REGULATION OF INTERSTITIAL CELL DIFFERENTIATION IN HYDRA ATTENUATA

III. EFFECTS OF I-CELL AND NERVE CELL DENSITIES

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SUMMARY

The interstitial cell (i-cell) of hydra, a multipotent stem cell, produces two classes of differentiated cell types, nerve cells and nematocytes, throughout asexual growth. Using a new assay, the regulation of i-cell commitment to either nerve cell or nematocyte differentiation was investigated. This assay was used to determine the fractions of i-cells differentiating into nerve cells and nematocyte precursors in a variety of in vivo cellular milieus produced by hydroxyurea treatment, differential feeding, and reaggregation of dissociated cells.

Nematocyte commitment was found to be positively correlated with the size of the i-cell population and independent of the axial position of the i-cells along the body column. This indicates that i-cell commitment to nematocyte differentiation may be regulated by feedback from the i-cell population. Nerve cell commitment was found to be correlated with regions of high nerve cell density. This suggests that nerve cell commitment is regulated by feedback from the nerve cell population or dependent on axial position. Implications of such mechanisms for the regulation of i-cell population size and distribution are discussed.

INTRODUCTION

The interstitial cell (i-cell) of hydra is a multipotent stem cell, producing two classes of cell types, nerve cells and nematocytes (e.g. Davis, 1974; Slautterback & Fawcett, 1959), throughout asexual growth. The multipotency of individual i-cells has been demonstrated by David & Murphy (1977), who cloned i-cells in reaggregates of mitotically inactive cells derived from nitrogen mustard-treated hydra. Individual clones were found to produce both nerve cells and nematocytes (David, 1975; David & Murphy, 1977). Thus individual stem cells have at least 2 differentiative pathways open to them, raising the question of what is responsible for i-cell commitment to nerve cell or nematocyte differentiation.

David & Gierer (1974) have calculated that under steady-state conditions 30% of the stem cells are committed daily to nematocyte differentiation, 10% are committed to nerve cell differentiation, and the remaining 60% divide to replenish the stem cell population. Evidence from a number of sources has indicated, however, that the fractions of the stem cell pool committed to nerve cell and nematocyte differentiation can vary from these steady-state values. Bode, Flick & Smith (1976) determined that the fraction of the stem cell population dividing and remaining stem

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cells rises to 70% following experimental reduction of the i-cell population using hydroxyurea. Furthermore, hydra fed either 1, 6 or 20 shrimp larvae per day differ in the relative sizes of their nerve cell and nematoblast populations (Bode, Flick & Bode, 1977). Thus the commitment of stem cells to nerve cell and nematocyte differentiation can vary under different conditions, suggesting that the process is subject to regulation.

It is very likely that i-cell differentiation is regulated by interactions with other cell types (cf. Bode & David, 1978). Asexual hydra are composed of only a few cell types: the epithelio-muscular cells of the ectodermal and endodermal cell sheets; 3 types of gland and mucous cells; and finally i-cells and their derivatives: 1–3 types of nerve cells, and 4 types of nematocytes and their differentiation intermediates (e.g. David, 1973). Since the total number of cell types is so small, the possible number of cell-cell interactions governing i-cell behaviour need not be very large. We have therefore examined the possible dependence of i-cell differentiation on the several cell types in hydra. In this paper we present evidence indicating that i-cell commitment to nematocyte differentiation is dependent on i-cell density, whereas nerve cell commitment is correlated with regions of high nerve cell density.

MATERIALS AND METHODS

Culture of animals

*Hydra attenuata* were cultured at 18 ± 1 °C in media consisting of 1 x 10⁻³ M CaCl₂ and 1.25 x 10⁻⁴ M Na₂ EDTA in spring water (Arrowhead) or of 1 x 10⁻³ M MgCl₂, 1 x 10⁻³ M KCl, 1.25 x 10⁻³ M Na₂ EDTA, 1 x 10⁻³ M CaCl₂ and 5 x 10⁻⁴ M NaHCO₃ in distilled H₂O. Stock cultures were fed 6–7 days per week with *Artemia* nauplii (3–8 nauplii/hydra) and washed approximately 8 h after feeding. Animals maintained on particular feeding regimes were individually fed the specified number (0–7) shrimp/day by placing the shrimp directly on the tentacles. Those fed 20 shrimp/day were flooded with shrimp; this results in an average ingestion of 20 shrimp (Bode *et al.* 1977). Feeding regime animals were maintained at the designated feeding level for a minimum of 9 days prior to experimental use.

Cellular composition

The cellular composition of whole hydra or of specific regions was analysed using the maceration technique of David (1973). In all experiments, 5–10 hydra or tissue fragments were macerated together. Cells were classified according to David's (1973) terminology. The nomenclature used here for the i-cells, however, will be modified as follows: the term i-cell, unless otherwise noted, will refer to those big i-cells (David, 1973) occurring singly or as pairs; this class includes the multipotent stem cells and i-cells committed to nerve cell or nematocyte differentiation (David & Gierer, 1974). Clusters or 'nests' of 4, 8, 16, or 32 i-cells, formerly referred to as little i-cells, are destined to form nematocytes (David & Gierer, 1974); these cells will be referred to as dividing nematoblasts. Those cells in which the developing nematocyst capsule can be seen will be termed postmitotic nematoblasts. Additionally, the term gland cell will be used to refer to all 3 types of gland and mucous cells (cf. David, 1973).

To estimate the density of any cell type (i.e. the ratio of that cell type to the epithelial cells), a differential cell count of at least 1000 cells of the appropriate types was made per determination. In addition, where total cell numbers per animal or region were determined, the total number of epithelial cells was determined using a Neubauer cell counter. The total numbers of other cell types were then calculated from their ratios to the epithelial cells.
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[3H]thymidine administration and autoradiography

Hydra were radioactively labelled by injection into the gastric cavity of [3H]thymidine (50 μCi/ml; 6 Ci/mM; New England Nuclear) using a polyethylene needle (David & Campbell, 1972).

Slides of macerated, radioactively labelled animals or fragments were processed for autoradiography as follows: slides were washed in distilled H2O, dipped in 1 : 1 distilled H2O : Kodak NTB, Nuclear Track Emulsion, and dried. After 7 days exposure at 4 °C, autoradiographs were developed for 7 min with Kodak D-19, rinsed in distilled H2O, fixed for 5 min with Kodak Rapid-Fix, and washed thoroughly with distilled H2O.

