EXPRESSION OF THE CAPACITY TO RELEASE [³H]NOREPINEPHRINE BY NEURAL CREST CULTURES¹

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Received January 3, 1983; Revised April 11, 1983; Accepted April 14, 1983

Abstract

Cultures of trunk neural crest cells from quail embryos were tested for their ability to release [³H]norepinephrine ([³H]NE) in response to depolarization. After 7 days *in vitro*, exposure of the cultures to either the alkaloid veratridine or 40 mM K⁺ results in the evoked release of [³H]NE. The release evoked by veratridine is blocked in the presence of tetrodotoxin. The release evoked by increased K⁺ is blocked by the calcium antagonist cobalt. Release in response to the nicotinic cholinergic agonist 1,1-dimethyl-4-phenylpiperazine was also observed. The amount of evoked release is highly correlated with the number of histochemically demonstrable catecholamine-containing cells in a given culture. Autoradiography reveals that the radioactivity taken up by these cultures is located in a subpopulation of cells whose morphology resembles that of the histochemically detectable catecholamine-containing cell population. Whereas capacity for the release of [³H]NE is readily detectable after 7 days *in vitro*, it is detectable only with difficulty after 4 days *in vitro*. There is a greater than 6-fold increase in uptake capacity over the period of 4 to 7 days *in vitro*. These results demonstrate that neural crest cultures grown without their normal synaptic inputs or targets can exhibit the capacity for stimulus secretion coupling characteristic of synaptic neurotransmitter release.

The development of a neuron from an undifferentiated precursor cell requires changes in many aspects of cellular metabolism and structure. Embryonic neural crest cells are the precursors of a wide spectrum of cell types in the adult including the neurons and supportive cells of the autonomic and most sensory ganglia, the cells of the adrenal medulla, the melanocytes of the skin, skeletal and connective tissues of the head and face and some neuroendocrine cell types (Weston, 1970; Noden, 1978; Le Douarin, 1980).

Some derivatives of the neural crest possess differentiated features that can serve as phenotypic markers of development. For example, melanocytes contain melanin pigment in their cytoplasm, and many principal neurons of sympathetic ganglia use norepinephrine as a neurotransmitter. Previous experiments have shown that, at

least with respect to catecholamine biosynthesis and melanogenesis, trunk neural crest cells *in vivo* and *in vitro* initially appear not to express differentiated properties (Enemar et al., 1965; Cohen and Konigsberg, 1975; Maxwell 1976; Allan and Newgreen, 1977; Cochard et al., 1978; Teitelman et al., 1979; Maxwell et al., 1982). While neural crest cultures initiated from the trunk region of avian embryos initially appear undifferentiated, after several days *in vitro* several neuronal and non-neuronal differentiated properties characteristic of neural crest-derived cells are expressed under certain culture conditions. Some neural crest cells differentiate into melanocytes and others acquire the capacity for the synthesis of acetylcholine and the catecholamines, norepinephrine and dopamine (Kahn et al., 1980; Fauquet et al., 1981; Maxwell et al., 1982). These capabilities appear and develop significantly during the period of 3 to 7 days *in vitro*, and this development can take place in the absence of the normal synaptic inputs and targets of autonomic neurons (Cohen, 1977; Kahn et al., 1980; Maxwell et al., 1982). Evidence from light and electron microscopic studies shows that these neural crest cultures eventually contain a heterogeneous cell population and that the observed increase in catecholamine biosynthesis is probably mediated by a subpopulation of cells developing within these cultures (Cohen, 1977; Sieber-

¹ We thank S. J. Potashner, S. J. Cooperstein, and A. Pappano for helpful discussions and F. Napolitano for typing the manuscript. This work was supported by Grant NS16115 and a Research Career Development Award from the National Institutes of Health, and Basil O'Connor Starter Grant 5-289 from the March of Dimes Birth Defects Foundation to G. D. M. Some of these results were presented at the 12th Annual Meeting of the Society for Neuroscience (Maxwell and Sietz, 1982).

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Blum and Cohen, 1980; Fauquet et al., 1981; Sieber-Blum et al., 1981; Maxwell et al., 1982).

In addition to neurotransmitter biosynthesis, developing neurons also acquire specific voltage-sensitive ion channels that are linked to other metabolic events including neurotransmitter release (Spitzer, 1979). In order to determine whether these neural crest cultures could express neuronal properties other than neurotransmitter biosynthesis and storage, we tested them for the development of the capacity to release [^3H]norepinephrine ([^3H]NE) in response to depolarization. The results show that these culture do develop the capacity for transmitter release. This release has many of the characteristics seen in more mature sympathetic neurons in tissue culture (Burton and Bunge, 1975; Patterson et al., 1976; Greene and Rein, 1978).

