0270-6474/84/0405-1361$02.00/0
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The Journal of Neuroscience
Vol. 4, No. 5, pp. 1361–1369
May 1984

QUANTITATIVE LINEAGE ANALYSIS OF THE FROG'S NERVOUS SYSTEM

I. Lineages of Rohon-Beard Neurons and Primary Motoneurons[1]

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Received August 29, 1983; Revised November 8, 1983; Accepted November 29, 1983

Abstract

After injection of horseradish peroxidase into single blastomeres in *Xenopus* embryos at 2- to 512-cell stages, all of the descendants could be traced and counted at tailbud stages. Progenitors of Rohon-Beard neurons and of primary spinal motoneurons were identified, and all neurons of each type that originated from individual progenitors were counted. From these data were derived the geometric mean of the number (N) of Rohon-Beard neurons (or primary motoneurons) that descended from a single progenitor at each generation from the first to ninth, the mean number of progenitors of each generation, and the probability that, following mitosis, daughter cells continue in the Rohon-Beard (or primary motoneuron) lineages (the continuation probability). On a log-log plot a straight line fitted to the N values intercepts the abscissa at the 13th generation for Rohon-Beard progenitors and the 16th generation for primary motoneuron progenitors. This indicates the number of generations before the entire set of each type of neuron is finally produced. The Rohon-Beard neurons and primary motoneurons each originated from a separate group of progenitors at the 9th generation (512-cell stage), but those progenitors gave rise to mesodermal, endodermal, and ectodermal cells in addition to neurons. Nevertheless, the continuation probability significantly greater than 0.5 shows that there is a bias toward recruitment of progenitors into lineages leading to production of those two types of neurons. This bias is considered to be a form of commitment.

The main purposes of this paper are to report the results of a new method of lineage analysis in the CNS of a vertebrate embryo and to discuss the significance of the findings in relation to the determination of nerve cell fates and the time of neuron origin.

Lines of descent of various types of nerve cells from ancestral cells in the early embryos of vertebrates are completely unknown. The suggestion that the lineages of neurons and glial cells have separated in the neuroepithelium of the developing vertebrate brain (His, 1889) has recently been confirmed (Levitt et al., 1981), but it is not known when this separation occurs. That it starts at early embryonic stages in the frog's brain is indicated by the finding that several types of neurons, including primary spinal motoneurons and Rohon-Beard neurons, originate as postmitotic cells during gastrulation in *Xenopus* (Lamborghini, 1980). Their lineages have probably separated at even earlier stages. In theory there are several possible patterns by which lineages may branch, but it is not known how this actually occurs in a vertebrate embryo.

Tracing cell lineages in embryos of vertebrates was greatly facilitated by the introduction of horseradish peroxidase (HRP) as a tracer of cell lineages (Jacobson and Hirose, 1978; Weisblat et al., 1978; Hirose and Jacobson, 1979). The advantages of this method are that the HRP can be injected selectively into individual cells of frog embryos at 2-cell to 512-cell cleavage stages and can be detected in all of the descendants at later stages when many types of differentiated nerve cells can be recognized (Hirose and Jacobson, 1979; Jacobson and Hirose, 1981; Jacobson, 1981a, 1983). The HRP does not

[1] Dedicated to the memory of Giro Hirose who died on August 15, 1983.

This work was supported by Grant BNS 8116768 to M. J. from the National Science Foundation. S. A. M. was supported by National Research Service Award NS 06955 from the National Institutes of Health. We would like to acknowledge the technical assistance of Hannelore Mueller, the secretarial assistance of Karen Evans, and assistance with statistical analysis from Dr. James Reading, Division of Biostatistics, Department of Family and Community Medicine, University of Utah School of Medicine.

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appear to alter normal cell proliferation, migration, or differentiation. Although it should be possible to trace the lineage of any identifiable type of cell by this method, there are advantages in looking first at Rohon-Beard neurons and primary motoneurons. The entire population of each of these cell types originates and differentiates early in development, the size of the population is small, and the cells are large and easily recognizable. They start functioning in the tailbud embryo: Rohon-Beard neurons are the first-order sensory neurons which receive input from the epidermis (Hughes, 1957), while the primary spinal motoneurons, which innervate axial somitic muscles, mediate the earliest escape and swimming movements (Hughes, 1959; Blight, 1978; Stehouwer and Farel, 1980; Roberts et al. 1981).

The accuracy with which Rohon-Beard neurons can be identified and counted in *Xenopus* made it possible to determine the numbers of Rohon-Beard neurons that descended from individual blastomeres of the 16-cell embryo (Jacobson, 1981a). By starting labeling of cells at successively later ancestral cell generations it was found that the Rohon-Beard neurons and primary spinal motoneurons originated from groups of ancestral cells at the 32-, 64-, 128-, 256-, and 512-cell stages (Jacobson and Hirose, 1981; Jacobson, 1983).