Hydroxyurea treatment

Hydra were treated with 10^{-4} M hydroxyurea in hydra medium (Bode et al. 1976). The length of exposure to the drug is described in each experiment. Animals were washed several times with fresh hydra medium following treatment.

Formation of reaggregates

Reaggregates of dissociated cells of whole hydra or of specific regions were prepared as described by Gierer et al. (1972). The dissociation medium used consisted of 5 x 10^{-4} M CaCl2, 1 x 10^{-3} M MgSO4, 2.8 x 10^{-3} M KCl, 1 x 10^{-4} M TES, 3.3 x 10^{-4} M Na2HPO4, 5 x 10^{-3} M Na-pyruvate, 5 x 10^{-3} M Na3 citrate, and 50 mg/l. rifampicin; the pH was adjusted to 6.9.

'Head' reaggregates were formed from cell suspensions prepared from heads including the apical half of the 1-region (apical one-eighth of the gastric region; Wolpert, 1969) pooled with the apical halves of buds with tentacles (stages 6-10, Otto & Campbell, 1977a). 'Body' reaggregates consisted of cells from the entire body column except the head, the apical half of the 1-region, and the apical halves of stage 6-10 buds. Reaggregates were allowed to develop 24 h before assaying either cellular composition or stem cell commitment. Four to eight aggregates were used per determination. For commitment studies aggregates were labelled by inserting a polyethylene needle directly through the epithelia to the centre of the hollow aggregates. All other procedures were as described for intact hydra.

Statistical analysis

In each case curves were fitted to the data by the method of least squares (e.g. Snedecor & Cochran, 1967). For each set of data the best curve was selected from among linear, logarithmic, exponential, or asymptotic regression curves by means of either (1) the minimum residual sum of squares (e.g. Snedecor & Cochran, 1967) or (2) the correlation coefficient, r, with the minimum probability, P, of arising solely by chance (e.g. Fisher, 1958).

RESULTS

Assay for i-cell commitment

To study stem cell commitment to nerve cell and nematocyte differentiation, an assay was developed to analyse i-cell differentiation at a time close to the point of commitment. The assay for commitment was defined on the basis of a knowledge of the i-cell differentiation pathways and the kinetics of i-cells traversing each pathway. The pathways an i-cell can undergo are as follows. I-cells occur as single cells or in pairs (David & Gierer, 1974). I-cells not committed to product cell differentiation divide and remain i-cells. Nerve cells differentiate directly from committed stem cells following a final mitosis (David & Gierer, 1974). Labelled nerve cells are found starting...
18 h after pulse-labelling hydra with [3H]thymidine and occur in large numbers by 26 h (David & Gierer, 1974). In contrast, a stem cell committed to nematocyte development undergoes a more complex development. It first undergoes 2–5 cell divisions. The resulting cells remain together in a ‘nest’ as a syncytium, and subsequently undergo synchronous differentiation to form 4–32 nematocytes of one type (Slatterback & Fawcett, 1959). During the earlier, proliferative stage these cells are termed dividing nematoblasts, while once the developing nematocyst capsule can be seen they are referred to as postmitotic nematoblasts. [In an earlier terminology (David, 1973) i-cells and the morphologically similar nests of 4 cells were referred to
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as big i-cells, whereas nests of 8, 16, or 32 cells were termed little i-cells.] The cell cycle durations for the i-cells and nests of 4 cells, although variable, average to around 24 h (Campbell & David, 1974).

Thus, in hydra pulse-labelled with [3H]thymidine the progeny of the cohort of labelled i-cells (1's and 2's) would be found subsequently as follows. One day after labelling, labelled i-cells, labelled nests of 4 dividing nematoblasts and labelled nerve cells would all be derived from the pulse-labelled i-cells (see Fig. 1). Larger nests of labelled cells would have been derived by the division of nests of 4 cells, and hence from labelled nematoblasts rather than from i-cells. Two days after labelling, the progeny of the labelled i-cell cohort would be found as labelled i-cells, labelled nests of 4 and 8 dividing nematoblasts, and labelled nerve cells (Fig. 1); larger nests again would have resulted from pulse-labelled nematoblasts. On the basis of these kinetics, i-cell commitment to nerve cell or nematocyte differentiation can be identified 1 or 2 days later, respectively. Thus the fraction of the i-cells committed to nerve cell differentiation within a single cell cycle (~ 1 day) is defined as the number of labelled nerve cells at 26 h divided by the total labelled progeny of the i-cells 26 h after pulse-labelling (see Fig. 1). Commitment of i-cells to nematocyte differentiation can be defined in a similar manner. Nests of 4 and 8 cells are the earliest distinguishable nematocyte precursors. Therefore, the nematocyte commitment fraction is defined as the fraction of the labelled cells derived from the pulse-labelled i-cells which are in nests of 4 and 8 dividing nematoblasts 48 h after labelling. The total size of the labelled cohort at 26 or 48 h is invariant to the fraction of the cells following each pathway; the labelled i-cell cohort doubles by 26 h and quadruples by 48 h. Since the total size of the analysed population is independent of the choice of pathway, the fraction of the cells in each compartment reflects the differentiation behaviour of the i-cell population.

This assay was used to analyse i-cell commitment in a variety of situations. Routinely hydra were pulse-labelled with [3H]thymidine, samples macerated at 26 h (for measuring nerve cell commitment) and at 48 h (for nematocyte commitment) and the labelled cell populations analysed with autoradiography. From 300-1000 labelled cells of the appropriate categories were scored per determination.

Since the labelled i-cell population consists of the multipotent stem cells as well as i-cells already committed to nerve cell or nematocyte differentiation (David & Gierer, 1974), the assay does not strictly measure new stem cell commitment. However, one can show (Appendix A, Yaross, 1978) that the results obtained by assaying the i-cell population are valid when restricted to the fraction representing the stem cell population.

