Gene Misexpression and Cell-Specific Lesions Reveal Functionality of Homeotically Transformed Neurons

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Hox genes are critical for establishing the segmental pattern of the vertebrate hindbrain. Changes in their expression can alter neural organization of hindbrain segments and may be a mechanism for brain evolution. To test the hypothesis that neurons induced through changes in Hox gene expression can integrate into functional neural circuits, we examined the roles of ectopic Mauthner cells (M-cells) in the escape response of larval zebrafish. The activity of the paired Mauthner cells in rhombomere 4 (r4) has been shown to be critical for generating a high-performance startle behavior in response to stimulation of the tail (Liu and Fetcho, 1999). Previous studies have found that misexpression of particular Hox genes causes ectopic M-cells to be generated in r2 in addition to the r4 cells (Alexandre et al., 1996; McClintock et al., 2001). With calcium imaging, we found that the homeotically transformed neurons respond to startle stimuli. To determine the roles of ectopic and endogenous M-cells in the behavior, we lesioned the r2, r4, or both M-cells with cell-specific laser lesion and examined the effect on startle performance. Lesion of the normal M-cells did not decrease escape performance when the ectopic cells were present. These results indicate that the homeotically transformed Mauthner cells are fully functional in the escape circuit and are functionally redundant with normal M-cells. We suggest that such functional redundancy between neurons may provide a substrate for evolution of neural circuits.

Key words: hindbrain; Hox genes; homeosis; Mauthner cell; startle; escape response

Introduction

Hox genes are critical for appropriate segmental patterning of the anteroposterior axis of the hindbrain. Alteration of Hox gene expression in the early embryo can result in changes in hindbrain segment identity and may be a mechanism for brain evolution (for review, see Lumsden and Krumlauf, 1996; Moens and Prince, 2002). Although the morphology of homeotically induced neurons has been examined, the functions of such cells have just begun to be determined. Recent work (del Toro et al., 2001) has demonstrated that in Hoxa1−/− mutant mice, loss of cells in the respiratory rhythm circuit is compensated in part by ectopic neurons that are integrated into novel breathing circuits. These data indicate that the brain can incorporate Hox-induced morphological changes at many levels of organization to generate a functioning organism.

Here, we used Hox gene misexpression to investigate the function of homeotically induced reticulospinal neurons and the integration of normal and ectopic cells in neural circuits. Ectopic expression of the zebrafish hoxb1b gene causes a posteriorizing transformation of the segmentally organized hindbrain at the level of rhombomere 2 (r2) (Alexandre et al., 1996; McClintock et al., 2001). In response to ectopic hoxb1b, r2 takes on multiple aspects of the character of the more posterior r4. These include expression of r4 marker genes and alterations in segment-specific neuronal identity. In particular, the large paired r4-characteristic Mauthner cells (M-cells) are duplicated in r2 (Alexandre et al., 1996; McClintock et al., 2001).

The bilateral Mauthner cells play a dedicated role in the initiation of the escape response (for review, see Zottoli and Faber, 2000). The M-cells and the startle behavior they elicit have provided a useful model for examining neural control of movement. The excitation of this circuit results in strong muscle activity contralateral to the stimulus and inhibition of ipsilateral activity (Fetcho and Faber, 1988). The resulting behavior is a rapid, forceful bend of the body and tail into a characteristic C-shape, turning away from the stimulus direction. We examined the effect of homeotic transformations in the escape circuit on function to investigate how new cells are integrated into pre-existing neural circuits.

We selectively lesioned the r2, the r4, or both M-cells on one side of the brain and examined the effect of the lesion on the kinematics of startle behavior. We found that the ectopic r2 M-cell can elicit a normal, high-performance startle response in the absence of the r4 M-cell. The presence of both cells did not confer higher startle performance than either r2 or r4 M-cells alone. Thus, the r2 and r4 Mauthner cells appear to be functionally redundant, suggesting that a homeotic transformation may add operative cells to a pre-existing neural circuit without chang-
ing the functionality of that circuit. We suggest that such cells may provide a substrate for circuit evolution.

