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ROHON-BEARD NEURON ORIGIN FROM BLASTOMERES OF THE 16-CELL FROG EMBRYO[1]

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Abstract

Clonal origins of Rohon-Beard neurons in *Xenopus* were determined quantitatively by injecting horseradish peroxidase into individual blastomeres at the 16-cell stage and later counting labeled and unlabeled Rohon-Beard neurons. Two different patterns of cleavage were selected. In pattern X, all Rohon-Beard neurons originated from three blastomeres (V1.1, V1.2, and V2.2) on each side; in pattern Y, all Rohon-Beard neurons originated from two blastomeres (V1.2 and V2.2) on each side. Counts of Rohon-Beard neurons at larval stages 32 to 34 showed that 96 to 100% (mean 99%) originated from blastomeres on the same side; of these, 68 to 90% (mean 75%) descended from V1.2, 20 to 31% (mean 24%) descended from V2.2, and 0 to 7% descended from V1.1. The significance of the regionally restricted origin of Rohon-Beard neurons is discussed.

The relation between cell lineage and differentiations of various types of neurons in the vertebrate central nervous system is not known. For example, it is not known whether the Rohon-Beard neurons always develop at characteristic positions in the dorsal spinal cord because their differentiation at those sites is controlled by local conditions or because their descent is from a specific ancestral cell or cells and is followed by a stereotyped pattern of cell migration to their final positions.

Rohon-Beard neurons in *Xenopus* are especially favorable for this type of investigation because they are large, easily identified cells and because they are among the first cells to originate in the central nervous system. Lamborghini (1980) has shown that 80% of the Rohon-Beard neuron precursors have completed their final DNA synthesis before the end of gastrulation in *Xenopus* (stage 13 of Nieuwkoop and Faber, 1967), 12 to 14 hr after fertilization.

Determining the embryonic ancestry of Rohon-Beard neurons has been made possible by the use of horseradish peroxidase (HRP) as an intracellular label for tracing cell lineages (Jacobson and Hirose, 1978, 1981; Weisblatt et al., 1978; Hirose and Jacobson, 1979). We have shown that HRP injected into individual blastomeres of *Xenopus* embryos does not alter normal development, is transmitted to all descendants of the injected cell, and can be detected in those descendants, including Rohon-Beard neurons, in the central nervous system at later stages of development (Hirose and Jacobson, 1979; Jacobson, 1980; Jacobson and Hirose, 1981). Here, I have used that technique to establish the embryonic ancestry of Rohon-Beard neurons more precisely and quantitatively. This investigation also served as a necessary preliminary to the studies of the effects of removal of individual blastomeres reported in the following paper (Jacobson, 1981).

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Materials and Methods

Embryos were obtained from matings of *Xenopus laevis* induced by chorionic gonadotropin. Embryos with well marked pigment gradients in the animal-vegetal and dorsal-ventral axes and with regular and symmetrical patterns of cleavage were selected for injection at the 16-cell stage. Two types of frequently seen patterns of cleavage were selected: type X with radial convergence of cleavage furrows at the animal pole and type Y with anticlinal cleavage furrows meeting the midline some distance dorsal and ventral to the animal pole. For ease of description, the injected blastomeres are designated as shown in Figure 1 and defined in Hirose and Jacobson (1979). An intracellular injection of horseradish peroxidase (HRP) solution (Type IX, Sigma; 20% in distilled water or Steinberg solution, 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 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% for 3 hr, 50% for 3 hr, and 25% until fixation). When they reached larval stages 32 to 34 of Nieuwkoop and Faber (1967), the embryos were fixed (0.5% paraformaldehyde, 2.5% glutaraldehyde, in phosphate buffer, pH 7.4) for 3 hr at 4°C and were washed twice (5%