Materials and Methods

Neural crest cultures. Cultures of neural crest cells were prepared from the trunk region of Japanese quail embryos (*Coturnix coturnix*) as described previously (Maxwell et al., 1982). Briefly, neural tubes containing the neural crest were isolated free of surrounding tissues and were plated and grown in a medium containing 37.5 ml of Dulbecco's modified Eagle's medium with 4.5 gm/liter of glucose, pH 7.4 (Grand Island Biological Co.), 37.5 ml of F-12, pH 7.4 (Grand Island Biological Co.), 15 ml of horse serum (Colorado Serum Co.), 10 ml of 9-day chick embryo extract (Cahn et al., 1967), 10 mg of gentamycin sulfate (Schering), 10 mg of kanamycin sulfate (Grand Island Biological Co.), 0.5 μg of 2.5 S nerve growth factor (Collaborative Research), 1 ml of 0.2 M L-glutamine (Sigma Chemical Co.), and 1 ml of a $100\times$ stock vitamin mix. Vitamin mix stock contained 1 mg of dimethyltetrahydrobiopterin (Calbiochem), 100 mg of ascorbic acid (Sigma), 10 mg of glutathione (Sigma), and 20 ml of distilled water, final pH, 6.0 (Mains and Patterson, 1973). Cultures were grown at 36.5°C in a 5% CO_2 atmosphere in 35-mm tissue culture dishes (Falcon) coated with a thin film of rat-tail collagen polymerized by ammonia vapors (Ehrmann and Gey, 1956). The neural tubes were removed from the cultures 42 to 48 hr after plating using tungsten needles, and immediately thereafter they were washed and fed with fresh medium. Cultures were then examined using phase contrast microscopy to ensure that the neural tubes had been completely removed, leaving behind only those cells that had migrated away from the tube. Cultures used in experiments after 3 to 4 days *in vitro* contained 15 neural crest outgrowths, while those used at later times contained 6 to 8 outgrowths per culture. This was done in order to equalize the number of cells used in experiments with cultures of different ages.

Cell counts. Cell counts on neural crest cultures were performed by detaching cells from the substrate using 0.025% trypsin (Grand Island Biological Co.) and 0.01% EDTA in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (Grand Island Biological Co.) monitored under a phase contrast microscope. Trypsinization was stopped by the addition of 100 $\mu\text{g}/\text{ml}$ of soybean trypsin inhibitor in growth medium. Cells were then

centrifuged at $600\times g$ and resuspended in 0.1 ml of Hanks' balanced salt solution buffered with 15 mM HEPES. Cell counts were performed with a hemocytometer using trypan blue dye exclusion as the criterion for viable cells.

Neurotransmitter release. Growth medium was removed from the cells, and the cultures were washed once and pre-incubated for 10 min in 0.65 ml of medium containing Dulbecco's modified Eagle's medium with 4.5 gm/liter of glucose without serum or embryo extract, 5 μM pargyline, and 5 mM ascorbate. Cultures were then incubated for 30 min at 36.5°C in 5% CO_2 in the same medium containing 1.3 μM [^3H]NE. This radioactive medium was made by evaporating appropriate amounts of L-[7- ^3H (N)]norepinephrine, 2.7 Ci/mmol, and L-[ring 2,5,6- ^3H]norepinephrine, 44.7 Ci/mmol (New England Nuclear) in a Speedvac (Savant), followed by addition of the appropriate volume of liquid medium. The purity of [^3H]NE used in these experiments was greater than 90%, as monitored by high voltage paper electrophoresis (Hildebrand et al., 1971).

At the conclusion of the 30-min labeling period the radioactive medium was replaced with HEPES-buffered Krebs-Ringer saline at 36.5°C in air (Greene and Rein, 1977a). This saline was collected and replaced at 5-min intervals. These collections were centrifuged for 1 min at $2000\times g$, and the radioactivity in 0.4 ml of the supernatant was measured in a liquid scintillation spectrometer using ACS cocktail (Amersham). Some collections contained veratridine (Sigma), tetrodotoxin (TTX) (Calbiochem), 1,1-dimethyl-4-phenylpiperazinium (DMPP) iodide (Aldrich), 40 mM K^+ , or 100 mM K^+ (osmotically adjusted) and were likewise pre-equilibrated at 36.5°C . Veratridine and tetrodotoxin solutions were prepared from fresh 5 times and 1000 times stock solutions, respectively, on the day of each experiment.