Materials and Methods

Hoxb1b mRNA and embryo injection. Synthetic capped hoxb1b mRNA was synthesized from linearized pCS2hoxb1b DNA template (McClintock et al., 2001) using the Ambion (Austin, TX) Megascript kit according to the instructions of the manufacturer. mRNA was microinjected into one to two cell-stage zebrafish embryos at 50 ng/μl in phenol red buffer (0.25% phenol red, 120 mM KCl, and 20 mM HEPES—NaOH, pH 7.5). Fish were maintained in embryo medium (until ~48 hr) and 10% HBSS (after ~48 hr).

Labeling Mauthner neurons in larval zebrafish. At 3 d postfertilization (dpf), zebrafish were visually screened for morphological abnormalities that occasionally result from early embryonic injections. Fish that appeared normal were injected with 50% fluorescein dye (molecular weight of 10,000 MW; Molecular Probes, Eugene, OR) in 10% HBSS for lesioning experiments or with a combination of 44% calcium green dextran (MW of 10,000; Molecular Probes) and 5.5% Texas Red dextran (MW of 10,000; Molecular Probes) for calcium imaging. The injection procedure followed that of Fetcho and O’Malley (1995). Fish were anesthetized with 0.02% 3-aminobenoic acid ethyl ester (Sigma, St. Louis, MO). A fine-tipped microelectrode connected to a Picospritzer (General Valve, Fairfield, NJ) was used to pressure inject dye into the caudalventral spinal cord to hit the M-cell axons. After injection, fish were returned to 10% HBSS to recover. At 4 dpf, fish were screened for labeled M-cells in r2 and r4. Fish with either unilateral or bilateral M-cells in r2 were selected for physiological and behavioral experiments. Note that because our retrograde labeling was performed with an unusually fine-tipped microelectrode to avoid morphological damage, fish with apparent unilateral duplications may have had bilateral duplication that was not detected.

Calcium imaging. The activity of r2 M-cells in response to startle stimuli was assessed by calcium imaging on the confocal microscope (Fetcho and O’Malley, 1995) in five fish. At 5 dpf, fish were anesthetized as described previously, rinsed in 10% HBSS, and embedded in a 1.2% agar solution dorsal-side down on a coverslip that formed the floor of a 3.5 cm Petri dish. The live mounted fish was covered with HBSS to prevent drying. The dish was placed on the stage of an inverted confocal microscope (model 510; Zeiss, Thornwood, NY), and a fine glass tapper anteriorly with an electric current was positioned against the side of the body on the ipsilateral side to the M-cells being investigated, as described previously (Liu et al., 2003). A time series of images was recorded beginning at the application of the startle stimulus and lasting until the fluorescence levels in the cell returned to baseline. By simultaneously examining both the response of calcium-sensitive dye and the response of the calcium-insensitive Texas Red dextran, we could correct the change in fluorescence for any movement artifact during the response.

Laser lesioning of Mauthner cells. To lesion M-cells, fish were first anesthetized and embedded in a light agar solution, as described for calcium imaging. The general lesion protocol of Liu and Fetcho (1999) was used. An argon laser at full strength was focused on the middle of the r2 or r4 M-cell axons. Axons of r2 and r4 Mauthner cells (blue arrows) in the trunk region of the spinal cord. Scale bar, 50 μm. C, Axons of r2 and r4 Mauthner cells (blue arrows) in the trunk region of the spinal cord. Scale bar, 5 μm.

Results

Ectopic gene expression as an approach for examining neural circuit function

Methodological approaches pioneered for studies of developmental genetics can provide powerful tools for altering nervous system morphology and function. Here, we made use of an ectopic gene expression strategy to produce altered brain anatomy such that ectopic Mauthner cells form in r2, simulating homeosis. We then used imaging and lesioning approaches to examine the consequences of altering neuronal disposition on both neural circuitry and behavior. In combination, these approaches not
only provide insight into homeotic processes but also a simple model for examining basic principles of neural circuit organization and function.

