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Determination of transmitter function by neuronal activity

(sympathetic neurons/cell culture/catecholamines/acetylcholine/development)

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ABSTRACT **The role of neuronal activity in the determination of transmitter function was studied in cultures of dissociated sympathetic neurons from newborn rat superior cervical ganglia. Cholinergic and adrenergic differentiation were assayed by incubating the cultures with radioactive choline and tyrosine and determining the rate of synthesis and accumulation of labelled acetylcholine and catecholamines. As in previous studies, pure neuronal cultures grown in control medium displayed much lower ratios of acetylcholine synthesis to catecholamine synthesis than did sister cultures grown in medium previously conditioned by incubation on appropriate nonneuronal cells (conditioned medium). However, here we report that neurons treated with the depolarizing agents elevated K^+ or veratridine, or stimulated directly with electrical current, either before or during application of conditioned medium, displayed up to 300-fold lower acetylcholine/catecholamine ratios than they would have without depolarization, and thus remained primarily adrenergic. Elevated K^+ and veratridine produced this effect on cholinergic differentiation without significantly altering neuronal survival. Because depolarization causes Ca^{2+} entry in a number of cell types, the effects of several Ca^{2+} agonists and antagonists were investigated. In the presence of the Ca^{2+} antagonists D600 or Mg^{2+}, K^+ did not prevent the induction of cholinergic properties by conditioned medium. Thus depolarization, either steady or accompanying activity, is one of the factors determining whether cultured sympathetic neurons become adrenergic or cholinergic, and this effect may be mediated by Ca^{2+}.**

Recent evidence strongly suggests that individual sympathetic neurons taken from the superior cervical ganglia (SCG) of newborn rats can either secrete catecholamines (CA) and form adrenergic synapses or secrete acetylcholine (AcCh) and form cholinergic synapses, depending on the culture environment in which they develop. When grown in the virtual absence of other cell types in an appropriate medium, the dissociated neurons develop many of the properties expected of adrenergic neurons. In contrast, when cocultured with appropriate non-neuronal cells, the neurons develop striking cholinergic properties (for reviews see refs. 1 and 2). The neurons do not have to be in contact with the non-neuronal cells for these cholinergic changes to occur; medium that has been conditioned by cultures of appropriate non-neuronal cells (conditioned medium, CM) also causes dramatic increases in (*i*) choline acetyltransferase activity as measured in neuronal extracts, (*ii*) AcCh synthesis and accumulation from [^{3}H]choline by living cells, and (*iii*) cholinergic synapse formation between the neurons (3–6). At the same time, however, growth in CM causes a marked reduction in adrenergic properties: CA synthesis and the proportion of synaptic vesicles containing small dense cores following permanganate fixation are considerably decreased (5, 6). Similarly, coculture with appropriate non-neuronal cells reduces the proportion of small granular vesicles observed after loading with exogenous CA (7).

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These effects of CM are rather specific. Doses of CM that increase the ratio of AcCh/CA production 100- to 1000-fold do not appreciably affect neuronal survival or growth (6). The constancy in survival and growth is not because only a few neurons in the population are involved in these transmitter changes: experiments on single neurons have shown that most, if not all, of the neurons are influenced in this manner by the culture environment. It is possible to grow single neurons in microcultures containing various concentrations of CM or non-neuronal cells (8, 9); as many as 80–90% of individual neurons grown on heart or skeletal muscle can be cholinergic, while as few as 0% are cholinergic under "control" conditions (10, 11). Furthermore, by growing cultures in various concentrations of CM, it is possible to obtain populations of single cells in which almost every cell synthesizes CA or in which a high proportion of cells synthesize AcCh (10). Finally, there is no evidence for a significant population of "silent" neurons that make no detectable transmitter under these conditions (10). These observations, taken together with the constancy of neuronal number and growth in the mass cultures with and without CM, argue that CM affects the choice of transmitter and type of synapses formed by individual sympathetic neurons but does not select certain predetermined cell types for survival.

Because it is possible in culture to cause virtually all of the immature sympathetic neurons to become cholinergic, why does only a small proportion of these same neurons become cholinergic *in vivo* (12–14)? One possibility is that *in vivo* some factor prevents most of the neurons from responding to the cholinergic cue, and this factor is absent from the cultures. One such potential influence, which is known to be important for postnatal adrenergic development, is the innervation of sympathetic ganglia by preganglionic afferents from the spinal cord (15–19). This paper describes the consequences on the adrenergic–cholinergic decision of mimicking the electrical effects of spinal cord innervation by chronically treating the cultured sympathetic neurons with the depolarizing agents elevated K^+ or veratridine, or by chronically evoking action potentials with a new method of direct electrical stimulation.

