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Sympathetic neuron density differentially regulates transmitter phenotypic expression in culture

(superior cervical ganglion/tyrosine hydroxylase/substance P/choline acetyltransferase/aggregation)

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ABSTRACT **The effects of cell density and aggregation on expression of transmitter traits were examined in dissociated, pure sympathetic neuron cultures, grown in fully defined, serum-free medium. After 1 week at a density of 7–8 × 10^3 neurons per 35-mm dish, moderate levels of tyrosine hydroxylase (tyrosine 3-monooxygenase, EC 1.14.16.2) activity and substance P were detected. When neuron density was increased 4-fold, a 4-fold increase in tyrosine hydroxylase activity was observed; i.e., there was no change in tyrosine hydroxylase activity per neuron. In contrast, substance P increased 30-fold, corresponding to a 7-fold increase in substance P per neuron. Choline *O*-acetyltransferase (EC 2.3.1.6) activity, not detected at low cell densities, was first detectable at a concentration of 15,000 neurons per dish and increased 6-fold when this cell concentration was doubled. Medium conditioned by high-density cultures failed to reproduce these effects on low-density cultures, suggesting that diffusible factors are not involved in the density-dependent differential regulation. Time-lapse phase-contrast microscopy of high-density cultures showed neuronal migration and progressive aggregation, which did not occur in low-density cultures. Our observations suggest that cell contact may mediate differential expression of transmitter traits.**

Development of the nervous system consists of a set of seemingly discrete, reproducible processes including cellular migration, aggregation, transmitter phenotypic expression, and synaptogenesis. Although a number of these processes have been defined in some detail, potential mechanistic relationships among the processes are unclear. For example, it is well recognized that aggregation generates the stable formation of nuclei in the brain and ganglia in the periphery. What, however, is the relationship of aggregation to transmitter expression?

One hint may derive from observations in the embryonic rat *in vivo*: initial expression of the catecholamine biosynthetic enzymes tyrosine hydroxylase (tyrosine 3-monooxygenase, EC 1.14.16.2) and dopamine-β-hydroxylase (dopamine β-monooxygenase, EC 1.14.17.1) and of catecholamines coincides with cellular aggregation to form the primitive sympathetic ganglia (1). The temporal association of cellular aggregation and transmitter expression raised the possibility that these processes are causally related. This possibility has gained indirect support from the observations that growth of bovine adrenal chromaffin cells (2) or PC12 rat pheochromocytoma cells (3) in high-density cell culture selectively increased tyrosine hydroxylase specific activity.

To examine the relationship of cell aggregation and transmitter expression, we are growing virtually pure, neonatal rat sympathetic neurons in fully defined, serum-free medium at various cell densities. We now report that increasing cell density (with attendant neuronal aggregation) differentially affects levels of tyrosine hydroxylase; choline *O*-acetyltransferase (EC 2.3.1.6), a cholinergic enzyme; and substance P, a putative peptide transmitter, in these neurons. The differential effects appear to be mediated by cell aggregation and contact and not through elaboration of diffusible factors.

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MATERIALS AND METHODS

Experimental Animals and Culture Techniques. Sprague–Dawley rat pups less than 24 hr old were used in all experiments. Methods of dissociation and culture have been described (4). Dissociated ganglionic single-cell suspensions were seeded at various concentrations, ranging from 10.5 to 48 × 10^3 cells (7–34 × 10^3 neurons) per 35-mm dish, in Ham's nutrient mixture F-12 supplemented with transferrin, putrescine, insulin, selenium, and progesterone (5). After 24 and 72 hr in culture, 10 μM 1-β-D-arabinofuranosyl cytosine (araC) was added to kill the dividing non-neuronal cells. Medium was replaced 24 hr after each addition of araC. Efficacy of this procedure was confirmed by examination by phase-contrast microscopy.

Determination of Cell Number. Cultures were rinsed with Ca^{2+}- and Mg^{2+}-free Puck's saline G and then incubated at 37°C for 30 min in 1 ml of a solution of the same composition but containing 1 mM EGTA and 0.1% trypsin. Neurons were gently removed from the dish bottom by pipetting and counted in a hemocytometer. All cell counts were done in duplicate.