Those findings suggested the research strategy that was used in the experiments reported here. Labeling individual ancestral cells with HRP made it possible to count all descendants of any recognizable type. The number of labeled descendants as a function of the stage at which the label was first injected could be obtained by starting labeling at progressively later cell generations from the first (2 cells) to the ninth (512 cells) generation. Knowing the mean number of cells of a specific type that originated from blastomeres at each generation and knowing the total number of cells of that type that finally developed made it possible to calculate the mean number of ancestral cells at each stage that gave rise to that cell type (assuming that all counts were made before any cell death occurred). Finally, knowing the mean number of Rohon-Beard or primary motoneuron ancestral cells at each generation made it possible to calculate the probability that, after each cleavage, the daughter cells remained in the lineages leading to Rohon-Beard neurons or primary motoneurons.

Materials and Methods

The specimens used for counting labeled neurons in this study were the same as those prepared for previous studies ($n = 507$; Jacobson and Hirose, 1978, 1981; Hirose and Jacobson, 1979; Jacobson, 1981a, 1983), supplemented by 78 specimens (see below: "*Tissue analysis*"). Methods of producing embryos, of HRP injection, histochemistry, and the controls were the same as reported earlier and will be summarized below.

Embryo selection and tissue processing. Embryos of *Xenopus laevis*, obtained from matings induced by human chorionic gonadotropin, were selected with well marked pigment gradients and with regular and symmetrical patterns of cleavage. An intracellular injection of HRP solution (type IX, Sigma, about 5% in distilled water, 1 to 2 nl) was given by pressure lasting a few seconds into a selected blastomere by means of a glass micropipette with a tip diameter of about 1 to 5 μm. In order to avoid the passage of HRP from the injected cell to its sister cell, the injection was made only after cleavage had been fully completed and shortly before the onset of the next cleavage.

Embryos were raised singly in Petri dishes at 20°C in Steinberg solution (100% diluted gradually to 20%). At stages 30 to 39 of Nieuwkoop and Faber (1967), embryos were fixed (0.5% paraformaldehyde, 2.5% glutaraldehyde, in phosphate buffer, pH 7.4, for 3 to 6 hr at 4°C). Frozen serial sections were cut coronally at 24 μm and were reacted histochemically for peroxidase activity as described previously (Hirose and Jacobson, 1979).

Control experiments. These were the same as those reported by Hirose and Jacobson (1979) and showed that injections did not interfere with normal development in the majority of cases, that no label appeared in animals injected with Steinberg solution alone, and that, in animals receiving HRP injections, the label was confined exclusively to the descendants of the injected blastomere (Hirose and Jacobson, 1979).

Tissue analysis. All specimens ($n = 585$) were examined for the presence of Rohon-Beard neurons and primary motoneurons. Embryos were selected for cell counting procedures if their development was normal, if the tissue processing resulted in few lost sections, and if the histochemical processing resulted in heavily labeled neurons and low background precipitate. In seven animals Rohon-Beard neurons or primary motoneurons constituted less than 1% of the total number of labeled neurons of other types, and these cases were considered aberrant and excluded from statistical analysis. Counts were made of Rohon-Beard neurons in 98 specimens and of primary motoneurons in 142 specimens. Criteria for identification of those neurons will be given below. Cells were counted at × 200, using the criteria of Konigsmark (1970) for somatic cell counts, in all sections from the caudal border of the otocyst to the section at which the curvature of the tail and the undifferentiated state of the spinal cord made neuron identification unreliable (usually at the level of the proctodeum). The diameters of the somata of all labeled Rohon-Beard neurons and all primary motoneurons were measured by means of an eyepiece fitted with a filar micrometer in four specimens cut coronally and two specimens cut horizontally at stage 32. It had been shown previously that there are no significant differences in the sizes of Rohon-Beard neurons stemming from different ancestral blastomeres (Jacobson, 1981a, b). The mean soma diameter was used to calculate the correction factor for split cell counting errors (Abercrombie, 1946). All cell counts reported here have been corrected.

Neuron identification. Rohon-Beard neurons were easily recognizable at stages 30 to 39, labeled or unlabeled, as the largest neurons situated at the dorsal or dorsolateral margin of the spinal cord (Hughes, 1957; Lamborghini, 1980). They were ovoid or pear shaped in coronal section, and in many cases the label extended into the distal neurite which could be traced to the epidermis. Identification of Rohon-Beard neurons was unambiguous in almost all cases. In the few cases in which it was

difficult to distinguish Rohon-Beard cells from other large neurons in the dorsal spinal cord, a positive identification was not made. Wide spacing between Rohon-Beard neurons and their distinctive appearance made it easy to count them, even in specimens injected at the 2-cell stage in which all of the cells in the dorsal part of the spinal cord were labeled.