The amount of evoked release was computed as the difference between the radioactivity collected during stimulation and the mean of the radioactivity in collections before and after stimulation. At the conclusion of some experiments the cells were scraped from the dish into 100 μl of Hanks' balanced salt solution (Grand Island Biological Co.) and solubilized with 0.5 ml of Protosol (New England Nuclear) at 60°C for 30 min. The radioactivity in this extract was measured in a liquid scintillation spectrometer using Econofluor cocktail (New England Nuclear). Radioactivity in this extract plus any evoked release during the experiment was defined as uptake.

The identity of the radioactivity released in response to depolarization was determined by descending paper chromatography using the method of Levin (1973). A 0.2-ml aliquot of a collection in which 100 mM K^+ was present was applied to a 4-cm-wide lane of Whatman P81 phosphocellulose chromatography paper. The paper was allowed to develop in *N*-butanol:95% ethanol:distilled water (1:1:1, v:v:v) until the solvent front advanced about 30 cm from the origin. Nonradioactive standards for norepinephrine and its metabolites were located on adjacent lanes and the radioactive lanes cut into rectangles 1 cm $\times$ 4 cm which were extracted in 0.3 N HCl and processed for liquid scintillation counting. A

0.1-ml aliquot of the collection was used to determine total ^3H release in the collection.

Glyoxylic acid-induced catecholamine fluorescence. At the conclusion of some release experiments catecholamine-containing cells were visualized using a method adapted from Furness and Costa (1975). Cultures were washed once with 2% glyoxylic acid, 0.2% formaldehyde in 0.1 M sodium phosphate buffer, final pH 7.0, and then allowed to incubate in fresh solution for 10 min at room temperature. The glyoxylic acid solution was then removed and the cultures were air dried for 40 min. The cultures were then heated in an oven at 100°C for 4 min, coverslipped using mineral oil, and observed using a Zeiss IM 35 microscope equipped for viewing catecholamine fluorescence.

Autoradiography. At the conclusion of other release experiments, cultures were fixed for 2 hr in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, with 3% sucrose (Rothman et al., 1978). Since the presence of numerous melanocytes made detection of silver grains difficult in some cultures, a bleaching procedure was used to reduce the intensity of the melanin pigment (Humason, 1961). Cultures were bleached for 1.5 hr with 4.7% performic acid and washed in running water for 10 min. They were then washed with 70% alcohol and processed for autoradiography using NTB-2 emulsion (Kodak). After exposure of autoradiographs for 2 weeks at 4°C in the dark, they were developed using D-19 developer (Kodak) and counterstained with hematoxylin and eosin.

Figure 1. Evoked release of ^3H NE by a neural crest culture after 7 days *in vitro* in response to $100\ \mu\text{M}$ veratridine (V) and $40\ \text{mM}$ K^+ . Neural crest cultures were incubated with $1.3\ \mu\text{M}$ ^3H NE and tested for their ability to release radioactivity above base line as described under "Materials and Methods." Evoked release was seen in six of seven cases after 7 days *in vitro* and in seven of seven cases after 9 days *in vitro*. The amount of evoked release was proportional to the number of catecholamine-containing cells in a culture (see Fig. 7). The abscissa indicates time elapsed after the conclusion of the ^3H NE incubation. Black horizontal bars indicate when veratridine or $40\ \text{mM}$ K^+ was present.

Figure 2. Inhibition of veratridine-stimulated release by tetrodotoxin (TTX). Neural crest cultures after 7 days *in vitro* were incubated with $1.3\ \mu\text{M}$ ^3H NE and tested for their ability to release radioactivity above base line in response to $100\ \mu\text{M}$ veratridine (V) plus $0.3\ \mu\text{M}$ TTX or to $100\ \mu\text{M}$ veratridine alone. A representative experiment is shown. In a series of such experiments TTX inhibited $96.6 \pm 3.2\%$ ($N = 3$) of the release evoked by veratridine. Black horizontal bars indicate when veratridine was present; the white horizontal bar indicates when TTX was present.