METHODS

SCG were removed from newborn rats, mechanically dissociated, plated in collagen-coated dishes, and grown in L-15 CO_2 medium with the additives glucose, glutamine, penicillin, streptomycin, vitamins, 5% rat serum, 7S NGF at 1 μg/ml, and Methocel at 6 mg/ml, as previously described (6, 20). Non-neuronal cells were eliminated by administration of 10 μM cytosine arabinonucleoside on days 2–4 and days 6–8 *in vitro* (6), and the culture medium was changed every 2–3 days.

To prepare the elevated K^+ medium, KCl-free, NaCl-free L-15 medium was obtained from Grand Island Biological Co. KCl and NaCl were added back to obtain the desired concen-

Abbreviations: AcCh, acetylcholine; CA, catecholamines; CM, conditioned medium; SCG, superior cervical ganglia.

Table 1. Effects of elevated K^+ and veratridine

	Additions		AcCh/CA	AcCh, fmol/neuron	CA, fmol/neuron	Neurons
			Experiment A			
	Days 1–20	Days 21–30				
1.	—	—	0.71 ± 0.09	15.8 ± 1.0	22.6 ± 1.6	5610 ± 720
2.	K^+	K^+	0.00 ± 0.01	0.2 ± 0.1	42.8 ± 1.7	4040 ± 160
3.	—	CM	11.93 ± 0.57	119.7 ± 1.1	10.1 ± 1.4	6460 ± 320
4.	K^+	K^+ + CM	0.30 ± 0.13	8.5 ± 3.3	30.0 ± 3.6	4590 ± 380
5.	K^+	CM	3.33 ± 0.77	58.6 ± 6.5	19.2 ± 5.3	5620 ± 130
6.	CM	—	14.36 ± 3.82	173.2 ± 18.3	13.7 ± 4.6	2840 ± 150
7.	CM	K^+	12.37 ± 2.24	195.4 ± 47.6	14.4 ± 3.8	1980 ± 800
			Experiment B			
	Days 1–10	Days 11–20				
	—	CM	8.78 ± 1.38	9.80 ± 2.0	1.1 ± 0.1	1420 ± 100
	Ver	Ver + CM	0.03 ± 0.02	0.05 ± 0.1	1.5 ± 0.2	1910 ± 430

The culture period was divided into two intervals, and different additions were made to the medium during each. Triplicate cultures were assayed for transmitter synthesis with 4-hr isotopic incubations at day 30 (experiment A) or day 20 (experiment B) as described in *Methods*, and expressed as fmol per neuron or as a ratio of the rate of synthesis of AcCh to CA. Neuronal somas were counted by phase microscopy before assay. In experiment A, the control KCl concentration was 4 mM, and the elevated KCl (K^+) concentration was 20 mM. In experiment B, veratridine (Ver) was added at a final concentration of 1 μg/ml. The data are expressed ± SEM.

trations, with NaCl adjusted to maintain constant osmolarity. This medium was made into L-15 CO_2 by addition of $NaHCO_3$ (20) and contained the same additives as the usual L-15 CO_2 medium. For experiments with divalent cations, small aliquots of sterile concentrated solutions of $CaCl_2$, $MgCl_2$, and $BaCl_2$ were added to 20 mM K^+ medium or 53 mM K^+ medium (see below) to obtain the desired concentrations. (The actual concentrations of Ba^{2+} and elevated Ca^{2+} in these experiments is not known because precipitates formed in the dishes and accumulated over the period of addition.) Veratridine and tetrodotoxin were obtained from Sigma and D600 from Knoll AG Chemische Fabriken.

CM was made by incubating L-15 CO_2 medium on monolayer cultures of rat heart or skeletal muscle cells as previously described (6). This CM was diluted to 60% with fresh L-15 CO_2 medium before administration to neuronal cultures. For 20 mM K^+ CM, CM was diluted with fresh 53 mM K^+ L-15 CO_2 to obtain a final concentration of 20 mM K^+.