Correlation of i-cell commitment with cellular distribution

To determine the normal regional distribution of nerve cell and nematocyte production in hydra, i-cell commitment was assayed as described above in steady-state animals which had been maintained on 6 shrimp/day. Following pulse-labelling with [3H]thymidine, animals were removed at 26 or 48 h and cut into the 5 regions indicated in Fig. 2. The fragments from each region were then macerated separately to
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determine the nerve cell and nematocyte commitment fractions at each level along
the body column. I-cell commitment was found to differ along the apical-basal axis
(Fig. 2). The nerve cell commitment fraction was highest at the extremities and lowest
in the gastric region. In contrast, nematocyte commitment was highest in the gastric
region and much lower in the head and lower peduncle.

![Graph showing nerve cell and nematocyte commitment fractions](image)

**Fig. 2.** I-cell commitment analysed by region. Nerve cell and nematocyte commit-
ment fractions were assayed in each of the 5 regions indicated: Head region included
the apical half of the 1-region (apical one eighth of the gastric region, Wolpert, 1969).
Each bar represents the mean value of 2 experiments; error bars indicate the range of
the data. Ten fragments of the appropriate region were macerated together for each
sample.

The regional pattern of i-cell differentiation suggests that stem cells are influenced
by local differences in some parameter. As the cellular composition of the animal is
known to vary along the length of the body column (Bode et al. 1973), cell types whose
axial distributions are similar to that of nerve cell or nematocyte commitment may be
interacting with i-cells to affect differentiation to the particular class of product cells.
To examine this possibility, the cellular composition of each region assayed for i-cell
commitment was also determined in hydra maintained on 6 shrimp/day. These data
are presented in Table 1 as the ratios of the number of cells of each cell type to the
epithelial cells, a convenient way to express the density of a given cell type within the
hydra epithelia (Bode et al. 1973). The relationship between the density of each non-
epithelial cell type throughout the hydra and either the nerve cell or nematocyte
commitment distribution was examined by calculating the correlation coefficients
(Fisher, 1958). As shown in Table 2, both nerve cell and nematocyte commitment
Stem cell differentiation in Hydra correlate with the distribution of many of the cell types within the animal at a significant level \( (P < 0.05) \). Both direct and inverse correlations were found to exist. The regional pattern of nerve cell commitment was directly correlated with the nerve cell and nematocyte populations and inversely correlated with the i-cells and the nematoblasts. Nematocyte commitment was found to be directly correlated with the regional distribution of the i-cells and nematoblasts and inversely correlated with the nerve cell and nematocyte populations. In an attempt to determine whether these correlations were causal or coincidental in nature, experiments were devised to vary the density of these cell types in hydra, and to measure the effect of these altered densities on nerve cell and nematocyte commitment.

### Table 1. Cell densities in different regions of hydra

<table>
<thead>
<tr>
<th>Region</th>
<th>I-cells</th>
<th>Post-dividing nematocytes</th>
<th>Post-mitotic nematocytes</th>
<th>Nematocytes</th>
<th>Nerve cells</th>
<th>Gland cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head</td>
<td>0.044</td>
<td>0.094</td>
<td>0.019</td>
<td>1.042</td>
<td>0.343</td>
<td>0.228</td>
</tr>
<tr>
<td>Gastric region</td>
<td>0.485</td>
<td>1.176</td>
<td>0.795</td>
<td>0.264</td>
<td>0.106</td>
<td>0.425</td>
</tr>
<tr>
<td>Budding region</td>
<td>0.351</td>
<td>0.773</td>
<td>0.462</td>
<td>0.080</td>
<td>0.063</td>
<td>0.207</td>
</tr>
<tr>
<td>Upper peduncle</td>
<td>0.201</td>
<td>0.646</td>
<td>0.517</td>
<td>0.100</td>
<td>0.238</td>
<td>0.138</td>
</tr>
<tr>
<td>Lower peduncle</td>
<td>0.030</td>
<td>0.101</td>
<td>0.071</td>
<td>0.071</td>
<td>0.357</td>
<td>0.946</td>
</tr>
<tr>
<td>Whole animal</td>
<td>0.223</td>
<td>0.584</td>
<td>0.368</td>
<td>0.120</td>
<td>0.114</td>
<td>0.194</td>
</tr>
</tbody>
</table>

Values are the means of 2 experiments; 10 regions or 5 whole hydra were analysed together per determination.

### Table 2. Correlations between i-cell commitment and cell densities

<table>
<thead>
<tr>
<th></th>
<th>Ratio to epithelial cells</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I-cells</td>
<td>Post-dividing nematocytes</td>
<td>Post-mitotic nematocytes</td>
<td>Nematocytes</td>
<td>Nerve cells</td>
<td>Gland cells</td>
</tr>
<tr>
<td>A. Nerve cell commitment fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r = )</td>
<td>0.691 (−)</td>
<td>0.726 (−)</td>
<td>0.777 (−)</td>
<td>0.707 (+)</td>
<td>0.708 (+)</td>
<td>0.174 (−)</td>
</tr>
<tr>
<td>( P &lt; )</td>
<td>0.05</td>
<td>0.02</td>
<td>0.01</td>
<td>0.05</td>
<td>0.05</td>
<td>—</td>
</tr>
<tr>
<td>B. Nematocyte commitment fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r = )</td>
<td>0.771 (+)</td>
<td>0.789 (+)</td>
<td>0.871 (+)</td>
<td>0.774 (−)</td>
<td>0.733 (−)</td>
<td>0.357 (+)</td>
</tr>
<tr>
<td>( P &lt; )</td>
<td>0.01</td>
<td>0.01</td>
<td>0.005</td>
<td>0.01</td>
<td>0.02</td>
<td>—</td>
</tr>
</tbody>
</table>

\( r = \) correlation coefficient (e.g. Fisher, 1958; cf. Materials and methods).

(+) indicates positive (direct) correlation.

(−) indicates negative (inverse) correlation.