Results

When neural crest cultures after 7 to 9 days *in vitro* were incubated with ^3H NE, washed, and exposed to test pulses of $100\ \mu\text{M}$ veratridine or $40\ \text{mM}$ K^+ , evoked release was observed in response to both stimuli (Fig. 1). In the presence of $0.3\ \mu\text{M}$ TTX, the veratridine-stimulated release of radioactivity was blocked (Fig. 2). Similarly, the K^+ -stimulated release was abolished in the presence of the Ca^{2+} antagonist, Co^{2+} (Fig. 3). The nicotinic cholinergic agonist DMPP also stimulated evoked release from neural crest cultures (Fig. 4).

In contrast to the response seen after 7 to 9 days *in vitro*, evoked release was not detected in response to either $100\ \mu\text{M}$ veratridine or $40\ \text{mM}$ K^+ after 3 to 4 days *in vitro*, although $100\ \text{mM}$ K^+ did stimulate release (Table I). When the amount of uptake was measured after 7 days *in vitro*, a value of 7641 ± 1601 cpm per 10^5 cells (mean $\pm$ SEM, $N = 8$) was observed, while uptake of 1101 ± 153 cpm per 10^5 cells (mean $\pm$ SEM, $N = 6$) was seen after 4 days *in vitro*. These values differed with a level of significance of $p < 0.005$ in the Student's t test. The identity of the radioactivity released during stimulation of neural crest cultures was examined after 7 days *in vitro*. When a $100\ \text{mM}$ K^+ pulse was used to maximize the response, 91% (mean of duplicate determinations) of the released radioactivity comigrated with norepinephrine.

In order to identify the cell population in the cultures that was probably responsible for the observed transmitter release, we first examined autoradiographically the distribution of radioactivity in cultures permitted to take

Figure 3. Inhibition of K⁺-stimulated release by cobalt. Neural crest cultures after 8 to 11 days *in vitro* were incubated with 1.3 μ M [³H]NE and tested for their ability to release radioactivity above base line in response to 40 mM K⁺ alone or in the presence of 3 mM CoCl₂ (Co⁺⁺) and 40 mM K⁺. A representative experiment is shown. Cobalt inhibited $98.6 \pm 1.5\%$ ($N = 5$) of the release evoked by 40 mM K⁺ in a series of such experiments. Black horizontal bars indicate when 40 mM K⁺ was present, the white horizontal bar indicates when Co⁺⁺ was present.

Figure 4. Evoked release of [³H]NE in response to DMPP. Neural crest cultures after 9 to 10 days *in vitro* were incubated with 1.3 μ M [³H]NE and tested for their ability to release radioactivity above base line in response to 100 μ M DMPP or 40 mM K⁺ as described under "Materials and Methods." A representative experiment is shown. In a series of such experiments evoked release was seen in five of five cases. The amount of DMPP-evoked release was proportional to the number of catecholamine-containing cells in the culture (see Fig. 8). Black horizontal bars indicate when DMPP or 40 mM K⁺ was present.

compared the morphology and number of catecholamine-containing cells detected histochemically with the amount of evoked release by a given culture. The morphology and incidence of the fluorescent catecholamine-containing cells resembled that seen in cultures examined autoradiographically (Fig. 6). Cell bodies and short processes were seen and longer processes were sometimes visible. When the amount of release evoked by veratridine and 40 mM K⁺ was compared with the number of catecholamine-containing cells, an excellent correlation ($r = 0.97$) was observed (Fig. 7). Release evoked by the cholinergic agonist DMPP was also proportional to the number of fluorescent catecholamine-containing cells in the culture (Fig. 8). Conversely, release is not related to the total cell population (all cell types) in a culture. The total number of cells in seven sister cultures after 7 days *in vitro* showed less than 35% variation, whereas differences in the amount of release and number of catecholamine-containing cells varied over a much wider range (Figs. 7 and 8).

Discussion

Characteristics of neurotransmitter release by neural crest cultures. Our findings support the hypothesis that some neural crest cells in tissue culture can acquire the ability to take up [³H]NE and release it into the medium in response to depolarizing stimuli. This depolarization-dependent release of radioactivity has many of the properties associated with the stimulus secretion coupling mechanism present in embryonic and neonatal sympathetic neurons and PC12 cells *in vitro* (Burton and Bunge, 1975; Patterson et al., 1976; Greene and Rein, 1977a, b, 1978).