Consistent identification of primary motoneurons relied upon cell morphology and location. Primary motoneurons, which are the first spinal motoneurons to innervate the adjacent somites (Coghill, 1913; Youngstrom, 1940; Hughes, 1959; Muntz, 1975), were identified initially by retrograde labeling after HRP application to myotomes (Moody and Jacobson, 1983). Their round somas were large (10 to 15 μm) and were not attached to the central canal. Their large dendritic trees extended laterally to the pial surface, and their axons, which frequently branched from a ventromedial dendrite, coursed 50 to 100 μm caudally before entering the myotome (Hughes, 1959; Blight, 1978; Roberts et al., 1981; Moody and Jacobson, 1983). However, since mature dendritic arbors and peripheral axons growing from the cell body were not always apparent in our preparations, any large round soma that was located within the ventral part of the spinal cord and had lost its connection with the central canal was included in the count.

Statistical analysis. The geometric mean of the corrected cell counts of the Rohon-Beard neurons and primary motoneurons for each injection age was calculated. The geometric means were used to generate a least squares line with a 95% confidence interval on log-log scales. This regression line allowed us to estimate, from the intercept of the ordinate, the expected total population of Rohon-Beard neurons (or primary motoneurons) on one side in the tailbud embryo; to determine, from the intercept of the abscissa, the cell generation of the final progenitors of Rohon-Beard neurons (or primary motoneurons); and to estimate the number of progenitor cells at each generation.

Results

The number of Rohon-Beard neurons descended from individual blastomeres at the 2-cell and 16- through 512-cell stages are reported in Table I, and the number of primary motoneurons descended from individual blastomeres at the 16- through 512-cell stages are reported in Table II. These numbers are localized on their blastomeres of origin at the 128-, 256-, and 512-cell stages in Figures 1 to 3. The progenitors of both Rohon-Beard neurons and primary motoneurons at these stages were confined to the dorsal-ventral equatorial region; Rohon-Beard progenitors were found ventrally and the primary motoneuron progenitors were found dorsally.

From these cell counts the geometric mean number (N) of each type of neuron that originates from a single progenitor was calculated at each stage (Tables III and IV). A least squares line was generated from the geometric means at each stage and plotted on log-log scales (Figs. 4 and 5). The points of this regression line are reported in Tables III and IV, as are the points for the upper and lower limits of the 95% confidence interval. The intercept of the ordinate represents the total number of neurons present on one side of the embryo (T). The intercept of the abscissa represents the cell generation of the lineage at which each individual progenitor gives rise to one neuron (Figs. 4 and 5).

TABLE I

The frequency of descendants from single progenitors: Rohon-Beard Neurons

No. of Rohon-Beard Neurons Originating from One Progenitor	Stage of Progenitor						
	2-cell	16-cell	32-cell	64-cell	128-cell	256-cell	512-cell
1							1
2					1		3
3			1		1	4	1
4		1				1	2
5		2	1	1	3	3	2
6				1	2	1	1
7				2		1	2
8				2	1	1	2
10					1	1	
11				1			
12				1		1	
13			1			1	
14		1		1			1
15			1				
16		2					
17		1		1	1		
18			1		1	1	
20		1		1	1		
26		1					
27			1				
34				1			
35			1				
37		1					
46		1					
48		1					
51		1					
53	1						
54	1						
55	1	1					
56	1	1					
58		1					
62	1						
63	1						
64	1						
65	2						
67	3						
68	2						
69	2						
70	1						
71	1	1					
72	1						
90	1						

Knowing the mean total number of each type of neuron (T) and the geometric mean number of each cleavage stage (N), we can calculate the mean number of progenitors (Gn) at each cleavage stage ($Gn = \frac{T}{N}$; Tables III and IV). The mean number of progenitors (Gn) is plotted on a log-log scale in Figure 6. The probability that the daughter cells will continue in the lineage leading to the production of Rohon-Beard neurons or primary motoneurons can be calculated (Tables III and IV). Because all of the progenitor cells up to and including the 512-cell stage give rise to many types of cells in the CNS,

TABLE II

The frequency of descendants from single progenitors: primary motoneurons

No. of Primary Motoneurons from One Progenitor	Stage of Progenitor						
	2-cell	16-cell	32-cell	64-cell	128-cell	256-cell	512-cell
2				2		1	1
3				1	1	3	
4			1		5	2	
5			1	1	2	1	
6			1	2		4	2
7				3		1	1
8		1	3	1		4	
9				2			
10		1	4	1	1	1	1
11					2	3	1
12			1	1	2	3	
13		1	2	2	1	2	1
15		1	3		3	3	
16			2	5	4	1	1
17			2	3		1	
18					2	1	
19					1		
20			1				
21			1		1		
22			1				
23		1		2	2		
24				1	1		
25		1		2		1	
26			2	1	1		
27					1		
28				1		1	
29		1					
31		1					1
32			2				
33					1		
34			1	1			
36				2			
40				1			
42				1			
43					1		
44			1				
45			1				
47				1			
48			1				
50		1					
51		1					
53			1				
58			1				
62			1				
63		1					
69	1						
71		1					
78		1					
81		1					
82		1					
83	1						
84	1						
91	1						
92		1					

mesoderm, endoderm, and epidermis, partitioning of lineages of those various cell types will occur at some stages. Knowing the number of progenitor cells at each generation (G1, G2, G3, . . . Gn) and the number of descendants of each type that originates from a single progenitor, we