Correlation coefficients were calculated using the data from the experiments in Table 1 and Fig. 2.
**Nematocyte commitment**

One of the cell types whose distribution was found to correlate with the regional pattern of nematocyte commitment is the i-cell population. If this correlation is causal rather than coincidental, reduction of the overall i-cell density in the animal should result in lowered nematocyte commitment. I-cell density can be reduced by treating hydra with hydroxyurea (Bode et al. 1976). Animals were treated with $10^{-2}$ M hydroxyurea (HU) for 24 h and periodically thereafter their i-cell density determined. I-cell density was reduced from $0.233 \pm 0.054 \, \text{(S.D.)}$ to $0.055 \pm 0.007$ by day 4, followed by recovery approaching control levels by day 10. Nematocyte commitment and i-cell density were assayed in control and HU-treated hydra at intervals during the recovery period. These data are presented in Fig. 3. Also in Fig. 3 are the results of nematocyte commitment and i-cell density measurements for hydra treated with $10^{-2}$ M HU for two 24-h periods separated by 12 h in hydra medium. In these

![Graph](image_url)
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In hydra, the i-cell density decreased to 0.004 and did not recover. As the results in Fig. 3 indicate, there was again a positive correlation ($r = 0.963$, $P < 0.001$) between i-cell density and nematocyte commitment. This suggests that nematocyte commitment is a function of i-cell density, since the reduction in i-cell density produced by HU treatment was accompanied by decreased nematocyte commitment.

![Graph showing correlation between i-cell density and nematocyte commitment](image)

**Fig. 4.** Correlation of nematocyte commitment with i-cell density in hydra maintained at different feeding levels (see text). Each point represents 5-10 hydra fed the same number of shrimp/day and analysed together.

As another means of varying i-cell density animals were placed on different feeding regimes. The cellular composition has been found to differ markedly between animals fed different amounts of food daily (Bode et al. 1977). Separate groups of hydra were fed 0, 1, 3, 4, 6, 7, or ~20 shrimp/day for 9–22 days. The i-cell density and nematocyte commitment fraction of animals at each feeding level were periodically assayed beginning on day 9. As shown in Fig. 4, the different feeding regimes resulted in a 3-fold variation in i-cell density. Again nematocyte commitment was positively correlated ($r = 0.543$, $P < 0.05$) with i-cell density.

To determine if different regions of hydra are equivalent in the extent to which i-cell density and nematocyte commitment are correlated, animals maintained on
different feeding regimes were also analysed by region. Hydra maintained on 0, 3, 7, or ~20 shrimp/day were assayed for i-cell density and nematocyte commitment. Just prior to maceration these animals were cut into 3 pieces, head, gastric region, and peduncle, and each region analysed separately. The results of this experiment are in Fig. 5. When the data from the 3 regions assayed are plotted together, there is a logarithmic correlation \( r = 0.833, P < 0.001 \) between i-cell density and nematocyte commitment. This relationship suggests that nematocyte commitment might be more sensitive to i-cell density at low i-cell densities. Since the slope of the curve is steepest at low i-cell density and decreases with increasing i-cell density the magnitude of the change in nematocyte commitment predicted by a given change in i-cell density also decreases with increasing i-cell density.

The data for each region examined in this experiment were found to be clustered in a different portion of the curve. Thus when each region was statistically analysed individually, i-cell density and nematocyte commitment were found to be correlated...
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in head regions \( r = 0.788, P < 0.02 \), but no significant correlation existed in either gastric regions or peduncles. These results coupled with the regional correlations shown in Fig. 2 could indicate that nematocyte commitment is more dependent on axial position than on i-cell density. This could explain why no correlation with i-cell density was found for the gastric region (Fig. 5). Alternatively, nematocyte commitment could be insensitive to i-cell density at high i-cell densities. If so, i-cell density

![Graph showing correlation of nematocyte commitment with i-cell density](image)

Fig. 6. Correlation of nematocyte commitment with i-cell density in the gastric region of hydroxyurea-treated hydra. Each point represents the gastric region of 10 animals analysed together.

and nematocyte commitment may have failed to correlate in this experiment because of the high i-cell density found in the gastric region. If the latter explanation is correct, then further reducing the i-cell density in the gastric region should again produce a reduction in nematocyte commitment. This was tested by examining the gastric regions of HU-treated animals. Hydra were exposed to \( 10^{-2} \text{M} \) HU for either 24 h only, or for 24 h followed by 12 h in hydra medium and re-exposure to HU for 12 h. On days 2, 4, 6, and 8 after the beginning of the HU treatment, i-cell density and nematocyte commitment were assayed in the gastric regions of control hydra and hydra subjected to both HU regimes. As demonstrated in Fig. 6, when the i-cell density was reduced, the nematocyte commitment fraction also decreased. In this experiment there was again a positive logarithmic correlation \( r = 0.796, P < 0.01 \)
between i-cell density and nematocyte commitment. This indicates that nematocyte commitment is correlated with i-cell density in the gastric region as well as in the head. Therefore nematocyte commitment cannot be solely a function of axial position.

Several other cell populations were found to be correlated with the regional pattern of nematocyte commitment in the animal (Table 2). The relationships between these cell types and the nematocyte commitment fraction were also examined in HU-treated hydra. Neither nerve cell, nematocyte, nor postmitotic nematoblast densities correlated with nematocyte commitment in this experiment, although the density of

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**Fig. 7.** Correlation of nerve cell commitment with nerve cell density in reaggregates of heads, bodies, or whole hydra. Each point represents 4–8 reaggregates analysed together.

**Fig. 8.** Correlation of nerve cell commitment with nerve cell density in whole hydra maintained at different feeding levels. Each point represents 5–10 hydra fed the same number of shrimp/day and analysed together.
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dividing nematoblasts was positively correlated with nematocyte commitment ($r = 0.567, P < 0.01$). However, previous work has indicated that the dividing nematoblasts are not likely to be involved in the regulation of i-cell differentiation (Bode et al. 1976). These authors found dividing nematoblasts to be virtually absent from hydroxyurea-treated hydra at the time during the recovery of the i-cell population when nematocyte differentiation resumes, which is inconsistent with a positive effect of dividing nematoblast density on nematocyte commitment. The evidence therefore suggests that the differentiated product cells and their intermediates do not affect i-cell commitment to nematocyte differentiation.

**Nerve cell commitment**

The regional distributions of several cell types were found to be either positively or inversely correlated with that of the nerve cell commitment fraction (Table 2). The first of these cell types to be investigated for a possible role in nerve cell determination was the nerve cell population itself. The positive correlation between nerve cell commitment and nerve cell density was examined by varying the nerve cell density in the animal in experiments similar to those for nematocyte commitment.

