeling experiments provide the first direct demonstration that cells in the regenerate can arise not only from the contralateral side, but also from the rostral and caudal neighboring rhombomeres. Although the data in Table 2 suggest that the ventral and lateral portions of the contralateral side are much more likely to contribute to the regenerate than the dorsal portion of the contralateral side, caution should be exercised in interpreting these data. Because of geometrical constraints, the ventral and lateral Dil applications were targeted to the ventricular surface, providing a direct approach to neural tube progenitor cells, whereas the dorsal applications were targeted to the outer (pial) surface, with a higher risk of labeling non-neural cells and neural crest cells destined to leave the neural tube.

The occurrence of regulative regeneration following bilateral ablation, though relatively rare, stands in contrast to earlier studies that have failed to observe such extensive regeneration, both in the chicken embryo (Birge and Hilleman, 1953;
Källén, 1955; Birge, 1959) and other species (reviewed in Cowan and Finger, 1982). A potential explanation for the discrepancy lies in the small number of experiments performed in earlier studies: Källén (1955) made bilateral ablations of the hindbrain in only 3 embryos; Birge and Hilleman (1953) made bilateral ablations of the metencephalon in only 4 embryos; and Birge (1959) made bilateral ablations of the mesencephalon in only 11 embryos. In all 3 studies, the ablations were performed over a range of stages which may not have been optimal for supporting a regenerative response. The conclusion that has been promulgated on the basis of these studies is that the neural tube rostral and caudal to an ablation does not contribute to the regenerative response. Support for this idea also comes from bilateral ablations of the mesencephalic alar plate made by Birge (1959) and Cowan and Finger (1982) (but see Scherson et al. 1993). Our experiments demonstrate unequivocally that a contribution from neighboring rhombomeres as seen for unilateral regenerates can be of a magnitude sufficient to support regeneration of an entire rhombomere.

Pattern formation in the regenerated hemi-rhombomere

The past few years have witnessed tremendous progress in elucidating how spatial patterns of neuronal differentiation are generated in the nervous system. It is now evident that diffusible, inductive signals emanating from the notochord, floor plate, and roof plate influence the phenotypic fate of nearby neuroblasts in a spatially graded fashion (Yamada et al., 1993; Basler et al., 1993). This is believed to occur through the establishment of gradients of the signals within the transverse plane of the neural plate and neural tube. How does this relate to the situation during regeneration? As noted above, our histological snapshots of regeneration in the hindbrain suggest that the discontinuity in the floor plate-roof plate axis created by the ablation continues for several hours as the ventral and dorsal fronts of neural tissue grow towards each other. The sequence that we have reconstructed from the most typical of these snapshots is similar to that reported earlier for hindbrain regeneration (Harrison, 1947; Källén, 1955; Scherson et al. 1993) and to that reported by Birge (1959) for the regenerating optic tectum, but conflicts with that observed by others. For example, Cowan and Finger (1982) observe that the first morphogenetic event during regeneration of the tectum is the establishment of a thin proliferative epithelium spanning the dorsoventral extent of the lesion. This epithelium then functions much as a normal ventricular zone, growing thicker as new cells are generated and migrate outwards. Other descriptions bear similarities to both of these scenarios (Harrison, 1947), and we have observed less typical variants that might fit the Cowan and Finger model as well (see Fig. 5).

The different interpretations of the morphogenetic sequence underscore the problems of reconstructing reality from a series of timepoints obtained from different preparations. Clearly, to determine whether the signals responsible for patterning are constrained to the regenerating dorsal and ventral fronts, or can bridge the gap, which is not cell-free but typically is filled to varying degrees with mesenchymal cells.

Deficiencies in specific neuron types have been observed previously following regenerative regeneration (Detwiler, 1944). The more frequent deficiency of alar plate neurons in the hindbrain of the chicken embryo contrasts, however, with observations in the spinal cord of urodeles, where Holtzer (1951) reported just the opposite: that basal plate neurons were more often deficient than alar plate neurons following regenerative regeneration. This was correlated with the stage of ablation; with increasing stage, the intact half-spinal cord lost the ability to restore neuron populations sequentially, in the order (1) motor neurons, (2) commissural (lateral sensory) interneurons, (3) ventral association interneurons and (4) dorsal association interneurons. This, Holtzer (1951) felt, might be causally tied to the normal order of neuronal generation, such that the intact half-spinal cord, if having already spent its potential to produce motoneurons when the ablation occurred, would be obliged to contribute only the potentials it had remaining. Birthdating studies in the hindbrain of the chicken embryo show that reticular neurons, motoneurons, and vestibular neurons of the hindbrain are generated in that order, but all 3 neuron types are still being generated at stage 11 and the motoneurons and vestibular neurons are still being generated well after stage 12* (McConnell and Sechrist, 1980). It seems therefore unlikely that the differential regulation of the reticular, motoneuron, and vestibular neuron populations is due to a progressive loss of potential in the hindbrain neural tube. Rather, we feel that it represents a disturbance of the inductive signals that pattern the floor plate-roof plate axis.

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