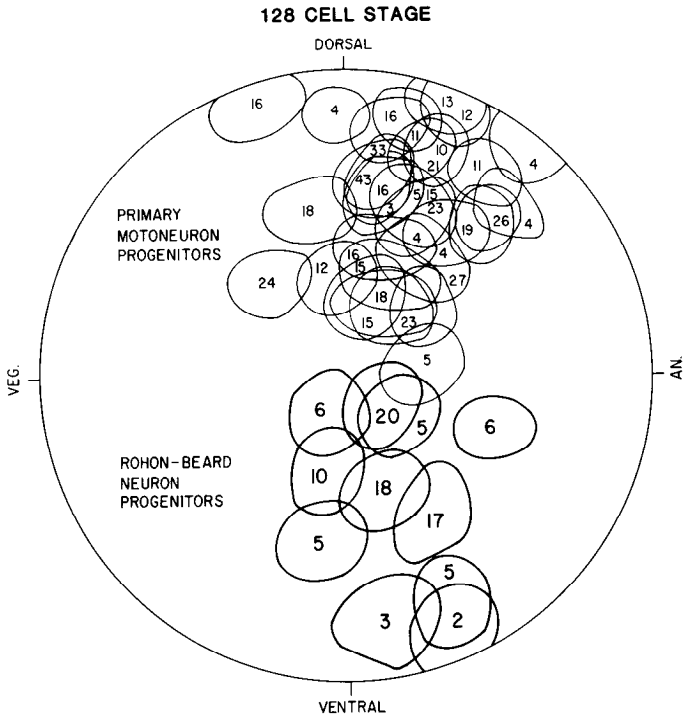

Figure 1. Map of the positions of blastomeres at the 128-cell stage (right lateral view) that gave rise to Rohon-Beard neurons (*thick outlines*) and primary motoneurons (*thin outlines*). The corrected number of neurons of the particular phenotype that were counted in the embryonic spinal cord after that particular blastomere was injected with HRP is shown within the outlines of each blastomere. The poles of the embryo, as determined by pigmentation gradients at the time of injection, are noted on the circumference of the embryo: *AN.*, animal pole; *VEG.*, vegetal pole. The diameter of the embryo is approximately 1.3 mm.

can derive the probability with which a single progenitor at any generation will give rise to each type of descendant in the final population of descendants. For example, knowing the mean number of Rohon-Beard progenitors at one generation (G), we can calculate the number of progenitors that would be formed at the next generation (Gn + 1) if all of the daughter cells continued in the lineage leading to Rohon-Beard neurons, i.e., (2Gn), and we can compare that number with the number of Rohon-Beard progenitors at Gn + 1. The ratio $\frac{Gn + 1}{2Gn}$ is called the continuation probability. The mean continuation probability was found to be close to 0.7 for the lineages of both Rohon-Beard neurons and primary motoneurons (Tables III and IV).

Discussion

These results demonstrate the power of the prospective method of tracing cell lineages by injecting a heritable tracer into progenitor cells at successive cell generations in the early embryo and later identifying and counting specified types of labeled cells that had originated from single progenitors. This method has made it possible to perform a quantitative lineage analysis for the first time in a vertebrate. After the identification had been made of all of the ancestral cells at each stage that gave rise to

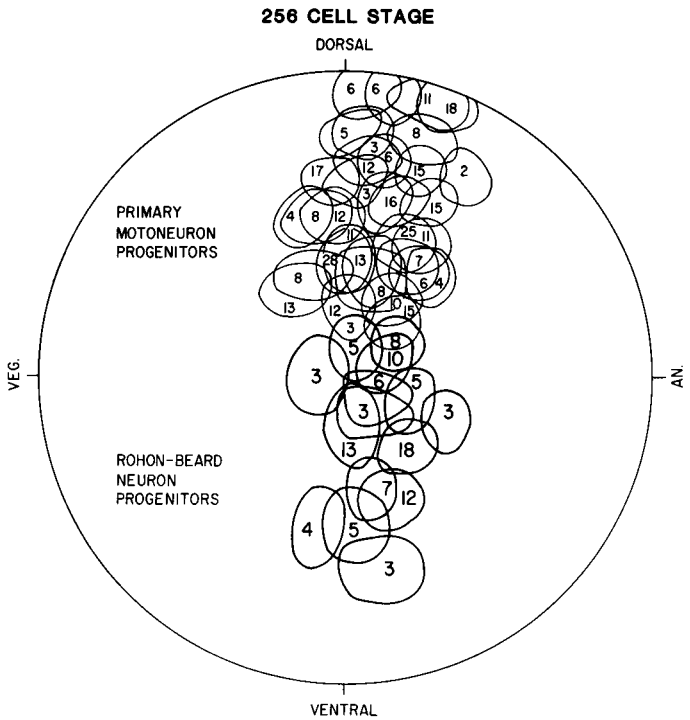

Figure 2. Map of the positions of blastomeres at the 256-cell stage (right lateral view) that give rise to Rohon-Beard neurons and primary motoneurons. The conventions are the same as those described in Figure 1.