For assay of transmitter synthesis, cultures were incubated in the presence of [^{3}H]tyrosine (New England Nuclear, 46 Ci/mmol) and [^{3}H]choline (New England Nuclear, 4.2 Ci/mmol) for 4–8 hr as previously described (20). All incubations were performed in medium containing 4 mM K^+. Before transmitter synthesis was assayed, cultures exposed to veratridine and their controls were treated with 3 μM tetrodotoxin for 2 days to allow repolarization; they were then assayed in the presence of 3 μM tetrodotoxin. For multi-chamber cultures (see below), isotope was applied only to the axons in the side chambers. Cultures were harvested and the transmitter products were separated by electrophoresis as previously described (20).

Some cultured neurons were stimulated electrically for a period of 1 to 2 weeks. These neurons were grown in a three-chamber tissue culture system (21); they sent their axons from a central fluid-filled chamber across tightly sealed partitions into two separate fluid-filled side chambers. The partitions formed a sufficiently tight seal around the crossing axons so that current passed between the chambers flowed through the axons and gave rise to action potentials (22). Square current pulses were delivered by a pair of 32-gauge platinum wires cemented through holes in the culture dish lid and immersed in the medium in the left and right side chambers. Pulse frequency was constant at 1/sec, pulse amplitude was 3 V, pulse duration was 1 msec, and the polarity was reversed each second to minimize possible polarization effects.

RESULTS

As a first approximation to the effects of neuronal activity on sympathetic development *in vivo*, we exposed dissociated sympathetic neurons in culture to the depolarizing agents elevated K^+ or veratridine (Table 1). The culture period was divided into two intervals with different additions during each, in order to assess the effects of depolarization and CM independently. Experiment A shows that chronic treatment with 20 mM KCl lowered the ratio of AcCh/CA synthesis more than 70-fold, primarily by depressing the rate of AcCh synthesis (lines 1 and 2). CA synthesis almost doubled in 20 mM K^+, which may be analogous to the increase in tyrosine hydroxylase activity accompanying full adrenergic maturation *in vivo*. This increase in CA synthesis in elevated K^+ was variable, however, and will be studied in more detail. As previously reported, addition of CM caused a large increase in the AcCh/CA ratio (lines 1 and 3). However, even in the presence of the cholinergic influence of CM, cultures maintained in 20 mM K^+ remained primarily adrenergic by the criterion of the AcCh/CA ratio (lines 3 and 4). Elevated K^+ did not have to be continuously present to exert a significant suppression of AcCh synthesis; pretreatment with elevated K^+ before CM addition also lowered AcCh synthesis (lines 3 and 5). Thus, the suppression of cholinergic characteristics by K^+ probably does not reflect a direct inhibition of AcCh synthesis itself, but rather a change in developmental fate. Some cultures were pretreated with CM for 20 days to access the ability of raised K^+ to reverse the cholinergic induction. It can be seen in lines 6 and 7 that elevated K^+ has little effect on the AcCh/CA ratio after CM pretreatment. CA production was not stimulated in this paradigm.

A study of the dependence of AcCh/CA ratio on KCl concentration chronically present in the medium (Fig. 1) showed that increasing K^+ from 20 to 80 mM did not result in significantly greater suppression of AcCh production. Furthermore,

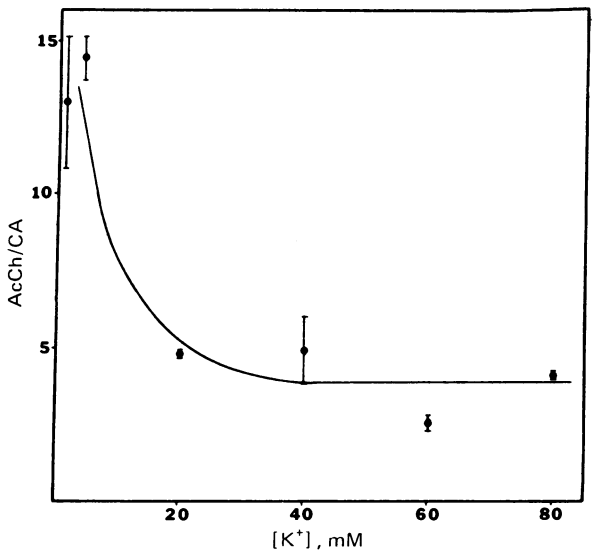

FIG. 1. Triplicate cultures were grown for 10 days in various K^+ concentrations, then for 10 days in CM, and then assayed for transmitter production with 4-hr isotopic incubations as described in *Methods*. The bars represent the ± SEM.

the enhanced CA production in cultures exposed only to 20 mM K^+ (no CM) was not further enhanced by increasing K^+ to 80 mM (data not shown). Although others have found that elevated K^+ increases neuronal survival in culture (23, 24), we did not find increased survival at 20 mM K^+ (Table 1) or at K^+ concentrations up to 80 mM (data not shown).