Extraction and Assay Procedures for Substance P, Tyrosine Hydroxylase, and Choline Acetyltransferase. Substance P was extracted from cultures and quantified by radioimmunoassay as described (6). For tyrosine hydroxylase extraction, cultures were rinsed with ice-cold Puck's saline G, following which 200 μl of 20 mM potassium phosphate buffer, pH 7.6/0.2% Triton X-100 was added. Dishes were then scraped as for substance P extraction. A 10-μl aliquot of extract was assayed for activity according to the method of Black and co-workers (7, 8). Choline acetyltransferase was extracted similarly to tyrosine hydroxylase except that 100 μl of 10 mM EDTA, pH 7.4/0.5% Triton X-100 was used. A 2-μl aliquot of extract was assayed for activity according to the method of Fonnum (9).

Statistics. Data were analyzed with a one-way analysis of variance and the Newman–Keuls test.

RESULTS

Dependence of Tyrosine Hydroxylase and Substance P on Neuronal Density. Sympathetic neurons of the neonatal rat superior cervical ganglion normally express both tyrosine hydroxylase and substance P (10, 11). To define the effect of increasing neuronal density on these different phenotypic characters, virtually pure, dissociated, ganglionic neuron cultures were grown at various densities. At relatively low densities (7–8 × 10^3 neurons per dish) moderate levels of

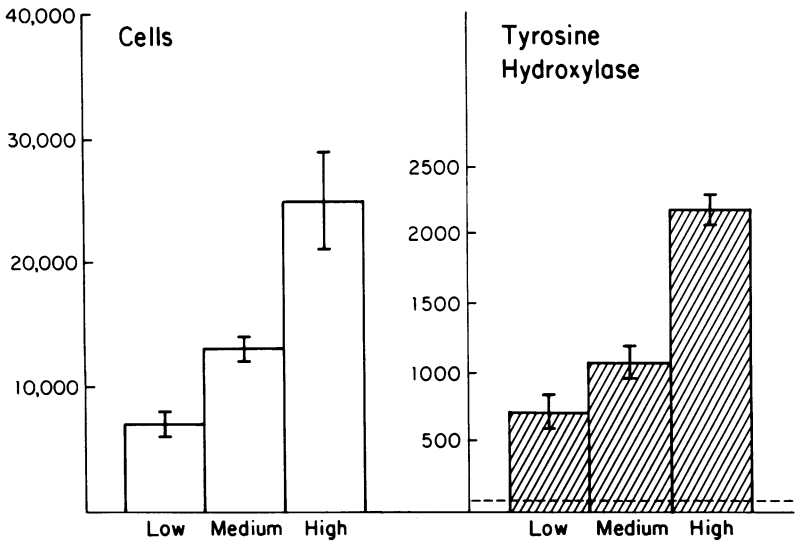

FIG. 1. Neuronal density and tyrosine hydroxylase activity. Cell suspensions were seeded at low, medium, and high densities (10,500, 21,000, and 42,000 cells per dish, respectively; for each experiment described in this paper, "medium" and "high" densities indicate initial cell densities 2 and 4 times greater, respectively, than "low" density). After 1 week, neuronal number (six dishes) and tyrosine hydroxylase activity (six dishes) were determined in sister cultures at all densities. Neuronal number is expressed as cells per dish (mean ± SEM); tyrosine hydroxylase activity is expressed as pmol of product per dish per hr (mean ± SEM). Dashed line represents mean tyrosine hydroxylase activity for high cell densities 6 hr after seeding.

tyrosine hydroxylase activity (728 pmol of product per dish per hr) were detectable after 1 week (Fig. 1). Increasing cell density yielded a linear increase in tyrosine hydroxylase activity to a maximum of 2200 pmol per dish per hr for 25,000 neurons per dish (Fig. 1). Thus, tyrosine hydroxylase activity per neuron was constant over a broad range of cell densities.

In contrast, cell density had a profound, nonlinear effect on substance P content. Low-density cultures contained 20 pg of substance P (Fig. 2). A 2-fold increase in cell density increased substance P 3-fold, whereas a 4-fold increase in cell density yielded a 30-fold increase in the peptide (Fig. 2).