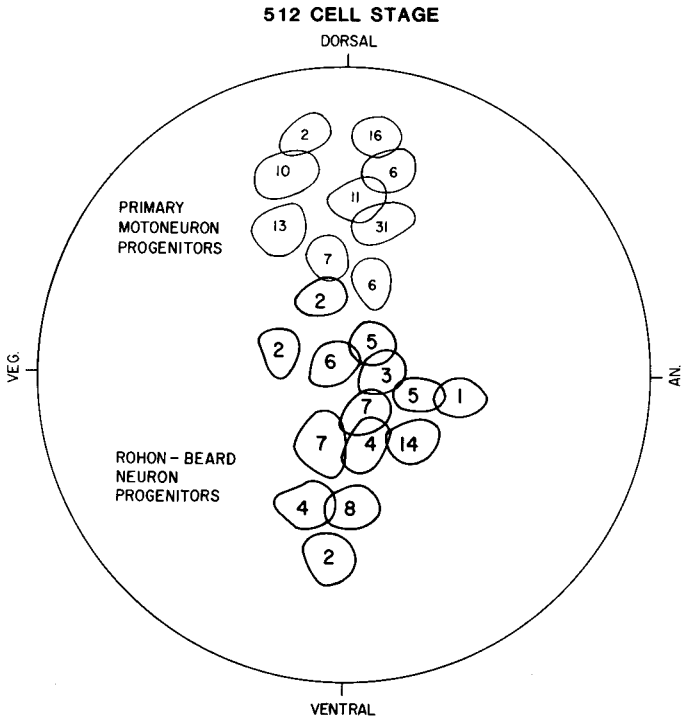

Figure 3. Map of the positions of blastomeres at the 512-cell stage (right lateral view) that give rise to Rohon-Beard neurons and primary motoneurons. The conventions are the same as those described in Figure 1.

TABLE III

Number of progenitors of Rohon-Beard neurons (RB) at each generation and the continuation probability calculated from the geometric mean, regression line, and 95% confidence interval (upper and lower limits)

Cell Generation	Mean[a]	RB Progenitors	Probability	Regression Line	RB Progenitors	Probability	Upper Limit	RB Progenitors	Probability	Lower Limit	RB Progenitors	Probability
1	65.3	1		62.4	1		80	1		48.7	1	
2												
3												
4	23.2	2.81		21.9	2.85		25.4	3.15		18.9	2.58	
			1.02			0.71			0.72			0.70
5	11.4	5.74		15.5	4.04		17.7	4.53		13.5	3.60	
			0.52			0.71			0.71			0.71
6	10.9	6.01		10.9	5.73		12.5	6.41		9.5	5.11	
			0.79			0.71			0.70			0.72
7	6.9	9.48		7.7	8.12		9.0	8.93		6.6	7.38	
			0.58			0.71			0.69			0.73
8	5.9	11.03		5.4	11.51		6.5	12.29		4.5	10.77	
			0.73			0.71			0.68			0.73
9	4.0	16.13		3.8	16.31		4.8	16.81		3.1	15.83	

[a] Geometric mean of the numbers of Rohon-Beard neurons that originated from single progenitors.

Rohon-Beard cells or primary motoneurons, it remained only to label individual ancestral cells at different stages and to count the number of labeled neurons at later stages of development after all neurons of that type had been formed. Because of the observed variability in the number of Rohon-Beard neurons that arise from individual ancestral cells at any one stage of development (Jacobson, 1981a, 1983), it was necessary to count the descendants of a sufficiently large number of ancestral cells and to sample from a sufficiently wide region of the embryo to obtain statistically significant results. We have labeled many cases of every blastomere of the 2- to 64-cell stages (Hirose and Jacobson, 1979, Jacobson and Hirose, 1981) and from the majority of blastomeres that are visible at the surface of the 128-, 256-, and 512-cell embryos (Jacobson, 1983). Those results showed that Rohon-Beard neurons originated from two or three blastomeres on each side of the 16-cell embryo and that, at the 512-cell stage, all of the primary motoneurons and all Rohon-Beard neurons originated from separate groups of about 15 blastomeres on each side (Fig. 3). At the 512-cell stage the lineages of Rohon-Beard neurons and primary motoneurons had separated completely, whereas the lineages were shared in some cases at the

TABLE IV

Number of progenitors of primary spinal motoneurons (PM) at each generation and the continuation probability calculated from the geometric mean, regression line, and 95% confidence interval (upper and lower limits)