Hydra of varying nerve cell density were produced using the technique of reaggregating dissociated cells (Gierer et al. 1972). Aggregates were made of cell suspensions prepared from isolated heads, bodies (hydra minus heads), or from whole
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hydra. Since the nerve cell density is highest in the head region and much lower in the remainder of the body column, this results in aggregates differing greatly in their nerve cell densities. By 24 h after reaggregation, aggregates have become hollow spheres composed of 2 tissue layers closely resembling those of normal hydra. At this time, aggregates were analysed for cell composition and pulse-labelled to assay nerve cell commitment. Nerve cell commitment was found to be positively correlated ($r = 0.872$, $P < 0.01$) with the nerve cell density (Fig. 7).

Nerve cell commitment was also assayed in hydra maintained on feeding regimes in experiments comparable to those described above for nematocyte commitment. Differential feeding leads to changes in the nerve cell density as well as in the i-cell density (Bode et al. 1977). Animals maintained on 0, 1, 3, 4, 7, or ~20 shrimp/day were assayed to determine nerve cell density and nerve cell commitment. There was again a significant positive correlation ($r = 0.633$, $P < 0.01$) between these parameters, as seen in Fig. 8. Thus as the nerve cell density of the intact hydra increased, the fraction of the stem cells committed to nerve cell formation increased as well.

The relationship between nerve cell density and nerve cell commitment in different

Fig. 10. Correlation of nerve cell commitment with i-cell density in whole hydroxyurea-treated hydra. Each point represents 5–10 hydroxyurea-treated (△) or control (▲) animals analysed together.
regions of hydra maintained on feeding regimes was then examined. Nerve cell
density and nerve cell commitment were analysed in heads, gastric regions, or ped-
uncles of hydra maintained on either 0, 3, 7, or ~20 shrimp/day. The results of 2 such
experiments are seen in Fig. 9. When the data from all regions were analysed together,
there is a positive, exponential relationship between nerve cell density and nerve cell
commitment ($r = 0.777$, $P < 0.005$). However, when the data from each region

![Graph showing correlation between nerve cell commitment and i-cell density in hydra maintained at different feeding levels and analysed by region. Each symbol represents a different region: head (square), gastric region (circle), and peduncle (triangle). Each point represents the pooled regions of 10 animals maintained at the same feeding level.](image-url)

Fig. 11. Correlation of nerve cell commitment with i-cell density in hydra main-
tained at different feeding levels and analysed by region. Each symbol represents
a different region: head (□), gastric region (●), and peduncle (△). Each point
represents the pooled regions of 10 animals maintained at the same feeding level.

were analysed separately, nerve cell density and nerve cell commitment were sig-
ificantly correlated only in the peduncles ($r = 0.756$, $P < 0.05$); these parameters
were independent in heads and gastric regions. This could indicate that nerve cell
commitment is influenced by the location of the i-cells along the body axis; this
possibility will be considered in the Discussion.

In the foregoing experiments the relationship between nerve cell commitment and
the densities of other cell types was also examined. No consistent positive correlation
was found. However, a significant inverse correlation was found in one case between
i-cell density and nerve cell commitment in HU-treated animals (Fig. 10; $r = 0.895$,
$P < 0.001$). These parameters were again inversely correlated when the data of the
3 regions analysed in hydra maintained an 0, 3, 7, or ~20 shrimp/day were examined
together (Fig. 11, $r = 0.813$, $P < 0.005$). However, when each region was analysed
individually, nerve cell commitment and i-cell density were not significantly correlated in any of the 3 regions. Furthermore, i-cell density and nerve cell commitment did not correlate in intact hydra maintained on different feeding regimes or in reaggregates of heads, body regions, or whole hydra. Thus nerve cell commitment was correlated with i-cell density only when very low i-cell densities were examined.

The regional distribution of the nematocytes and their differentiation intermediates (dividing and postmitotic nematoblasts) also correlated with nerve cell commitment in intact hydra (Table 2). But when the population densities of these cell types were measured in reaggregates of heads, bodies, or whole hydra, none of these cell types was found to correlate with nerve cell commitment. In a single experiment, when HU-treated hydra were examined, the density of dividing nematoblasts was inversely correlated ($r = -0.761, P < 0.02$) with nerve cell commitment; again the postmitotic nematoblast and nematocyte densities failed to correlate with nerve cell commitment. Thus it seems unlikely that i-cells, nematocytes, or their intermediates play major roles in the regulation of nerve cell commitment.

DISCUSSION

Assay for stem cell commitment

The assay for i-cell commitment used here was developed in order to be able to directly measure and quantitate the commitment to nerve cell and nematocyte differentiation of the stem cell population at any given point in time. Previous estimates of the fractions of the stem cells differentiating along each pathway have relied heavily on calculated turnover times for the differentiated cell types and on assumptions of exponential growth of all cell populations in steady state, exponentially growing hydra (David & Gierer, 1974). In contrast, we have pulse-labelled a cohort of i-cells within intact animals, and one or two days later examined the distribution of labelled cells to determine the fractions that had left the stem cell compartment.

The assay is based on the kinetics of i-cell proliferation and differentiation (Fig. 1). The validity of the assay rests upon two major assumptions. The first of these is that the effect of previous i-cell commitment is not large. Some i-cells are likely to be committed to nerve cell or nematocyte differentiation at the time of labelling (David & Gierer, 1974). As a result, cells previously committed to nematocyte differentiation, for example, will appear in the denominators of both the nerve cell and nematocyte commitment fractions as well as in the numerator of the nematocyte commitment fraction. Thus the nerve cell and nematocyte commitment fractions are not wholly independent. Extensive model calculations have shown that this type of interdependence may mildly distort the shape of relationships between the commitment fractions and other parameters, but not alter the basic nature of such correlations (cf. Appendix A, Yaross, 1978).

For example, the extent to which the assayed nerve cell commitment fraction is affected by previous nematocyte commitment can be seen by examining the relationship between the regional patterns of nerve cell density and nerve cell commitment. In the gastric region where nematocyte commitment is high, 32% of the cells belong-
ing to the labelled i-cell cohort are likely to be committed to nematocyte differentiation (David & Gierer, 1974). Therefore the fraction of the i-cells which are stem cells is lower than in the extremities where nematocyte commitment is low. As a result the assayed nerve cell commitment fraction is correspondingly reduced in the gastric region at equivalent nerve cell commitment per stem cell. For steady-state animals, the magnitude of the decrease this produces can be estimated by correcting for measured nematocyte production, and nerve cell commitment per stem cell calculated

![Graph showing nerve cell commitment per i-cell and nerve cell commitment per stem cell](image)

Fig. 12. Comparison between nerve cell commitment per i-cell and nerve cell commitment per stem cell (see text). The solid line is the relationship between the nerve cell commitment fraction and nerve cell density seen in Fig. 9. The dotted line represents nerve cell commitment per stem cell, corrected for the effect of nematocyte commitment (Yaross, 1978).