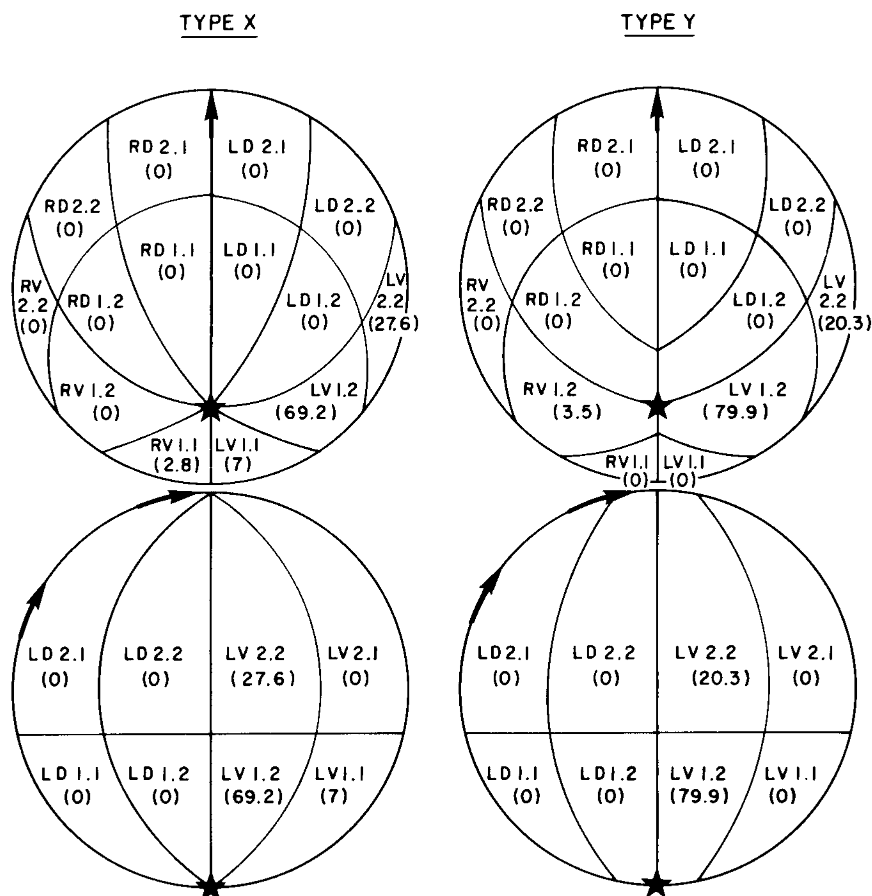

Figure 1. Cleavage patterns X and Y at the 16-cell stage. The animal pole view is shown in the *upper diagram* and the left lateral view is shown in the *lower diagram*. The *star* is at the animal pole and the *arrow* points along the midline to the vegetal pole. Designations of blastomeres are given in Hirose and Jacobson (1979): *LD*, left dorsal; *RD*, right dorsal; *LV*, left ventral; *RV*, right ventral. Blastomeres can be identified by the darker pigmentation at the animal pole and by the fact that ventral blastomeres are more darkly pigmented than those located dorsally. *Numbers in parentheses* show the mean percentage of Rohon-Beard neurons on the left side of the spinal cord originating from each blastomere. The diameter of the embryo is about 1.3 mm.

sucrose for 6 hr and 15% sucrose for 1 hr, both in phosphate buffer, pH 7.4, at 4°C). Frozen serial sections were cut coronally at 24 μm. Sections mounted on subbed slides were reacted histochemically for peroxidase by a modification of the method of Graham and Karnofsky (1966), using diaminobenzidine tetrahydrochloride as the chromagen as previously described (Hirose and Jacobson, 1979). The resulting brown reaction product will be referred to as the "label." All specimens (n = 46) were examined for the presence of labeled Rohon-Beard neurons and counts of labeled and unlabeled Rohon-Beard neurons were made in 14 specimens. Rohon-Beard cells were easily recognizable, labeled or unlabeled, as the largest neurons situated at the dorsal or dorsolateral margin of the spinal cord (Fig. 2).

Control experiments were the same as those reported in 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 found in all descendants of the injected blastomere and only in those descendants (Hirose and Jacobson, 1979). The diameters of the somata of all labeled Rohon-Beard neurons were measured in 4 specimens at stage 32 (after HRP injection of V1.1, V1.2, and V2.2) by means of an eyepiece fitted with a filar micrometer. These data were used to determine whether there are differences in sizes of Rohon-Beard neurons stemming from different ancestral blastomeres and to correct for split cell counting errors (Ambercrombie, 1946; Konigsmark, 1970).

Results

The results of the control experiments were the same as reported previously (Hirose and Jacobson, 1979).

Of the 77 embryos injected with HRP at the 16-cell stage, 13 died or were rejected because of abnormal development. The remaining 64 specimens developed normally and were fixed at stages 32 to 34 and processed as described above, but 18 failed to process properly. The remaining 46 were examined microscopically for labeled cells in the central nervous system. The types of labeled

cells and their regional distribution in the central nervous system were as previously described (Hirose and Jacobson, 1979; Jacobson and Hirose, 1981).