Cell Generation	Mean[a]	PM Progenitors	Probability	Regression Line	PM Progenitors	Probability	Upper Limit	PM Progenitors	Probability	Lower Limit	PM Progenitors	Probability
1	81.3[b]	1		66.3			97.9			44.9		
2												
3												
4	34.7	2.34		27.9	2.37		34.0	2.88		23.0	1.96	
			0.91			0.67			0.70			0.63
5	19.1	4.26		21.0	3.17		24.2	4.04		18.1	2.48	
			0.67			0.67			0.69			0.65
6	14.1	5.75		15.7	4.22		17.6	5.55		14.0	3.21	
			0.58			0.67			0.66			0.67
7	12.2	6.65		11.8	5.63		13.4	7.31		10.4	4.33	
			0.71			0.67			0.64			0.70
8	8.6	9.44		8.8	7.51		10.5	9.34		7.4	6.04	
			0.48			0.67			0.63			0.71
9	8.9	9.11		6.6	10.02		8.3	11.76		5.3	8.54	

[a] Geometric mean of the numbers of primary motoneurons that originated from single progenitors.

[b] This number is based on unilateral cell counts of normal embryos and not on counts of embryos injected with HRP at the 2-cell stage.

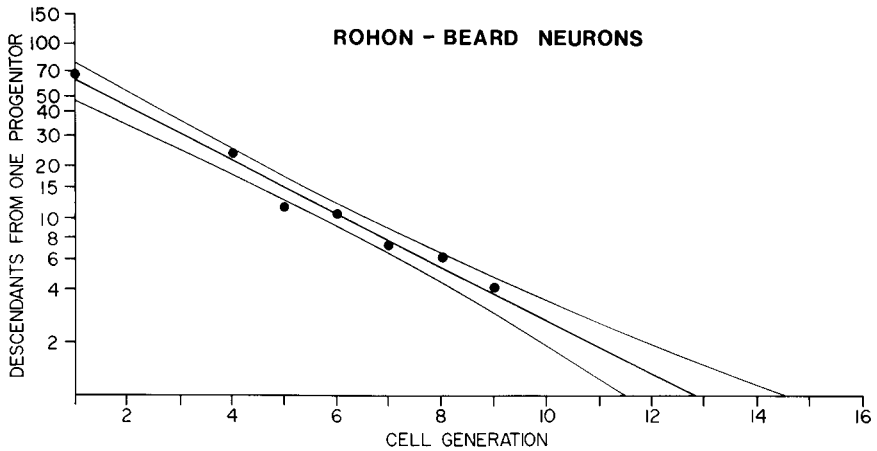

Figure 4. The numbers of Rohon-Beard neurons expected to descend from a single progenitor labeled during the first 15 cell generations are represented by a regression line (*thick central line*) plotted on log-log scales. The geometric means of the numbers of Rohon-Beard neurons derived from a single labeled progenitor at various cell generations (1, 4 to 9) are illustrated by *solid circles.* The regression line and 95% confidence intervals (*thin upper and lower lines*) were generated from the data presented in Table I. The intercept of the *ordinate* represents the total number of Rohon-Beard neurons present on one side of the embryo and the intercept of the *abscissa* represents the cell generation of the lineage at which each individual progenitor gives rise to one Rohon-Beard cell.

128- and 256-cell stages as shown in Figures 1 and 2 (Jacobson, 1983).

The main findings are, first, that the lineages of Rohon-Beard neurons and primary motoneurons of *Xenopus* diverge progressively from the 16-cell stage (Jacobson, 1981a), and those lineages have separated completely in the 512-cell blastula. Second, the results confirm that these types of neurons each originate from a separate group of ancestral cells (Figs. 1 to 3): the primary motoneurons originate from the posterior-medial ancestral cell group and the Rohon-Beard neurons originate from the posterior-lateral ancestral cell group (Jacobson, 1983). Each blastomere gives rise to a small percentage of the total population, and there is an uncertainty in the number of neurons of a specified type that descend from a single progenitor (Jacobson, 1983). Tables I and II show the frequency of origin of Rohon-Beard neurons and primary motoneurons from single progenitors belonging to successive generations up to the ninth generation; the range in these numbers indicates a lack of determinancy in the specific number of a particular type of neuron derived from a single progenitor.

Analysis of the results reveals that on a log-log plot there is a linear relationship between the geometric means of the numbers of neurons of each specified type that originate from single progenitors at successive generations (Figs. 4 and 5). Where the linear regression intercepts the abscissa indicates the cell generation of the lineage at which each individual progenitor gives rise to one Rohon-Beard neuron or primary motoneuron. In other words, the intercept at the abscissa shows the average generation at which all members of these neuron types became postmitotic. The intercepts at the abscissa