One hallmark of neuronal transmitter release is its sensitivity to extracellular calcium (Rubin, 1974). The evoked release induced by 40 mM K⁺ is virtually abolished by Co²⁺, which is a selective reversible antagonist of Ca²⁺. This strongly suggests that evoked release from these neural crest cultures is dependent on Ca²⁺ from the external medium. We could not test this by using medium without calcium because it promoted detachment of cells from the substrate. The alkaloid veratridine is also able to stimulate evoked release from neural crest cultures. Veratridine is thought to bind to and open the voltage-sensitive sodium channels involved in the action potential (Catterall, 1975, 1980). Our results are consistent with the presence of such channels in these neural crest cultures. The concentration of veratridine we have

TABLE 1

Development of [³H]NE release by neural crest cultures

Neural crest cultures were grown and analyzed as described under "Materials and Methods." The number of cultures giving release greater than 50 cpm of the total number tested is indicated. In the 7- to 9-day cultures the amount of evoked release was proportional to the number of catecholamine-containing cells present in the culture (see Fig. 7).

Time in Culture (days)	Incidence of Release Evoked by		
	100 μ M Veratridine	40 mM K ⁺	100 mM K ⁺
3-4	0/5	0/5	3/3
7-9	13/14	13/14	2/2

up [³H]NE. We found (Fig. 5) that silver grains were distributed over only a small subset of cells in the cultures. Both cell bodies and processes were seen. Sometimes silver grains were observed over processes without any obvious connection to cell bodies. These observations are consistent with the notion that only a small fraction of the cells in the culture release [³H]NE. We therefore

Figure 5. Autoradiographic location of radioactivity at the conclusion of a release experiment. A neural crest culture after 7 days *in vitro* was incubated with $1.3 \mu\text{M}$ [^3H]NE and tested for the ability to release radioactivity in response to $100 \mu\text{M}$ veratridine and 40 mM K^+ . Evoked release of 1378 cpm and 521 cpm was seen in response to veratridine and K^+ , respectively. The culture was then processed for autoradiography as described under "Materials and Methods." Both labeled cell bodies (C) and processes (P) can be seen in a subpopulation of cells in the culture. Since labeled processes without cell bodies are sometimes seen, it is difficult to accurately quantitate the number of labeled cells. However, the frequency and morphology of labeled cells appears similar to that of catecholamine-containing cells revealed by glyoxylic acid-induced fluorescence (Fig. 6). The appearance of the cells shown here is representative of that seen in 12 cultures after 7 to 12 days *in vitro*. Scale bar, $50 \mu\text{m}$.

used results in near maximal sodium ion flux in mouse neuroblastoma cells (Catterall, 1975). The action of veratridine is blocked by the presence of TTX, which acts as a noncompetitive inhibitor of veratridine and is known to bind selectively to sodium channels and acts to prevent the flux of sodium ions across the membrane (Catterall, 1975). The observed rise in the radioactivity following the washout of veratridine and TTX, prior to the second pulse of veratridine, is probably due to the fact that veratridine is lipid soluble and may be washed out relatively slowly.

Some cholinergic synaptic transmission in sympathetic ganglia is mediated by nicotinic acetylcholine receptors on principal ganglion cells (Feldberg and Gadum, 1934; Volle, 1969). DMPP has been shown to be a cholinergic agonist that is quite specific for ganglionic nicotinic cholinergic receptors (Haefely, 1974). DMPP has previously been shown to be able to evoke [^3H]NE release from day 11 embryonic chick sympathetic neurons in culture after 6 days *in vitro* and from the PC12 cell line (Greene and Rein, 1977b, 1978). When the agonist DMPP was used in our experiments, it evoked release from trunk neural crest cultures. Thus, at least

some of the release we observed may result from activation of nicotinic acetylcholine receptors. Taken together, our findings suggest that the release that develops in these neural crest cultures has many similarities with neuronal transmitter release and, in particular, with release of [^3H]NE from sympathetic neuron cultures from chick embryos and neonatal rats (Burton and Bunge, 1975; Patterson et al., 1976; Greene and Rein, 1978).

Since the neural crest cultures we have used are morphologically heterogeneous, it is difficult to ascertain directly what cell populations are responsible for the evoked release. However, the strong correlation of the amount of evoked release with the number of catecholamine-containing cells is consistent with the hypothesis that this cell population is responsible for the evoked release. We do not know whether the fluorescent cells contain endogenous catecholamine which they have synthesized, [^3H]NE taken up from the medium, or both. Our autoradiographic findings provide additional support for the notion that a subpopulation of cells with morphology similar to that of cells that can manufacture catecholamines is responsible for the uptake and release

Figure 6. Morphology of catecholamine-containing cells present at the conclusion of release experiments. A neural crest culture after 12 days *in vitro* was incubated in $1.3 \mu\text{M}$ [^3H]NE and tested for its ability to release radioactivity in response to $100 \mu\text{M}$ veratridine. Evoked release of 327 cpm above background was seen. The culture was then processed for glyoxylic acid-induced fluorescence as described under "Materials and Methods." Fluorescent cells both with and without processes are observed. The morphological appearance of the cells shown is representative of those seen in 21 cultures examined after 7 to 12 days *in vitro*. Scale bar, $50 \mu\text{m}$.

of [^3H]NE. The large variation in the number of catecholamine-containing cells generated in such cultures has been observed previously (Cohen, 1977; Maxwell et al., 1982) and is likely a result of microenvironmental differences between cultures.