(cf. Yaross, 1978). When the assayed nerve cell commitment per i-cell data for hydra maintained on different feeding regimes and analysed by region (Fig. 9) are transformed in this manner to nerve cell commitment per stem cell, the exponential relationship with nerve cell density is essentially unchanged (see Fig. 12). Thus the effect of previous commitment on the assay parameters is quite small. The data in this paper have been expressed as fractions of the i-cell population rather than of the stem cell population because the former is the manner in which the data are collected. Calculations of this type indicate that the conclusions drawn based on commitment per i-cell apply equally to commitment per stem cell (Yaross, 1978).

A second question arises as to how accurately the foregoing kinetic picture describes the behaviour of the i-cell population. Because of the cell cycle variability that
Campbell & David (1974) found to be characteristic of i-cells, the kinetics outlined in Fig. 1 do not describe all i-cells. For example, i-cells with relatively short cell cycles committed to nematocyte formation might be found as nests of 16 cells 48 h after labelling while those with relatively long cell cycles may still be in nests of 2 cells. There is no single time point at which all pulse-labelled i-cells which were committed to nematocyte differentiation would be found as nests of 4 and 8 cells; at 48 h the slow and fast 'tails' of the labelled i-cell cohort would be lost from the analysis. However, calculations using the known extremes for cell cycle parameters indicate that at least 50–80% of i-cells occurring singly or as pairs committed to nematocyte differentiation would still be found as nests of 4 or 8 cells at the time nematocyte commitment was assayed (Yaross, 1978).

Nerve cell differentiation requires 18–20 h from the time of the final S-phase to the completion of cytodifferentiation into a recognizable nerve cell (David & Gierer, 1974). Since the length of S-phase for i-cells is 12 h (Campbell & David, 1974), one half to two thirds (~ seven twelfths) of the pulse-labelled stem cells committed to nerve cell differentiation would be identifiable as labelled nerve cells 26 h after labelling. Later assay times to allow the remainder of the committed nerve cell precursors to differentiate were not practical as they would allow i-cells with very short cell cycles to be lost from the 1+2+4+ nerve cell population. However, as the time required for the differentiation of new nerve cells is uniform throughout hydra (David & Gierer, 1974), the percentage of committed nerve cells detected by the assay should also be invariant. Therefore comparative studies are not adversely affected by the one-third to one-half underestimate of nerve cell commitment.

The validity of the assay is further supported by calculations which show the obtained values for daily nerve cell and nematocyte commitment to be in close agreement with what David & Gierer (1974) found to be required for the steady-state maintenance of these populations. The total number of cells committed to each product cell type was obtained by multiplying the commitment fraction (cells committed/i-cell) by the total i-cells/region. Additionally, for nerve cell commitment it is necessary to correct for the underestimate produced by the assay (see above). This can be done by multiplying the total obtained by 12/7 (since seven twelfths of the total committed cells would be detected by the assay). The results of these calculations for steady-state hydra fed 7 shrimp/day are in Table 3. Also in Table 3 are the values predicted by David & Gierer (1974) on the basis of steady-state requirements for hydra of comparable size. Thus the assay, when applied to steady-state hydra, yields results quite similar to what David & Gierer (1974) have shown to be necessary for the maintenance of the nerve cell and nematocyte populations of such animals. It is therefore likely that any errors inherent in the assay are small in magnitude.

In addition, the number of stem cells produced daily can be calculated from the data and compared with the requirements for the maintenance of the stem cell population. The number of stem cells present in hydra maintained on 7 shrimp/day is the total i-cell number (Table 3) minus the fraction of the i-cells previously committed to differentiation (62% of i-cells committed to nematocyte differentiation; David & Gierer, 1974; Appendix A, Yaross, 1978). Thus there would be
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8969 - (0.62 x 5633) = 5477 stem cells per animal. To maintain the stem cell density after one day of exponential growth, assuming the tissue doubling time to be 4.5 days (Otto & Campbell, 1977b), the total number of stem cells would increase to 6391 as shown in Table 4. The number of stem cells produced daily can be calculated from the assayed commitment to product cell differentiation. If stem cells which do no

Table 3. Daily commitment of i-cells to product cell differentiation

<table>
<thead>
<tr>
<th>Total i-cells per animal</th>
<th>Nematocyte commitment fraction (a)</th>
<th>I-cells committed to nematocyte formation (b)</th>
<th>Nerve cell commitment fraction (c)</th>
<th>I-cells committed to nerve cell formation (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured</td>
<td>8969</td>
<td>0.628</td>
<td>5633†</td>
<td>0.024†</td>
</tr>
<tr>
<td>Calculated (David &amp; Gierer, 1974)</td>
<td>9040</td>
<td>—</td>
<td>4660‡</td>
<td>—</td>
</tr>
</tbody>
</table>

* Based on mean determinations for hydra fed 7 shrimp/day from feeding regime experiments presented in Figs. 4, 5, 8, 9.
† (a) x (b).
‡ This includes both single i-cells (1760) and i-cells in nests of 2 (2900) committed to nematocyte formation.
§ (a) x (c) x (12/7); see text for explanation.