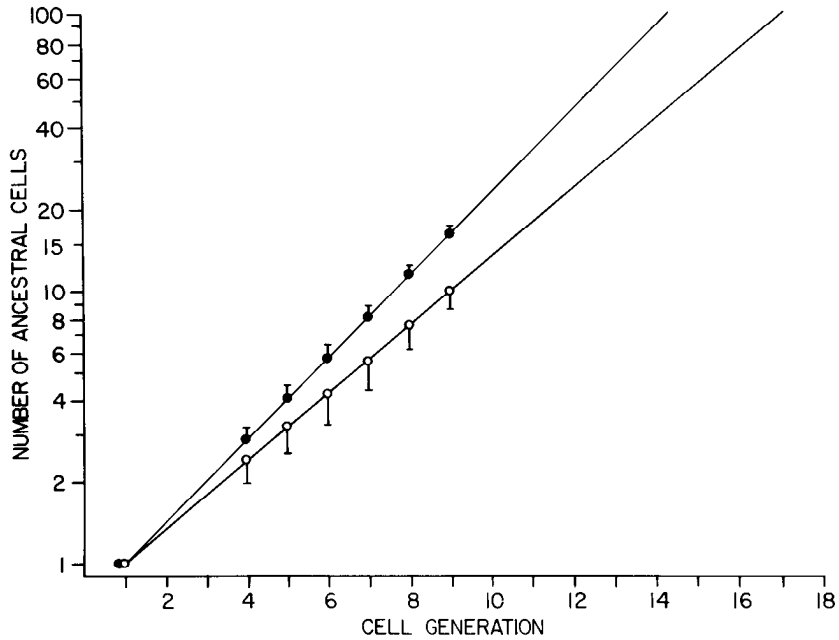

Figure 5. The numbers of primary motoneurons expected to descend from a single progenitor labeled during the first 18-cell generations are represented by a regression line (*thick central line*) plotted on log-log scales. The geometric means (*solid circles*), regression line, and 95% confidence intervals (*thin upper and lower lines*) were generated from the data presented in Table II.

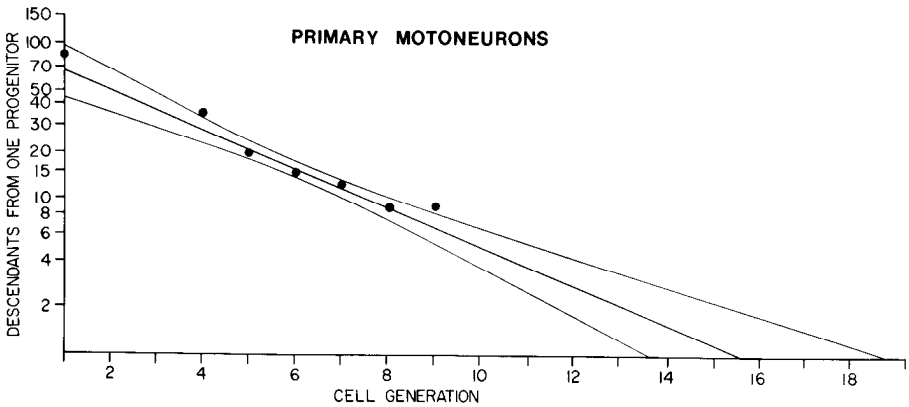

Figure 6. The mean numbers of ancestral cells for Rohon-Beard neurons (*solid circles*) and primary motoneurons (*open circles*) and the 95% confidence intervals (*bars*) increase linearly, on a log-log scale, with increasing cell generation. These data are presented in Tables III and IV.

of the 95% confidence limits indicate that 95% of these types of neurons originate within those limits, one or at most two cell generations on each side of the mean.

These results show the generation of the lineage at which each type of neuron originates but, because the generation times are not known, these results are not plotted in time. However, studies of neuronal birth dates, using tritiated thymidine as a label, have shown that more than 80% of the progenitors of the large neurons of the spinal cord have passed through the DNA synthesis phase of their terminal cell cycle at late gastrula stages 11½ to 12 (Lamborghini, 1980). This occurs at 16 to 18 hr after fertilization in our embryos (M. Jacobson, manuscript in preparation). From this it may be concluded that embryonic stages 11½ to 12 coincide with the 14th to 15th generation of the lineages of Rohon-Beard neurons and primary motoneurons. Note that this does not correspond to the same number of generations for the embryo as a whole. All cells in the *Xenopus* embryo divide almost synchronously and rapidly until the 12th generation, after which asynchrony and lengthening of the generation time occur (Newport and Kirschner, 1982a, b; M. Jacobson, manuscript in preparation). Throughout this discussion the numbers of cells are given in relation to the number of generations from the first cleavage; therefore, those numbers are independent of changes of cell generation time. This is necessary because of progressive lengthening of the duration

of the cell cycle during development and the gradual asynchrony of cleavages that occur after the 12th cell generation.