Veratridine depolarizes neurons by directly opening voltage-dependent Na^+ channels and is effectively antagonized by tetrodotoxin (25). As shown in Table 1, experiment B, veratridine at 1 μg/ml (1.5 μM) greatly reduced the AcCh/CA ratio in cultures exposed to CM. As with raised K^+, this depolarizing agent was effective without significantly increasing the number of neurons surviving in culture. At higher concentrations (10 μg/ml, present only every other day) veratridine was quite toxic, as evidenced by a decline in neuronal survival and a poor appearance of the neurons with phase microscopy. However, even at this toxic concentration, veratridine suppressed cholinergic development (AcCh/CA = 0.1). That the action of veratridine on the AcCh/CA ratio was due to its effect on the voltage-sensitive Na^+ channels was shown by a complete inhibition of the veratridine effect by the simultaneous presence of tetrodotoxin (3 μM; AcCh/CA = 24). Finally, as with raised K^+, pretreatment with veratridine reduced the subsequent response to CM, even though the depolarizing agent was present for only the first 10 days (AcCh/CA = 4).

Because steady depolarization is not identical to normal electrical activity, we have also employed a new method of direct electrical stimulation to intermittently depolarize the neurons. It was found that neurons grown in a three-chamber culture system could be stimulated by passing electrical current between the side chambers (22). Intracellular recordings were made from a few neurons in several cultures to assess the efficacy of the stimulation; pulses 1 msec in duration and only 1–5 V in amplitude produced action potentials in all of the neurons studied; the neurons responded to each pulse with a single action potential. After 7 days were allowed for a substantial number of neurites to cross the barriers and enter the side chambers (21), stimulation at 1/sec was begun in some cultures and continued for 7 days (Table 2; lines 3 and 4). After the 7-day stimulation period, treatment of cultures with CM was begun, and continued for 7 more days (lines 2, 3, and 4). At the time of addition of CM, stimulation was discontinued in one group of cultures (line 3) but was continued for an additional 7 days in another group (line 4). Thus, group 3 differed from group 4 in two respects: in 3, stimulation was for a shorter period and did not overlap with CM. Stimulation caused a significant reduction in the AcCh/CA ratio; the stimulated cultures were primarily adrenergic even though they were treated with CM. The lowering of the AcCh/CA ratio resulted from a reduction of AcCh synthesis with much less effect on CA production. The effects on the AcCh/CA ratios were greater when the stimulation period was prolonged (lines 3 and 4). In another experiment, stimulation was ineffective in suppressing cholinergic development if tetrodotoxin was present throughout the period of stimulation (data not shown).

Chronic depolarization or electrical stimulation could lead to changes in gene expression in developing neurons in several possible ways. Depolarization by elevated external K^+, veratridine, and action potentials causes Ca^{2+} entry in a variety of preparations (e.g., refs. 26 and 27; and P. H. O'Lague, E. J. Furshpan, and D. D. Potter, unpublished data). To investigate the possibility that Ca^{2+} entry is involved in the suppression of cholinergic differentiation caused by elevated K^+, neurons were grown in the presence of 20 mM Mg^{2+} or D600 [a methoxy derivative of verapamil, which blocks Ca^{2+} channels (40); 10 μM] to reduce Ca^{2+} influx. 5 mM Ca^{2+} and Ba^{2+} were also tested to see if the effects of elevated K^+ were enhanced. As shown in Table 3, both Mg^{2+} and D600 greatly inhibited the action of K^+ in suppressing cholinergic development. In contrast, addition of Ca^{2+} or Ba^{2+} tended to augment the effectiveness of K^+. These results taken together support the hypothesis that Ca^{2+} is involved in the suppression of cholinergic differentiation by raised K^+.