Ectopic expression of zebrafish hoxb1b has been shown previously to result in duplication of the M-cells and several other neurons typical of r4 in r2 (Fig. 1A,B) (Alexandre et al., 1996; McClintock et al., 2001). No other phenotypic consequences have been reported in response to ectopic expression of hoxb1b (Alexandre et al., 1996; McClintock et al., 2001). Using retrograde labeling, we found both unilateral and bilateral M-cell duplications in r2, as reported previously. We found that the ectopic M-cells are very similar morphologically to the r4 M-cells. Thus, a single cell lies in a hemisegment of r2, with laterally and ventrally projecting dendrites and a large commissural axon that descends the full length of the spinal cord. Using retrograde labeling, we found that the r2 and r4 Mauthner axons lie in close proximity to one another in the ventral spinal cord (Fig. 1C, arrows), suggesting that the ectopic r2 M-cells may be integrated into functional circuits.

The rhombomere 2 Mauthner cell responds to startle stimuli

Before examining the possible role of r2 Mauthner cells in startle behavior, we used calcium imaging to determine whether the r2 M-cell was active in response to a startle stimulus (a tail touch) that is known to elicit a response in an r4 M-cell. We retrograde labeled M-cells with calcium-sensitive dye and recorded their activity in response to a touch–startle stimulus using time-lapse confocal imaging (n = 5 fish). Figure 2A shows the typical response of an r2 M-cell to a startle stimulus; fluorescence in the cell increased by nearly 40% during the response (Fig. 2B), indicating that the neuron had fired an action potential. The ability of ectopic r2 M-cells to respond to the same startle stimulus as r4 M-cells shows that they receive appropriate sensory input and suggests that they may play a role in the behavioral response.

Larval zebrafish with ectopic r2 Mauthner cells show a normal startle behavior

Because misexpression of hoxb1b causes major changes in zebrafish hindbrain organization (McClintock et al., 2001), we investigated whether the homeotically transformed larvae show a normal startle response. We compared the behavioral responses of normal larval zebrafish with those with ectopic r2 M-cells and found that both performed a normal startle behavior, consisting of a rapid turn away from the stimulus and subsequent burst swimming (Fig. 3A). We further examined both the angle that the head turned through during the initial body bend and the peak angular velocity of that turn, because these two parameters have been shown previously to be significantly reduced in response to Mauthner cell lesion in normal larvae (Liu and Fetcho, 1999). We found that head movement angle and peak angular velocity did not differ significantly between normal zebrafish larvae and those with M-cell duplications resulting from hoxb1b misexpression (Table 1). Our results show that larvae with ectopic r2 M-cells show startle performance equivalent to that of normal larvae. We conclude that startle behavior is neither negatively nor positively impacted by the presence of ectopic r2 M-cells.

Simultaneous lesion of both r2 and r4 M-cells disrupts the startle response

To begin to explore the functionality of the M-cells, we used a laser lesion strategy to remove labeled M-cells while leaving surrounding cells intact and assessed how this deletion affected performance. To establish baseline responses for each individual before cell lesions, we recorded startle behaviors at 5 dpf (Table 1). We then lesioned both the r2 and the r4 M-cells from one side of the hindbrain. After recovery overnight, we assessed the startle behavior of each larva. As a control for developmental changes between 5 and 6 dpf, we compared prelesion and postlesion startles toward the intact side. We found no significant difference in either head angle or angular velocity (p = 0.143 and p = 0.2852, respectively). In contrast, we recorded significantly reduced head angle (p < 0.007) and peak angular velocity (p < 0.005) in turns to the side of the body normally controlled by the lesioned cells compared with postlesion trials of turns to the opposite side of
we selectively lesioned r2 or r4 M-cells to investigate their individual functions in the startle circuit. When we lesioned an r2 M-cell from a homeotically transformed larva, leaving intact the r4 M-cell, we found no significant difference \((p > 0.05)\) in head angle or angular velocity when compared between prelesion and postlesion trials or between postlesion turns to the intact and ablated sides (Fig. 5A). The unaltered startle response of these larvae in the absence of the ectopic r2 M-cell demonstrated that the r4 M-cell functions normally within the transformed hindbrain.