Table 4. Stem cell number during exponential growth

<table>
<thead>
<tr>
<th>Day</th>
<th>Epithelial cells, no.</th>
<th>Stem cells, no.</th>
<th>Stem cell density</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32039*</td>
<td>5477†</td>
<td>0.171</td>
</tr>
<tr>
<td>1</td>
<td>37373†</td>
<td>6391‡</td>
<td>0.171</td>
</tr>
</tbody>
</table>

* Based on mean determinations for hydra fed 7 shrimp/day from experiments presented in Figs. 4, 5, 8, 9.
† Calculated as described in text.
‡ Number predicted by exponential growth with tissue doubling time of 4.5 days (Otto & Campbell, 1977b).
§ Number predicted by measured commitment fractions (see text).

become committed to nerve cell or nematocyte differentiation divide to form 2 stem cells within 24 h, the stem cell number after 1 day would be 5934 (see Appendix F, Yaross, 1978, for details). As the number of stem cells on day 1 could range from 0 to 10 954, corresponding to 100 to 0% differentiation of stem cells on day 0, the agreement between the calculated production of stem cells and the requirement for maintenance of the stem cell population is quite good. This indicates that virtually all i-cells can be accounted for by stem cell proliferation and nerve cell and nematocyte differentiation.
I-cell density and nematocyte commitment

The experiments concerning nematocyte commitment strongly suggest that i-cell density and nematocyte commitment are tightly coupled in hydra. It is clear that it cannot be determined from correlations alone whether variations in one parameter are responsible for changes seen in a second or if, on the other hand, they are both responding in concert to a third, unknown variable. The fact that two different methods (hydroxyurea treatment and differential feeding) of varying i-cell density produced corresponding changes in the nematocyte commitment fraction does, however, lend credence to the hypothesis that stem cell commitment to nematocyte formation is regulated by i-cell density. The results of the regional experiments further suggest that i-cell density can affect nematocyte commitment in any region of hydra, thereby distinguishing it from positional effects on i-cell commitment.

No other cell type in the animal was found to be consistently correlated with nematocyte commitment in hydra. Thus the i-cell is most likely the only cell type involved in the regulation of stem cell commitment to nematocyte differentiation. The type of nematocyte formed, however, is correlated with the axial position of the i-cell (Bode & Smith, 1977) and may therefore represent another level of control.

The non-linear nature of the relationship between i-cell density and nematocyte commitment is most clearly demonstrated when the results of all the experiments in this paper concerning nematocyte commitment and i-cell density are plotted together (Fig. 13). Nematocyte commitment very closely approximates a saturable function of i-cell density. Thus if nematocyte commitment is responding to i-cell density, it is a more sensitive function at low i-cell density, and relatively insensitive to i-cell density at higher levels.

The relationship between i-cell density and nematocyte commitment (Fig. 13) in effect is formally equivalent to negative feedback loop regulating i-cell density. At high i-cell densities, a large fraction of the stem cells would be committed to nematocyte differentiation, thereby leaving the stem cell compartment, maintaining the i-cell density at a constant value. This could account for the observation that the i-cell : epithelial cell ratio is quite constant in normal hydra (Bode et al., 1973, 1976) even though the i-cell cycle time is one third that of the epithelial cells with both populations constantly in the mitotic cycle (David & Campbell, 1972; Campbell & David, 1974). At low i-cell densities, fewer stem cells would be committed to nematocyte differentiation, permitting a greater number of stem cells to divide and remain stem cells, thereby causing an increase in the overall stem cell population size and density.

Feedback regulation of the i-cell population size has been proposed previously (Bode, 1973; Bode et al., 1976; Yaross & Bode, 1976; David & Murphy, 1977). In particular, Bode et al. (1976) determined that in HU-treated hydra, more i-cells divided and fewer differentiated than in control animals, leading to the recovery of the i-cell population. As a result these authors suggested that high i-cell density might cause i-cells to leave the mitotic cycle, and perhaps to differentiate. The results presented in this paper allow this hypothesis to be refined. The relationship between i-cell density and nematocyte commitment suggests that the recovery of the i-cell
population in HU-treated animals is due to the specific decrease in nematocyte commitment since nerve cell commitment was found not to decrease in HU-treated hydra. Therefore the action of high i-cell density may be to positively influence stem cells to become nematocytes, rather than to negatively influence their proliferation.

![Graph](image)

Fig. 13. Correlation between nematocyte commitment and i-cell density. The data are the cumulative results of the experiments presented in Figs. 2-6. The dotted line represents the asymptotic regression curve \( y = A - B(e^{-x}) \); Snedecor & Cochran, 1967) fitted to the data by the method of least squares.

**Nerve cell density and nerve cell commitment**

David & Gierer (1974) found that the nerve cell populations in the hypostome, gastric region, and peduncle labelled at the same rate when continuously exposed to \(^{3}H\)thymidine. As the nerve cell density and total nerve cells vary considerably from region to region, this indicates that the production of new nerve cells must parallel the distribution of the existing population. The results presented here represent the first direct measurement of stem cell commitment to nerve cell differentiation and demonstrate that there is indeed differential nerve cell commitment in the various regions of hydra.

The axial pattern of nerve cell commitment was found to parallel that of nerve cell
density. Experimental manipulation to alter the nerve cell density with feeding regimes and in aggregates again showed the same positive correlation. These results are consistent with a positive feedback loop in which nerve cells promote the differentiation of i-cells to nerve cells. However a positive feedback loop regulating nerve cell commitment would be expected to lead to exponentially increasing nerve cell density (cf. Milsum, 1966). This raises the questions of why, if high nerve cell density leads to increased nerve cell commitment, all i-cells in the head region or peduncle do not become nerve cells, and why the nerve cell densities of the extremities remain constant, rather than increasing. Partial answers to these questions may be found in that: (1) the i-cell density of the extremities is, in fact, reduced as compared to the remainder of the body column (Table 1). (2) I-cell migration into the head region may replenish as much as 75% of the i-cell population each day (Yaross, Smith & Bode, unpublished observations). (3) Continuous tissue displacement from the hypostome into the tentacles and sloughing at the tips of the tentacles, hypostome, and basal disk (Campbell, 1967) causes removal of nerve cells that may balance the continuous production. (4) There may be an upper limit beyond which nerve cell commitment becomes insensitive to increased nerve cell density. This could explain why in heads of hydra maintained on different numbers of shrimp/day, nerve cell commitment remained at a constant high level despite 2-3-fold increases in nerve cell density (cf. Fig. 9).

Conversely, in the body column the question is why the nerve cell density remains low if there is a positive feedback loop. A similar explanation exists. Positive feedback would lead to nerve cell production, but this would be balanced by the continuous expansion of the epithelial tissue (Campbell, 1967). Thus the nerve cell density would not increase.