Expression of the capacity for neurotransmitter release. Our data on the expression of the capacity for neurotransmitter release indicate that, as with transmitter biosynthesis, the period of 3 to 7 days *in vitro* is a critical one in which maturation of uptake and release properties occurs. This is based on the finding that it is more difficult to detect evoked release from cultures at 3 to 4 days *in vitro* than at 7 to 9 days *in vitro*. There are several possible explanations for this result. Since the amount of uptake per cell after 4 days is one-seventh that after 7 days, the difficulty in detecting release at 3 to 4 days may be due to the smaller amount of [^3H]NE in releasable pools at this time. Thus it may be that stimulus secretion coupling machinery is present, but uptake and storage of NE into releasable pools is limiting. These data are consistent with the maturation of a catecholamine-metabolizing cell population occurring between 3 and 7 days *in vitro* (Cohen, 1977; Kahn et al., 1980; Maxwell et al., 1982). Since some sympathetic neuroblasts retain the ability to divide even after they begin to synthesize catecholamines (Cohen, 1974; Rothman et

al., 1978, 1980), this development of increased uptake activity may result from larger numbers of cells able to take up catecholamines as well as from the synthesis of new uptake systems by individual cells in the population. It is also possible that the greater release in older cultures is due to the fact that depolarization of cells may be more difficult in young cultures due to differences in membrane properties. Alternatively, the coupling between depolarization and secretion may be not as well developed in the younger cultures.

Although the temporal resolution of our experiments is over a period of days, our results are consistent with a model in which expression of neurotransmitter biosynthetic, uptake, and release capacity all mature over a similar time course under the particular conditions of our experiments. It should be noted, however, that under some *in vitro* conditions the development of individual adrenergic traits, such as uptake and synthetic ability, may be independently regulated in sympathetic neurons (Reichardt and Patterson, 1977; Wakshull et al., 1978).

Our results thus indicate that considerable adrenergic differentiation by neural crest cells can occur in tissue culture in the absence of their normal synaptic inputs and targets (although inappropriate synapses may form) and without the presence of embryonic cell types such as notochord and somitic mesenchyme. This differentiation

Figure 7. Relationship of evoked release to the number of fluorescent catecholamine-containing cells. Neural crest cultures after 7 to 9 days *in vitro* were incubated with $1.3 \mu\text{M}$ [^3H]NE and tested for their ability to release radioactivity in response to $100 \mu\text{M}$ veratridine and 40 mM K^+ as described under "Materials and Methods." At the conclusion of the release experiment the cultures were processed to reveal fluorescent catecholamine-containing cells as described under "Materials and Methods." A correlation of $r = 0.97$ is observed between the number of catecholamine-containing cells in the culture and the amount of evoked release, which is the sum of the release stimulated by separate 5-min pulses of $100 \mu\text{M}$ veratridine and 40 mM K^+ delivered as shown in Figure 1. A least squares fit of a regression line to the data gave a line with the equation $y = 0.68x + 71.7$ and a standard error of the estimate of y of 174.4 cpm .

Figure 8. Correlation of DMPP-stimulated release and the number of fluorescent catecholamine-containing cells. Neural crest cultures after 9 to 10 days *in vitro* were incubated with $1.3 \mu\text{M}$ [^3H]NE and tested for their ability to release radioactivity in response to $100 \mu\text{M}$ DMPP as described under "Materials and Methods." At the conclusion of the experiment the cultures were processed to reveal fluorescent catecholamine-containing cells as described under "Materials and Methods." A least squares fit of a regression line to the data gave a line with the equation $y = 0.09x + 60.7$ and a standard error of the estimate of y of 254.4 cpm . A correlation coefficient of $r = 0.84$ was observed.

includes the development of a functional stimulus secretion coupling mechanism. Whatever developmental cues are required must be provided in the medium, on the substrate, by other neural crest cells in the culture, or by the transient presence of the neural tube in the culture.

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