Although other types of labeled cells were apparent in the central nervous system, no labeled Rohon-Beard neurons were seen in specimens in which the following blastomeres had been injected (no significant differences were seen between the results of injecting homologous blastomeres on the left and right sides): types X and Y, D1.1 ($n = 4$); D1.2 ($n = 8$); D2.1 ($n = 4$); D2.2 ($n = 6$); V2.1 ($n = 4$). No labeled Rohon-Beard neurons were seen after injection of V1.1 type Y ($n = 3$).

Rohon-Beard neurons were labeled after injection of blastomeres V1.2 ($n = 8$) and V2.2 ($n = 6$) in types X and Y but were labeled in type X only after injection of V1.1 ($n = 3$). All labeled Rohon-Beard neurons were found on the injected side only, except after injection of V1.1, type X, and after injection of V1.2, type Y, in which a few labeled Rohon-Beard neurons were found contralaterally (Table I). In all cases, the labeled Rohon-Beard neurons were mingled with unlabeled Rohon-Beard neurons along the length of the spinal cord. Extramedullary neurons (Hughes, 1957) also were labeled along the entire length of the spinal cord. Other types of cells were labeled in the dorsal spinal cord and dorsal spinal ganglia as described by Hirose and Jacobson (1979).

TABLE I

Rohon-Beard neurons at larval stages 32 to 34 after injection of single blastomeres at the 16-cell stage

X and Y are the different types of cleavage patterns shown in Figure 1. LV and RV refer to left ventral and right ventral cells, respectively. A1 to A3, B1 and B2, and C1 and C2 refer to the experiments discussed in the text.

Type	Injected Cell	Uninjected Side[a]		Injected Side[a]	
		Labeled	Unlabeled	Labeled	Unlabeled
X (A1)	RV 1.2	0	64 (100%)	37 (69%)	17 (31%)
X (A2)	LV 1.2	0	62 (100%)	55 (71%)	23 (29%)
X (A3)	RV 1.2	0	68 (100%)	51 (68%)	24 (32%)
Mean X (A)	V1.2	0	65 (100%)	48 (70%)	21 (30%)
Y (A1)	RV 1.2	3 (4%)	68 (96%)	46 (69%)	21 (31%)
Y (A2)	LV 1.2	3 (4%)	70 (96%)	71 (90%)	8 (10%)
Y (A3)	RV 1.2	2 (3%)	63 (97%)	56 (80%)	14 (20%)
Mean Y (A)	V1.2	3 (4%)	67 (96%)	58 (81%)	14 (19%)
X (B1)	RV 2.2	0	90 (100%)	26 (31%)	59 (69%)
X (B2)	LV 2.2	0	54 (100%)	14 (24%)	44 (76%)
Mean X (B)	V2.2	0	72 (100%)	20 (28%)	52 (72%)
Y (B1)	RV 2.2	0	67 (100%)	16 (20%)	63 (80%)
Y (B2)	LV 2.2	0	71 (100%)	17 (21%)	65 (79%)
Mean Y (B)	V2.2	0	69 (100%)	16 (20%)	64 (80%)
X (C1)	LV 1.1	2 (3%)	65 (97%)	5 (7%)	65 (93%)
X (C2)	LV 1.1	1 (1%)	69 (99%)	4 (7%)	57 (93%)
Mean X (C)	V1.1	2 (3%)	67 (97%)	5 (7%)	61 (93%)
Y (C1)	LV 1.1	0	56 (100%)	0	53 (100%)
Y (C2)	RV 1.1	0	53 (100%)	0	60 (100%)
Mean Y (C)	V1.1	0	55 (100%)	0	56 (100%)

[a] Corrected for split cell counting errors.