Cell Density and Choline Acetyltransferase Activity. Although choline acetyltransferase activity is predominantly localized to presynaptic terminals of the superior cervical ganglion *in vivo* (12, 13), non-neuronal factors in culture elicit cholinergic expression in postsynaptic ganglion neurons (14). To determine whether increased neuronal density can also elicit cholinergic expression, we examined choline acetyltransferase activity in the cultures. At low cell densities, activity was not detected (Fig. 3). However, activity appeared at a density of 15,000 neurons per dish and increased 6-fold when cell density was doubled (Fig. 3).

Cell Density and Specific Transmitter Characters Per Neuron. To adequately evaluate the effects of cell density, the specific content (or activity) of each transmitter character was expressed per neuron for each neuron density examined. Tyrosine hydroxylase activity per neuron remained constant (Fig. 4). In contrast, choline acetyltransferase activity per neuron increased 4-fold and substance P content per neuron increased 7-fold at high neuronal densities, suggesting that cell density affected different traits differently.

Effect of Conditioned Medium. To determine whether the effects of increased density were mediated by diffusible neuronal factors, low-density cultures were exposed to

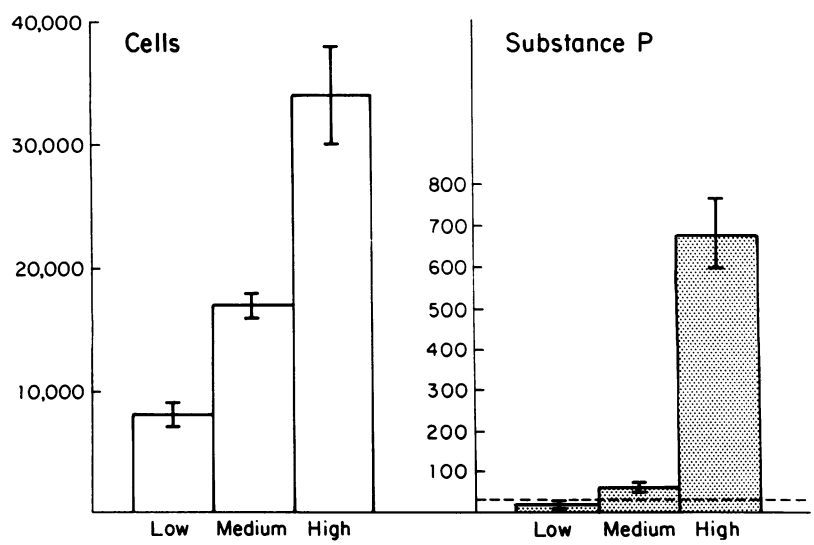

FIG. 2. Neuronal density and substance P content. Cell suspensions were seeded at various densities (low density, 12,000 cells per dish; see legend to Fig. 1). After 1 week, neuronal number (six dishes) and substance P content (six dishes) were determined in sister cultures at all densities. Neuronal number is expressed as cells per dish (mean ± SEM); substance P content is expressed as pg per dish (mean ± SEM). Dashed line represents mean substance P content for high cell densities 6 hr after seeding.