From these data we can calculate the mean number of each type of neuron (N) that originates from a single progenitor at each cleavage stage (Figs. 4 and 5) and the mean number of progenitors (Gn) at each cleavage stage (Fig. 6). However, we know that more than 80% of Rohon-Beard neurons and primary motoneurons complete their final DNA synthesis at late gastrula stages (Lamborghini, 1980). Therefore, the number of progenitors on each side cannot exceed the final total number of Rohon-Beard neurons or primary motoneurons on each side (mean, 65.75 and 81.75, respectively). Thus, for the majority of those types of neurons the lineage is terminated at the stage when the mean number of progenitors equals the final mean number of descendants, i.e., at the 13th cell generation in the case of Rohon-Beard neurons and at the 16th generation in the case of the primary motoneuron. The lineage analysis is likely to be more complicated after generation of the first neurons because after that time the lineages will no longer be regular and symmetrical.

The number of ancestral cells at each stage cannot be measured directly at present. The method used here is indirect and subject to limitations. We have calculated the mean number of ancestral cells at each stage by dividing the total number of differentiated neurons of each type by the mean number of neurons of that type which originates from a single ancestral cell. There are two reasons for believing that this procedure is justified. First, the fact that the number of ancestral cells increased linearly on a log-log plot (Fig. 6) indicates that the lineages were regular and symmetrical. Second, the increase of labeled cells in clones initially labeled at 16-, 32-, and 64-cell stages and counted at progressively later stages shows that all labeled cells continued dividing and no cell death occurred during the time before the 13th generation (M. Jacobson, manuscript in preparation). However, these findings do not exclude the possibility that a small percentage of unlabeled cells entered the lineages of Rohon-Beard neurons or primary motoneurons. However, that would have resulted in a reduction of the mean continuation probability closer to 0.5 when, in fact, the mean continuation probability was close to 0.7. There were variations in the continuation probabilities calculated at different generations (Table III and IV), but they varied randomly, presumably as a result of counting errors. The continuation probabilities did not show a progressive reduction closer to 0.5 with increasing cell generations as would be predicted on the hypothesis that some unlabeled cells entered the lineage.

The probability that the daughter cells will continue in the lineage leading to production of Rohon-Beard neurons or primary motoneurons is close to a mean of 0.7 for both lineages (Tables III and IV), indicating a biased commitment toward development of these specificied cell types. How the number of cells of a specific type is controlled and how the time of phenotypic commitment is controlled are two different, albeit related, questions. Cell determination or commitment (the terms are equivalent) has been defined in terms of the transplantation and explantation experiments that have been used to test the state of cellular commitment: if the cell's potency outside its normal environment is the same as the fate in the intact embryo it is said to be committed, but if potency exceeds cell fate it is not committed (Weiss, 1939). Not wishing to enter into a detailed critique of this definition of commitment, it is sufficient to point out that this definition is arbitrarily limited by the results of a certain type of experiment. When grafting experiments have shown that embryonic stem cells are multipotential, it has generally been assumed that there are equal probabilities of the several different phenotypes arising from the same progenitor. When different numbers of the various phenotypes are generated, this is believed to occur as a result of divergence of their lineages at different times and different rates of proliferation and possibly differential cell death in different lineages.

In those terms, commitment is an all-or-none event occurring at a specific time for a specified type of cell. However, it has been suggested that cell determination is gradual or progressive and that there may be a period of labile determination during which cells are gradually biased into one or another pathway of differentiation (Rossant, 1977). We may thus define cell commitment in terms of the experimental strategy used in these experiments: if the probability of a progenitor cell giving rise to a specified type of descendant is greater than chance, the progenitor is biased toward, and may be said to be committed to, the production of that specified cell type. In those terms commitment is not all-or-none and may be labile and reversible. This type of commitment would not necessarily be detected by grafting or explanting cells. Although such experiments have the advantage of being able to test the effects of the environment on cell fates, they are limited by the fact that the grafts contain many cells of unknown heterogeneity. In our experiments the possibility remains that the observed bias occurred because the cells shared the same environment. To study this further we are transplanting single labeled cells of known lineage from a labeled embryo to a different position in an unlabeled embryo.

This bias toward development of a specified type of cell within a lineage that also produces other types of cells has not previously been reported. The HRP method of lineage tracing has made it possible to measure the bias precisely in these experiments. It remains to be shown whether there is also a reciprocal bias against production of other types of cells. Presumably, if there is a higher probability of production of Rohon-Beard neurons, there may be a lower probability of production of other types of nerve cells and of non-neuronal cells that are all produced in the same clone. Or the biases in favor of the Rohon-Beard neurons and primary motoneurons may result in rapid production of all of those neurons early in development, after which the bias may shift in favor of another class or classes of cells.

This bias toward production of Rohon-Beard neurons or primary motoneurons appears to be necessary in order to produce those types of neurons at sufficiently early stages of development. We conceive of such early-forming neurons as preferred neurons in the sense that they could only be produced early in development if certain

progenitors were exclusively devoted to their production (which has not been observed) or if certain progenitors had a marked preference for production of such neurons although also producing other types of cells. Once those preferred neurons have been generated, the lineage may continue to produce other types of cells, possibly again showing a preference for the production of one type of cell. When all or most cells of that type have been generated, the bias may be directed toward production of yet another type of cell.

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