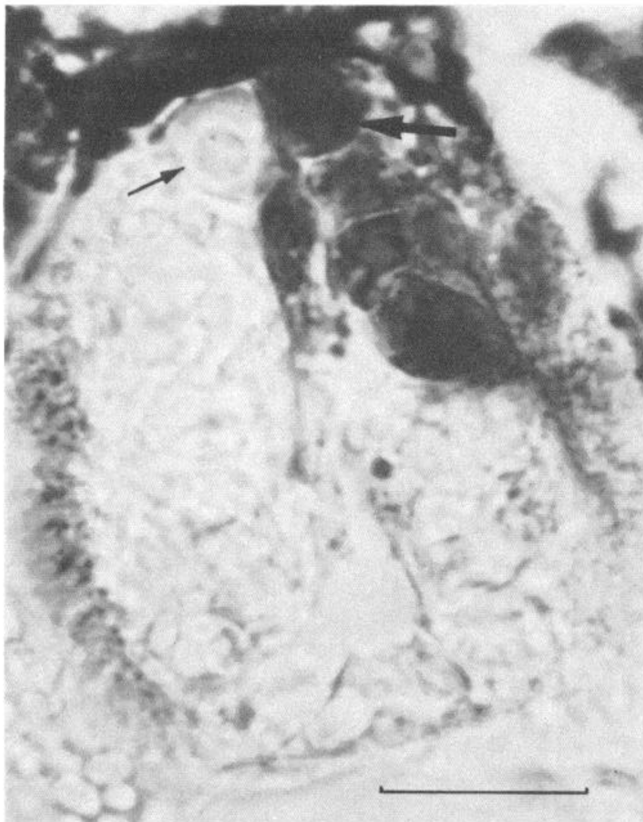

Figure 2. Coronal section through the spinal cord at stage 34 after injection of HRP into blastomere RV 1.2 at the 16-cell stage. There are several labeled cells in the dorsal half of the right side of the spinal cord. These include a labeled Rohon-Beard neuron, indicated by the *large arrow*, and several commissural neurons whose axons filled with label can be traced across the ventral commissure to the lateral funiculus on the opposite side. An unlabeled Rohon-Beard neuron at the dorsal margin of the cord on the left side is indicated by the *small arrow*. The dark material outside of the spinal cord, dorsal to the Rohon-Beard cells, is melanin. The *bar* is 50 μm.

The diameter of the somata of Rohon-Beard neurons showed a rostrocaudal gradient. At the rostral end of the spinal cord, which has a maximum diameter of about 140 μm, the diameters of Rohon-Beard neuron somata were about 30 μm, whereas caudally, where the spinal cord tapers down to a diameter of about 70 μm, the diameters of Rohon-Beard neuron somata were about 15 μm. Diameters of labeled Rohon-Beard neurons descended from different ancestral cells measured in 4 different animals were: V1.1, mean diameter, 25.4 μm (2 animals, $n = 19$); V1.2, mean diameter, 22.8 μm ($n = 108$); V2.2, mean diameter, 22.1 μm ($n = 49$). The differences between the diameters of Rohon-Beard neurons arising from different blastomeres are not statistically significant. These mean diameters were used to correct for split cell counting errors using the method of Abercrombie (1946) in which

$$N = n\left(\frac{t}{t + d}\right)$$

where N is the corrected cell count, n is the raw cell count, t is the section thickness, and d is the mean diameter of the Rohon-Beard neurons (Table I).

Discussion

These experiments show that Rohon-Beard neurons originate entirely from two blastomeres (V1.2 and V2.2) on each side in cleavage type Y and from three blastomeres (V1.1, V1.2, and V2.2) on each side in type X. The majority of Rohon-Beard neurons descended from blastomere V1.2 in both types of cleavage, but there were significant differences between the two types with respect to the percentages of these neurons that arose from a particular blastomere and with respect to the percentage of labeled Rohon-Beard neurons on the side opposite the injection. In cleavage type X, blastomere V1.2 gave rise to 68 to 71% (mean 70%) of Rohon-Beard neurons, all on the same side, whereas, in type Y, blastomere V1.2 gave rise to 69 to 90% (mean 81%) of Rohon-Beard neurons on the same side and 3 to 4% of Rohon-Beard neurons on the opposite side. In cleavage type Y, no Rohon-Beard neurons descended from V1.1, whereas, in type X, blastomere V1.1 gave rise to 7% of Rohon-Beard neurons on the same side and 1 to 2% on the opposite side. There were no significant differences between the percentage of Rohon-Beard neurons that descended from V2.2 in the two types of cleavage.