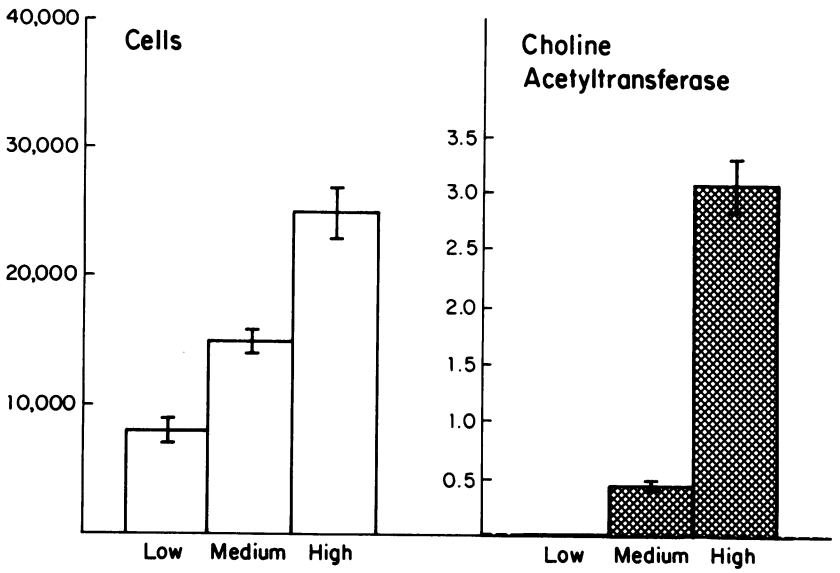

FIG. 3. Neuronal density and choline acetyltransferase activity. Cell suspensions were seeded at various densities (low density, 12,000 cells per dish; see legend to Fig. 1). After 1 week, neuronal number (six dishes) and choline acetyltransferase activity (six dishes) were determined in sister cultures at all densities. Neuronal number is expressed as cells per dish (mean ± SEM); choline acetyltransferase activity is expressed as nmol of product per dish per hr (mean ± SEM). Dashed line represents mean choline acetyltransferase activity for high cell densities 6 hr after seeding.

culture medium conditioned by 1-week-old, high-density cultures (Fig. 5). High-density cultures prepared from the same initial cell suspensions served as controls. Conditioned medium did not elicit any increase in either choline acetyltransferase activity or substance P, whereas high-density control cultures exhibited marked, nonlinear increases in both characters, as expected (Fig. 5). Consequently, some other concomitant of increased density, such as cell contact itself, might be critical.

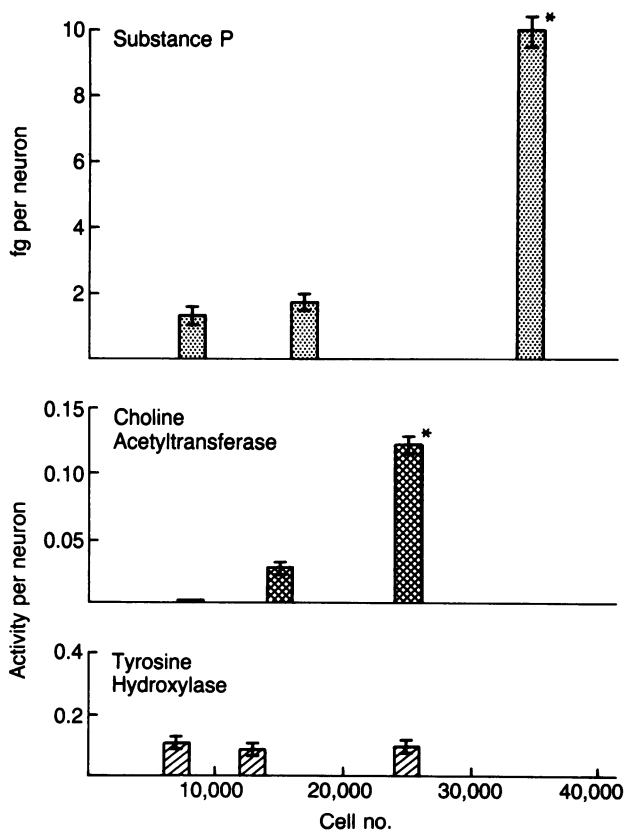

FIG. 4. Neuronal density and specific activity (or content) of tyrosine hydroxylase, choline acetyltransferase, and substance P. Data from Figs. 1–3 are expressed as activity or content per neuron for tyrosine hydroxylase and choline acetyltransferase (pmol of product per neuron per hr) and for substance P (fg per neuron) ± SEM. *Differs from all other groups at $P < 0.01$.

Neuron Density and Aggregation. To determine whether increased density actually did increase the incidence of neuronal aggregation over the range examined, cultures derived from the same cell suspension were examined by phase-contrast microscopy. Twenty-four and 48 hours after plating, cells in the high- and low-density cultures exhibited the same low incidence of aggregation. However, by 1 week, high-density cultures contained multiple cellular aggregates, whereas few if any were observed in low-density cultures. To define the dynamics of high-density aggregation in greater detail, culture dishes were marked, and time-lapse phase-contrast microscopy was performed over the course of 1 week (Fig. 6). No differences in aggregation between high- and low-density cultures were apparent at 24 hr, when perikaryal diameter was increasing (Fig. 6). However, between 48 and 96 hr in high-density cultures only, perikarya appeared to migrate toward one another, eventually forming large multicellular aggregates (Fig. 6). Thereafter, little further aggregation occurred. Aggregation was not observed at any time in low-density cultures.