We next investigated function of ectopic r2 M-cells in the absence of r4 cells by lesioning the r4 Mauthner cell. In normal larvae, this manipulation resulted in a decrease in head angle and angular velocity (Table 1) (Liu and Fetcho, 1999). In contrast, in fish with ectopic r2 M-cells, lesion of r4 M-cells caused no significant decrease \((p > 0.05)\) in either head angle or angular velocity when comparing postlesion with prelesion trials or postlesion turns to the ablated and intact side (Figs. 3C, 5B). These results demonstrated that activity of the ectopic r2 M-cell was sufficient to initiate a normal startle response, revealing that the ectopic M-cell had integrated into a fully functional neural circuit. We conclude that the functions of ectopic r2 and endogenous r4 M-cells are redundant: the r2 M-cell can elicit a startle behavior.

Both r2 and r4 M-cells can mediate startle responses

To further explore the roles of the r2 and r4 M-cells in behavior, we selectively lesioned r2 or r4 M-cells to investigate their individual functions in the startle circuit. When we lesioned an r2 M-cell from a homeotically transformed larva, leaving intact the r4 M-cell, we found no significant difference \((p > 0.05)\) in head angle or angular velocity when compared between prelesion and postlesion trials or between postlesion turns to the intact and ablated sides (Fig. 5A). The unaltered startle response of these larvae in the absence of the ectopic r2 M-cell demonstrated that the r4 M-cell functions normally within the transformed hindbrain.

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Figure 3. Time series illustrating postablation startles of larval zebrafish. The angle of the head in frames indicated by an asterisk shows the peak angle at the end of the initial lateral bend compared with the position of the body just before movement (0 msec). Fish are startled with a fine glass probe, and their behavior is recorded with high-speed video at 1000 Hz. A, A time series of images illustrating startle in a fish with both r2 and r4 M-cells. The fish turns 96° at 10 msec. The initial body bend is followed by continued swimming movements reflective of a typical larval zebrafish startle. B, Startle after both the r2 and r4 Mauthner cells are lesioned. The initial body bend is followed by rhythmic swimming. C, The same pattern of startle behavior is seen in fish after the r4 Mauthner cell is ablated with a turn away from the stimulus. However, the angle at the end of the initial bend is higher than that seen with both cells lesioned, 101° at 10 msec. The startle is followed by rhythmic swimming.

Table 1. Kinematic parameters of startle behavior before and after Mauthner cell ablation

<table>
<thead>
<tr>
<th>Ablation Regime</th>
<th>Head Angle (°)</th>
<th>Head Angular Velocity (°/msec)</th>
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<tr>
<td>Ectopic M-cells in R2b</td>
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<tr>
<td>R2 and R4 ablation</td>
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<tr>
<td>Preablation</td>
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<tr>
<td>Intact side</td>
<td>119.12 (5.80)</td>
<td>19.94 (1.02)</td>
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<tr>
<td>Ablation side</td>
<td>113.76 (4.07)</td>
<td>18.15 (0.79)</td>
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<tr>
<td>Postablation</td>
<td></td>
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<tr>
<td>Intact side</td>
<td>111.28 (4.42)</td>
<td>18.02 (1.02)</td>
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<tr>
<td>Ablation side</td>
<td>88.10 (4.49)</td>
<td>14.28 (0.86)</td>
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<tr>
<td>R2 ablation</td>
<td></td>
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<td>Preablation</td>
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<tr>
<td>Intact side</td>
<td>118.59 (6.01)</td>
<td>20.83 (0.74)</td>
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<tr>
<td>Ablation side</td>
<td>112.44 (5.95)</td>
<td>19.00 (0.89)</td>
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<tr>
<td>Postablation</td>
<td></td>
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<tr>
<td>Intact side</td>
<td>122.48 (4.91)</td>
<td>20.96 (0.92)</td>
</tr>
<tr>
<td>Ablation side</td>
<td>122.74 (4.85)</td>
<td>19.31 (0.89)</td>
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<tr>
<td>R4 ablation</td>
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<td>Preablation</td>
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<tr>
<td>Intact side</td>
<td>108.71 (6.18)</td>
<td>19.24 (0.73)</td>
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<tr>
<td>Ablation side</td>
<td>118.27 (3.22)</td>
<td>20.50 (0.81)</td>
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<td>Postablation</td>
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<tr>
<td>Intact side</td>
<td>109.02 (5.43)</td>
<td>18.62 (1.16)</td>
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<tr>
<td>Ablation side</td>
<td>106.69 (6.23)</td>
<td>17.47 (0.80)</td>
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<td>Normal fishc</td>
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<td>R4 ablation</td>
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<tr>
<td>Intact side</td>
<td>123.76 (4.44)</td>
<td>19.85 (0.42)</td>
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<tr>
<td>Ablation side</td>
<td>113.36 (4.51)</td>
<td>18.01 (1.25)</td>
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<td>Postablation</td>
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<tr>
<td>Intact side</td>
<td>108.59 (6.48)</td>
<td>19.93 (0.67)</td>
</tr>
<tr>
<td>Ablation side</td>
<td>80.96 (2.97)</td>
<td>14.56 (0.64)</td>
</tr>
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</table>