DISCUSSION

One approach to the investigation of the role of presynaptic input in the development of sympathetic neurons would be to coculture neurons from SCG with spinal cord, especially because cord explants innervate dissociated sympathetic neurons under culture conditions similar to those used here (28). How-

Table 2. Direct electrical stimulation

	Additions or treatment				
	Days 7–14	Days 14–21	AcCh/CA	AcCh, pmol/dish	CA, pmol/dish
1.	—	—	0.19 ± 0.04	0.084 ± 0.030	0.416 ± 0.076
2.	—	CM	1.16 ± 0.13	0.472 ± 0.170	0.437 ± 0.148
3.	Stim	CM	0.53 ± 0.07	0.291 ± 0.045	0.543 ± 0.054
4.	Stim	Stim + CM	0.03 ± 0.01	0.017 ± 0.011	0.550 ± 0.190

The culture period was divided into two intervals. The neurons were electrically stimulated (Stim) as described in *Methods* or grown with 60% conditioned medium (CM). Quadruplicate cultures were assayed for transmitter synthesis with 8-hr isotopic incubations at day 21 as described in *Methods*, and the data are expressed as pmol per dish ± SEM. The data are not expressed per neuron because the neurons were difficult to count in the chamber system.

Table 3. Role of Ca^{2+} in K^+ effect

Additions	AcCh/CA	AcCh, fmol/neuron	CA, fmol/neuron
—	5.60 ± 0.13	8.94 ± 0.80	1.57 ± 0.12
K^+	0.20 ± 0.06	0.48 ± 0.16	2.40 ± 0.35
$K^+ + Mg^{2+}$	3.97 ± 0.65	9.45 ± 0.36	2.45 ± 0.33
K^+ + D600	2.54 ± 0.42	4.58 ± 0.95	1.80 ± 0.21
$K^+ + Ca^{2+}$	0.07 ± 0.07	0.08 ± 0.09	1.78 ± 0.67
$K^+ + Ba^{2+}$	0.09 ± 0.03	0.50 ± 0.18	5.46 ± 0.27

Triplicate cultures were grown with the additives shown for 8 days, then with the same additive plus CM for an additional 8 days and then assayed for transmitter production with 4-hr isotopic incubations as described in *Methods*. The data are expressed as in Table 1. The elevated K^+ concentration (K^+) was 20 mM, and control medium contained 1.1 mM Ca^{2+} and 1.4 mM Mg^{2+}. The added ions were present at the following nominal concentrations: Ca^{2+}, 6.1 mM; Mg^{2+}, 22.4 mM; Ba^{2+}, 5 mM. The D600 concentration was 10 μM.

ever, included with the neurons in these cord explants are a large number of non-neuronal cells, and previous work has demonstrated that dissociated cell cultures of brain cells (6), Schwann cells (29), and ganglionic non-neuronal cells (30) are capable of inducing cholinergic differentiation in sympathetic neurons. Our preliminary studies of sympathetic neurons cocultured with cord explants revealed that a significant proportion of the former became cholinergic. To separate the effects of "cord CM" and synaptic activation, we employed three different means of stimulating the sympathetic neurons in the absence of other cell types.

The present results demonstrate that chronic treatment of developing sympathetic neurons with elevated K^+, veratridine, or direct electrical stimulation yields cultures with greatly reduced ratios of AcCh/CA synthetic rates. Under the weakly cholinergic conditions of 0% CM, the depolarizing agents produce virtually pure adrenergic cultures. Even in the presence of 60% CM, these agents can lower the AcCh/CA ratio about 100-fold. Because the AcCh/CA ratio has proven to be a good index of the proportion of neurons following adrenergic or cholinergic differentiative pathways (5, 6, 10), it is likely that the depolarizing agents lower the percentage of neurons that become cholinergic. During the first few days in culture, the sympathetic neurons begin to differentiate adrenergically, even in the presence of CM, as indicated by their ability to synthesize and store CA (20) and the high proportion of small granular vesicles visualized with permanganate staining (ref 7; S. Landis, unpublished data). The depolarizing agents appear to fix or stabilize this initial developmental choice so that subsequent addition of the cholinergic signal (CM) has a considerably reduced ability to reverse the adrenergic choice. It is possible that depolarization could stabilize a cholinergic developmental choice as well as an adrenergic one, but this has not yet been directly tested. It does not appear that activity directly inhibits AcCh synthesis or the interaction of the cholinergic signal with the neurons, because pretreatment with elevated K^+, veratridine, or electrical stimulation reduces the AcCh/CA ratios of cultures subsequently exposed to CM in the absence of these agents. This failure of the cells to respond to cholinergic induction by CM supports the idea that the onset of activity is the signal for final determination of transmitter choice.