DISCUSSION

Our observations indicate that neuronal density differentially regulates distinct transmitter traits. Since we employed virtually pure populations of dissociated neurons cultured in fully defined, serum-free medium, it is likely that neuron–neuron interactions governed this regulation. In fact, our studies suggest that neuronal aggregation and direct contact mediated the transmitter effects.

Increased cell density differentially altered noradrenergic, cholinergic, and peptidergic traits that are localized to sympathetic neurons (11, 14). Choline acetyltransferase activity increased at least 4-fold per neuron and the substance P level rose 7-fold, whereas tyrosine hydroxylase activity per neuron remained constant over the range tested (Fig. 4). Although we have not localized these traits to specific individual neurons in these particular cultures, previous work has indicated that cholinergic, noradrenergic, and peptidergic

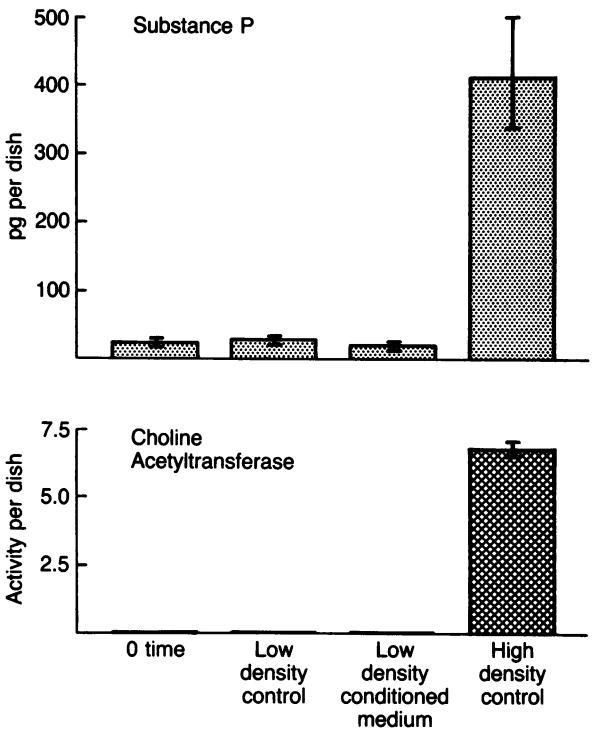

FIG. 5. Lack of effect of conditioned medium on substance P content and choline acetyltransferase activity. Cell suspensions were plated at low and high densities. Low-density control (six dishes) and high-density control (six dishes) cultures were fed with fresh medium. Low-density, conditioned medium cultures (six dishes) were fed with medium taken from 1-week-old high-density cultures. All experimental and control cultures were grown for 1 week. Substance P is expressed as pg per dish (mean ± SEM); choline acetyltransferase activity is expressed as nmol of product per dish per hr (mean ± SEM); 0 time, 6 hr after seeding.

characters are, in fact, present in the same sympathetic neurons under a variety of conditions (11, 15). Our observations confirm the general contention that phenotypic traits for different transmitters may be differentially regulated in the same neuronal population (10, 14–20), and, more specifically, our results indicate that cell density is one condition evoking differential effects. Moreover, the fact that substance P content and choline acetyltransferase activity increased per neuron while tyrosine hydroxylase activity remained constant suggests that there is no obligatory reciprocal relationship between the transferase and substance P on the one hand and the hydroxylase on the other.

Our studies may also be relevant to the general problem of conditions necessary for cholinergic and peptidergic expression in classical noradrenergic sympathetic neurons. Previous studies suggested that expression and increases of peptidergic and cholinergic traits required the presence of non-neuronal cells or factors elaborated therefrom (14, 15, 18–22). The present studies, performed with cultures that contained virtually no non-neuronal cells and that were grown in fully-defined serum-free medium, indicate that sympathetic neurons may express cholinergic and peptidergic characters in the absence of non-neuronal elements. In this context, high neuronal density elicited the same effects as the presence of non-neuronal cells. Consistent with this contention, in low-density cultures, only tyrosine hydroxylase showed a time-dependent increase.