Angle and angular velocity of head movement are shown before and after ablation in Mauthner cells in rhombomeres 2 and 4, rhombomere 2, or rhombomere 4, and before and after rhombomere 4 M-cell ablation in normal larval fish. Ablation side refers to the side of the body to which the targeted neurons project, whereas the intact side refers to the opposite side of the body. Ablation side 96°, Ablation side 80.96 (2.97), Ablation side 108.59 (6.23), Ablation side 111.28 (4.42), Ablation side 109.02 (5.43), Ablation side 106.69 (6.23), Ablation side 123.76 (4.44), Ablation side 113.36 (4.51), Ablation side 108.59 (6.48), Ablation side 80.96 (2.97). Normal fish: n = 3 fish; three trials per treatment per fish. Mean ± SEM.
with performance parameters equivalent to those elicited by co-active r2 and r4 M-cells or the r4 M-cell alone.

Discussion
We combined Hox gene misexpression with neural imaging, cell-specific lesion, and behavioral analysis to investigate how duplicated neurons are integrated into neural circuits and behaviors and, more specifically, how neurons generated by homeotic processes function. We demonstrate that a combination of molecular genetic approaches with neural and behavioral analysis provides a powerful approach for the examination of neural circuit organization and evolution. We find that alteration of the circuit by addition of a duplicated M-cell in r2 neither increases nor decreases startle performance. Rather, we demonstrated that the functions of the r2 and r4 M-cells are redundant, with either cell independently able to drive a normal startle. Our results indicate that major changes to the organization of a neural circuit need not be deleterious to its function. We suggest that cell duplication during evolution may be a mechanism for the diversification of neural circuits.

The role of multiple Mauthner cells in startle behavior
Our finding that startle behavior is not affected in zebrafish with ectopic Mauthner cells is consistent with previous work on zebrafish deadly seven mutants (des6\textsuperscript{420} and des6\textsuperscript{377}) (van Eeden et al., 1996), which develop up to eight Mauthner cells on each side of the hindbrain in rhombomere 4 (Gray et al., 2001). Liu et al. (2003) found that in the des6\textsuperscript{420}, all M-cells appear to respond to startle stimuli, and they respond simultaneously, suggesting that they are receiving similar sensory input. In addition, although multiple M-cells were active in response to a startle stimulus, the additional activity did not enhance behavioral performance (Liu et al., 2003). We found similar results for fish with ectopic M-cells generated by hoxb1b misexpression; although both r2 and r4 M-cells were active in response to startle stimuli, behavioral performance remained the same.

Liu and Fetcho (1999) found that the performance of startles elicited by a tail-directed stimulus decreased after a single M-cell was lesioned, demonstrating that the M-cell is critical for a high-performance startle under this stimulus regime. Using protocols for behavioral tests modeled after Liu and Fetcho (1999), we found a similar decrease in startle performance after lesion of
both the r2 and r4 M-cells on one side of the hindbrain. We also found that the activity of each M-cell alone is sufficient to drive a high-performance startle response. We conclude that the homeotically induced cells in these fish do not need to function together to maintain a normal, high-performance startle response.