The fact that up to 4% of labeled Rohon-Beard neurons were found on the side opposite labeled blastomeres V1.1 (type X) and V1.2 (type Y) probably results from a small geometrical disparity between the first cleavage furrow (shown by the *star* and *arrow* in Fig. 1) and the presumptive midsagittal plane (midline) of the central nervous system. The first cleavage in *Xenopus* usually coincides closely with the future midline of the embryo, but large deviations from this coincidence occur naturally and can be produced experimentally without affecting normal development (Morgan, 1927, pp. 158–168). Small disparities between the first cleavage plane and the presumptive midline of the embryo would result in the observed labeling of Rohon-Beard neurons on both sides of the midline after labeling those ancestral cells that extend to the first cleavage furrow.

These observations show that Rohon-Beard neurons originate from the same geographical region of the 16-cell blastula regardless of the pattern of cleavage. This region forms a band extending just below the horizontal meridian on both sides from the animal pole but does not reach the vegetal pole (i.e., does not include V2.1). The band thus includes blastomere V1.2 in both types of cleavage and includes V1.1 in type X but not in type Y where V1.1 lies too far below the horizontal meridian to include the region from which Rohon-Beard neurons originate. This raises the question of whether the Rohon-Beard neurons arise from this region because of an invariant pattern of localization of cytoplasmic determinants which are parceled out into blastomeres regardless of the pattern of cleavage, as Wilson (1925, pp. 1072–1076) so clearly pointed out.

The possibility of such determinants of Rohon-Beard neuron differentiation being localized in blastomeres V1.1, V1.2, and V2.2 in type X cleavage and in V1.2 and V2.2 in type Y cleavage and predominantly in V1.2 in both types of cleavage is dealt with in the following paper (Jacobson, 1981). Let it suffice to say that there is no evidence one way or the other to distinguish between the hypothesis of prelocalization of determinants of Rohon-Beard cell differentiation on the one hand and some other form of determination of Rohon-Beard neuron differentiation presumably involving cellular interactions and the acquisition of "positional information" (Wolpert, 1969) appropriate for differentiation of Rohon-Beard cells at their characteristic positions.

Regardless of how the developmental programming of Rohon-Beard neuron differentiation occurs, it has to take place before stage 13, the end of gastrulation, when 80% of these neurons precursors have completed their final DNA synthesis (Lamborghini, 1980). In the 8 to 10 hr that elapse between the 16-cell stage, at which the label was introduced in these experiments, and stage 13, at which most Rohon-Beard neurons originate, six to eight cell generations are produced (Hara, 1977; M. Jacobson, unpublished observations), i.e., 128 to 512 cells have been generated by stage 13 from each of the blastomeres that give rise to Rohon-Beard neurons. Estimates of the number of Rohon-Beard neurons vary somewhat: Hughes (1957) shows 70 to 80 on each side at a stage corresponding approximately to stages 37 and 38; Lamborghini (1980) reported more than 100 on each side after stage 40; I have counted 53 to 90 on each side with a mean of 68 at stages 32 to 34. These differences between the counts of Rohon-Beard cells at stages 32 to 34 and after stage 40 may be due to late differentiation of part of the Rohon-Beard cell population. In any case, Rohon-Beard neurons constitute only a fraction of the descendants of V1.2 alone. Other types of cells that descend from the same ancestral cells as the Rohon-Beard neurons are extramedullary neurons (Hughes, 1957), dorsal root ganglion cells, and some other types of neurons in the dorsal half of the spinal cord as well as cells of the olfactory placode and olfactory bulb derived from V1.1 in some cases, cells of the otic vesicle derived from V1.2 (Jacobson and Hirose, 1981), and a number of non-neural cells such as mesenchyme of the tail fin and various types of cutaneous ectodermal cells. Thus, the progenitors of the Rohon-Beard neurons also give rise to many of the types of cells that have been shown to arise from the neural crest. Thus, V1.1, V1.2, and V2.2 are definitely not committed to the production of Rohon-Beard neurons only and are not even committed, in any sense of the term, to the production of neurons only.

These experiments cannot be expected to answer the question of the extent of the multipotentiality of the ancestral cells that normally give rise to Rohon-Beard neurons because a limitation of labeling experiments such as these is that they do not give any information about the degree of determination or "commitment" at the time of labeling the cells but show only the prospective fates of the labeled cells. Removal of these cells may give information about the regulative capacities of the remaining cells and about the degree to which cells are committed to the formation of specific types of differentiated descendants. Such experiments are reported in the following paper (Jacobson, 1981), but, for their interpretation, they depend on the knowledge of prospective fates that has been obtained from the experiments reported here.

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