What consequences of high neuronal density actually elicited the expression of choline acetyltransferase and the increases in substance P? Medium conditioned by high-density cultures failed to reproduce these effects, suggesting

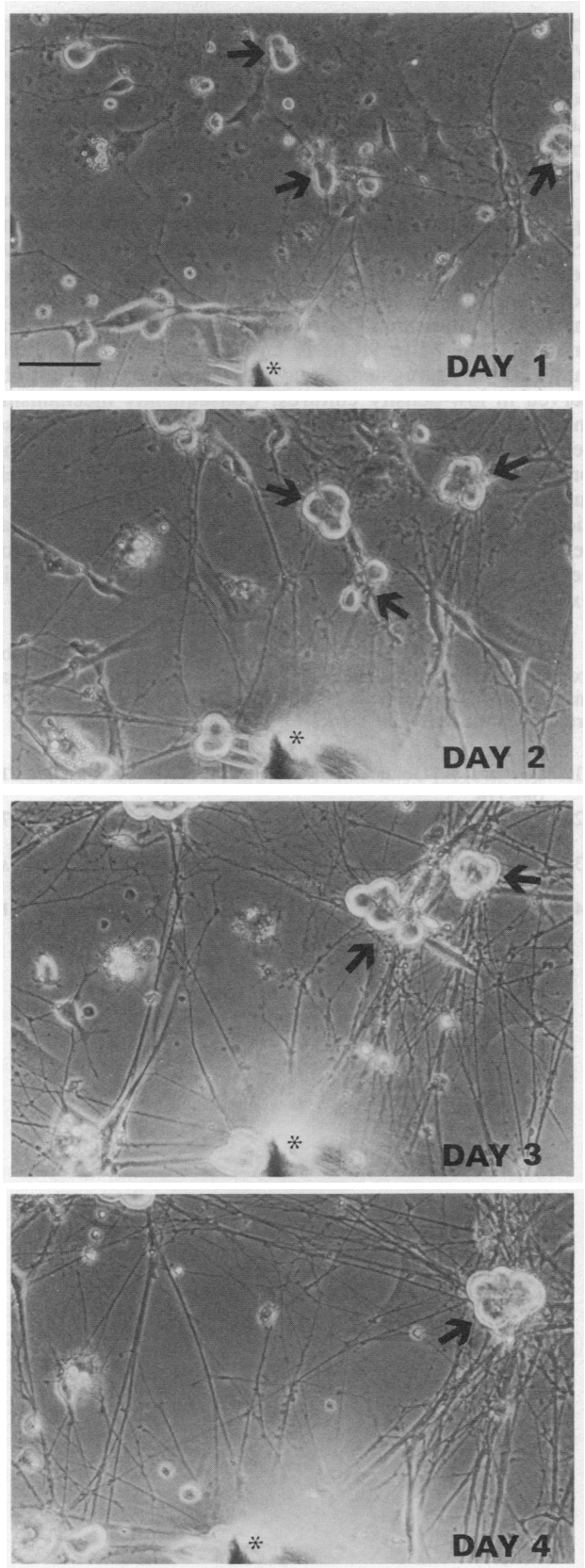

FIG. 6. Neuronal aggregation in high-density cultures. Cell suspensions were plated at high density. Time-lapse phase-contrast micrographs were taken of the same area of the culture dish at 24-hr intervals beginning 1 day after seeding. Marker on dish bottom is represented by *. Arrows indicate neurons which migrated to form the large neuronal clump seen at day 4. (Bar = 25 μm.)

that diffusible factors elaborated by high-density neurons were not responsible. Since medium was transferred directly from high- to low-density cultures, it is unlikely that any potentially labile elements were damaged. Further, the use of fully defined, serum-free medium precludes the possibility that high-density neurons modified an unidentified medium component.

Rather, several lines of evidence suggest that neuronal aggregation, and perhaps direct contact, may elicit the transmitter changes. Our observations indicate that active perikaryal migration occurred in the high-density cultures but not in the low-density cultures. Time-lapse, phase-contrast microscopy indicated that perikarya migrated toward one another from 24 to 96 hr in culture and formed multiple multicellular aggregates. Neuronal aggregation was not observed in low-density cultures. Consequently mechanisms involved in migration and/or aggregation (23–25) may also regulate transmitter phenotypic expression. In fact, the present system offers the opportunity to define the intercellular molecular interactions regulating transmitter expression. In this manner, it may be possible to define causal relationships among the apparently distinct ontogenetic processes of cellular migration, aggregation, and phenotypic expression.

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