If activity does make these neurons resistant to cholinergic induction by CM, what role does spontaneous activity in culture play in the determination of transmitter function? Although no detailed study of spontaneous activity in normal growth medium has yet been conducted, spontaneous excitatory postsynaptic potentials and complex waves of activity can be readily observed in cholinergic cultures (31, 32). Nevertheless, cultures grown in 60% CM have a very high proportion of cholinergic neurons as determined by cholinergic interactions between random pairs of neurons and the proportion of small granular vesicles (5). One might have expected that a few cholinergic neurons could stimulate the remainder of the neurons and keep them in the adrenergic state. There are several possible explanations for why this does not appear to occur: (*i*) the amount of spontaneous activity is too low to be effective; (*ii*) 60% CM is such a strong cholinergic influence that it overwhelms the effect of spontaneous activity; or (*iii*) by the time enough cholinergic neurons have differentiated to provide stimulation, most of the neurons have responded to CM and it is too late for activity to stabilize an adrenergic state.

Electrical activity and chronic depolarization cause extensive alterations in neuronal metabolism, any of which could influence the developmental fate of the cell (3, 26, 33, 34). Although a number of investigators have reported increased cell survival in elevated K^+ (23, 24), no significant increase in cell number was observed under our conditions. Although such metabolic effects could play a role in altering the development of the neurons, we can propose the relatively simple hypothesis that depolarization-induced ion fluxes couple changes in membrane potential to subsequent cytoplasmic events in development. Both intense electrical stimulation and exposure to veratridine lead to increases in intracellular Na^+ and decreases in intracellular K^+ (P. H. O'Lague, E. J. Furshpan, and D. D. Potter, unpublished data; ref. 25). On the other hand, exposure to 20 mM K^+ does not alter the cellular content of K^+ in intact SCG (35) and may lower intracellular Na^+ through increased activity of the Na^+,K^+-ATPase (36). The changes in membrane potential seen in L6 muscle cells maintained in 20 mM K^+ are consistent with the interpretation that little alteration of intracellular ions occurs (37). Because the changes in intracellular Na^+ and K^+ to be expected after these different methods of depolarization are qualitatively quite different, no investigation of their role was undertaken. On the other hand, electrical stimulation, veratridine, and elevated K^+ have all been found to increase Ca^{2+} entry in various preparations (refs. 26 and 27; P. H. O'Lague, E. J. Furshpan, and D. D. Potter, unpublished data) and so the role of Ca^{2+} was examined in the present study.

Ba^{2+} effectively replaces Ca^{2+} in supporting Ca^{2+}-dependent action potentials (38), muscle contraction, and transmitter release (39), while Mg^{2+} antagonizes these actions of Ca^{2+} (39). In K^+-depolarized cultures induced with CM, addition of Mg^{2+} to the medium allows cholinergic development while addition of Ca^{2+} or Ba^{2+} tends to further enhance adrenergic character. D600, an alkaloid that rather specifically blocks Ca^{2+} channels (40), also permits cholinergic differentiation in K^+-depolarized cells. These observations are consistent with the interpretation that influx of Ca^{2+} is a necessary step in the linking of depolarization to developmental events in the cell. There are at least two sites for Ca^{2+} entry during activity: (*i*) the Ca^{2+} current involved in transmitter release from presynaptic terminals (39) and (*ii*) the Ca^{2+} component of the action potential in the somata of sympathetic neurons in culture (P. H. O'Lague, E. J. Furshpan, and D. D. Potter, unpublished data). Ca^{2+} is known to exert control over a number of cellular processes, including cyclic nucleotide metabolism (41). Interestingly, Schubert *et al.* (42) have observed that cyclic AMP increases the cholinergic properties of the pheochromocytoma cell line PC 12 (43).

There is considerable evidence from studies of other systems that is consistent with the idea that changes in membrane potential or ion fluxes can influence development. For instance,

in muscle development, the localization of cholinergic receptors (44), maintenance of the contractile apparatus (45) and resting potential (44), and the resistance to hyperinnervation (46) are all affected by direct electrical stimulation in the absence of neuronal input. In sympathetic development, trans-synaptic stimulation during the first weeks of postnatal life *in vivo* plays a critical role in adrenergic differentiation, leading to an increase in ganglionic tyrosine hydroxylase and dopamine β-hydroxylase. The present paper extends the developmental role of neuronal activity to that of a final determinant of transmitter function. The onset of activity apparently fixes the transmitter choice of the neurons and thus ends the period during which transmitter function can be modified by developmental cues.

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