In every case nerve cell commitment also was correlated with axial position: nerve cell commitment was high in the head and lower peduncle and low in the gastric, budding, and upper peduncle regions. This suggests that nerve cell commitment may be position-dependent. Position dependence of stem cell commitment has been proposed previously (e.g., Bode & David, 1978; Bode, 1973; David, 1975; Bode & Smith, 1977), and other evidence exists indicating that positional effects play a major role in nerve cell commitment. In particular hydra undergoing head regeneration demonstrate large, rapid changes in nerve cell commitment at the regenerating surface that precede other changes in the cellular distribution (Yaross & Bode, 1978). Position dependence is, however, a formal description. At present the possibility that another position-related variable is responsible for the regional pattern of nerve cell commitment cannot be excluded. Nevertheless, the correlation between nerve cell density and nerve cell commitment suggests that nerve cell density may be the underlying basis of position-dependent nerve cell commitment in intact hydra.

Finally the inverse correlation between nerve cell commitment and i-cell density in HU-treated hydra was analysed. These parameters were significantly correlated in those experimental situations where low i-cell densities (< 0.15) were analysed. This relationship may result in part from the definition of the assay. The assay measures the fraction of the i-cells which differentiate to nerve cells. Therefore at low i-cell
density when nematocyte commitment is reduced, the fraction of the i-cells which are stem cells, rather than committed to nematocyte production, increases. Consequently the assayed nerve cell commitment fraction would increase somewhat without reflecting increased stem cell commitment (cf. Yaross, 1978). However, the possibility remains that the correlation between nerve cell commitment and i-cell density also reflects an additional influence on nerve cell commitment. The data formally resemble an inhibitory effect of i-cells on nerve cell commitment. This could reflect competition for stem cells by the nerve cell and nematocyte pathways at high i-cell density when nematocyte commitment is high.

**Regulation of i-cell differentiation**

The results presented here, namely that nematocyte commitment behaves as a function of i-cell density and that nerve cell commitment behaves largely as a function of nerve cell density, permit a first approximation as to how stem cell behaviour in hydra is regulated to be proposed: (1) Nerve cells positively influence i-cells to form nerve cells. When nerve cell density is high it is the dominant influence. (2) I-cells positively influence their own commitment to nematocyte production. This consequently produces a negative feedback effect on i-cell population size. When nerve cell density is low, i-cell density is the major determinative influence. At low i-cell density, i-cells preferentially divide. As the i-cell density increases, an increasing fraction is committed to nematocyte production. Thus i-cell behaviour would be regulated in part by a pair of feedback effects from the i-cells and nerve cells. (3) The type of nematocyte to be formed depends on the axial position of the i-cell (Bode & Smith, 1977). Unlike the position-dependence of nerve cell commitment, which can be interpreted in terms of nerve cell density, the basis of the position-dependence of nematocyte type is unclear.

This very simple explanation does describe the axial distribution of i-cells, nerve cell commitment, and nematocyte commitment in the animal, namely that nerve cell density and nerve cell commitment are high in the head and lower peduncle and low elsewhere, while i-cell density and nematocyte commitment are low in the extremities and high throughout the rest of the body column. This picture will very likely become more complex as more is learned about i-cell regulation.

It must be emphasized that the present experiments only indicate which cell populations may be interacting to affect i-cell differentiation; as yet there is no direct evidence for a specific mechanism for such interactions. For nematocyte commitment, the results are consistent with a mechanism whereby i-cells continuously release a substance which at sufficiently high concentrations results in nematocyte commitment. Similarly the positive feedback loop for nerve cell commitment suggests that nerve cells continuously release a substance which influences commitment to nerve cell differentiation.

Only one substance is known that might be involved in the feedback loops proposed here. Schaller (1976a,b) has extensively purified an activity, head activator, which has properties consistent with a positive feedback effect on i-cell differentiation to nerve cells. Head activator is predominantly localized in the nerve cells of hydra (Schaller
Incubation in head activator leads to increased nerve cell differentiation in both intact and regenerating animals (Schaller, 1976a,b). Results resembling positive feedback in the regulation of cell differentiation have been described elsewhere as well. Exogenous collagen (Kosher & Church, 1975) and chondrocyte-derived mucoprotein (Nevo & Dorfman, 1972) have been found to stimulate their own synthesis by chondroblasts in vitro. Also, investigations in other systems have revealed cell density effects analogous to that described for i-cell density and nematocyte differentiation. Studies of myogenesis in vitro suggest that myoblasts continuously alter the environment, resulting in cytodifferentiation and fusion into myotubes when they reach a critical density (Konigsberg, 1971; Konigsberg & Buckley, 1974). Thus feedback effects, both positive and negative, may be of widespread importance in development.

A final consideration with respect to a positive feedback loop for nerve cell commitment is the role it may play in pattern formation in hydra. Nerve cells and/or nerve cell-produced substances have been repeatedly proposed as the underlying basis of regional differences in hydra (Burnett, 1966; Bode et al. 1973; Gierer & Meinhardt, 1972; Meinhardt & Gierer, 1974; Berking, 1974; Schaller, 1976b). Meinhardt & Gierer (1974) have suggested that a nerve cell gradient is the gradient of activator source density they postulate to be the stable basis of pattern formation in hydra. In the continuously growing hydra tissue this gradient of activator sources would be maintained by a nerve cell-produced substance causing the differentiation of stem cells to nerve cells. The observed positive correlation between nerve cell density and nerve cell commitment reported here is consistent with such a model. Thus, a nerve cell positive feedback loop could be the basis of the maintenance of the activator source density gradient proposed in the Gierer-Meinhardt model for pattern regulation in hydra. The role of nerve cells in the control of hydra pattern polarity has recently been questioned by studies of the developmental capacities of nerve-free hydra produced by colchicine treatment (Marcum, Campbell & Romero, 1977). Marcum et al. (1977) have found that normal polarity reversal can occur when the gastric region of nerve-free hydra is experimentally inverted. However, these authors suggest the possibility that in nerve-free hydra the epithelial cells acquire functions normally performed by the nerve cells. This would be completely consistent with the evidence supporting a role for nerve cells in pattern formation. Thus mechanisms such as a nerve cell positive feedback loop may form a link between the morphogenetic behaviour of hydra tissue and the regulation of stem cell differentiation in normal hydra.

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