In deadly seven mutants, each of the multiple M-cells is thought to have only a limited output in the spinal cord; thus, individual M-cells may not be able to elicit a startle response at peak performance (Liu et al., 2003). Reduced output of individual M-cells is suggested by correlation between an increased number of Mauthner cells (three to six cells) and a decreased number of Mauthner axon collaterals (Liu et al., 2003). These collaterals are the sites of contact to spinal cord interneurons and motoneurons in the startle circuit (Fetcho and Faber, 1988). Furthermore, the M-cells of the des973 mutant tend to have collaterals with nonoverlapping distributions, indicating that the multiple M-cells are dividing their input among the startle interneurons and motoneurons in the spinal cord (Liu et al., 2003). Our contrasting finding, that lesioning one of two M-cells does not decrease performance, suggests that when there are only two M-cells on one side of the hindbrain, there is not significantly reduced output in the spinal cord. In support of this idea, Liu et al. (2003) noted that the one des973 mutant with two M-cells that they examined had axon collateral numbers at the low end of the range of collateral numbers for fish with one M-cell.

Homeosis as a mechanism for brain evolution
The term homeosis was coined by Bateson (1894) to describe naturally occurring phenotypes in which one body segment takes on the likeness of another. Subsequent work from Lewis (1978) revealed that homeotic phenotypes in the fruit fly (Drosophila melanogaster) can be generated by mutations in the homeotic (Hox) genes. Hox genes have since been isolated from a wide range of animal phyla and appear to represent an ancient and well conserved mechanism to regionalize the developing primary body axis (de Rosa et al., 1999). Comparative analysis of arthropods has allowed differences in Hox gene expression or function to be correlated with major alterations to the body plan. For example, changes in regulation of the Hox genes Ubx and AbdA correlate with evolution of modified feeding appendages in crustaceans (Averof and Patel, 1997). Similarly, changes in the protein structure of Ubx correlate with the transition from multilimbed crustacea to hexapod insects (Galant and Carroll, 2002; Ronshaugen et al., 2002). Furthermore, comparative analysis within vertebrates has revealed correlations between Hox gene expression domains and vertebral morphology (Burke et al., 1995). Such studies suggest that evolutionary changes in Hox genes could play a major role in the establishment of different body plans.

Although convincing links have been made between gene expression and morphology, the subsequent step of determining how homeotic changes in morphology affect animal function is just beginning to be addressed. del Toro et al. (2001) examined the respiratory circuit of Hoxa1–/– mice and found that in animals lacking Hoxa1 function, a novel neural circuit took over respiratory rhythm generation. This work demonstrates that novel circuits specified by Hox gene expression can function in behavior. We have now shown that a homeotic transformation in the zebrafish produces a functional segment: a transformed hindbrain segment generates a functional neural circuit, redundant with pre-existing neural circuits, that is capable of mediating behavior. Similar to del Toro et al. (2001), we suggest that production of localized novel circuitry may provide a neural substrate for evolution.

The reticulospinal neurons that lie in each zebrafish homberome have unique segment-specific morphologies and functions (Mendelson et al., 1986a,b; Hanneman et al., 1988). However, it has been suggested previously that the nonequivalent segments of the hindbrain evolved from a reiterated series of equivalent or “homonomous” segments (Hanneman et al., 1988; Lee and Eaton, 1991). In the ancestral condition, each rhombomere would have contained equivalent neurons, possibly with M-cell-like properties. Thus, our homeotically transformed animals may not represent a novel morphology but rather an atavistic change toward a more primitive condition.

Duplicated circuitry may represent a novel condition or an atavistic change, but in either case, we suggest that redundancy in function of circuits could facilitate functional specialization. In our specific case of duplicated M-cells, modification of either the r2 or r4 M-cell circuit would not decrease the startle performance. Thus, one of the duplicate circuits is freed to evolve a novel role, or alternatively, the two circuits could evolve complementary, nonredundant functions within the startle behavior. Such mechanisms may have led to the specialization of neural circuits. Although the evolution of neural circuits is thought to be highly constrained (Nishikawa et al., 1992; Tierney, 1996), we suggest that neuron duplications, which could arise from homeotic transformation, provide a viable mechanism by which large-scale changes in brain morphology and organization may occur.

References