PERSISTENCE OF AN AMINE UPTAKE SYSTEM IN CULTURED RAT SYMPATHETIC NEURONS WHICH USE ACETYLCHOLINE AS THEIR TRANSMITTER

E. WAKSHULL, M. I. JOHNSON, and H. BURTON

From the Departments of Physiology and Biophysics, and Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT

Cultures of dissociated rat superior cervical ganglion neurons (SCGN) were treated with the sympatholytic agent, guanethidine. When treated within the first couple of weeks in vitro, the neurons were rapidly destroyed. The cells grew less susceptible to the toxic effects of guanethidine with age in vitro. Moreover, the apparent affinity, K_m, of the transport molecule for norepinephrine (NE) and guanethidine remained essentially unchanged between 2 and 7 wk in culture, as did the maximum velocity of transport (V_{max}). This is at a time when previous studies have shown these neurons to be using acetylcholine (ACh) as their neurotransmitter. Cultures which were grown without supporting cells and from which cholinergic synaptic interactions were recorded physiologically were processed for autoradiography after incubation with [^{3}H]NE. All cell bodies and processes seen had silver grains accumulated over them.

These experiments show that sympathetic neurons in vitro maintain their amine uptake system relatively unchanged, even though they use ACh as their transmitter. The implications of these findings are discussed.

KEY WORDS tissue culture · sympathetic neurons · guanethidine · amine pump · cholinergic

It has been known for many years that target tissues perform a maintenance function necessary for the survival of innervating neurons. Until recently, little was known about how much environmental (cellular and humoral) interactions could influence the specific differentiated properties of a neuron. Over the last few years, however, it has become clear that at least the neurotransmitter that a neuron releases can be effected by external factors (41, 43, 51). We were interested in whether other neuronal properties were altered under conditions which induced a neuron to change its neurotransmitter.

Evidence for neurotransmitter plasticity has come from work on rat sympathetic neurons grown in vitro. Cultures of dissociated rat superior cervical ganglion neurons (SCGN) have been shown to form synapses with each other (6, 51, 56) and with co-cultured skeletal muscle cells (reference 48 and E. Wakshull, unpublished observations). These synaptic connections have been shown by pharmacological criteria to be cholinergic (13, 41, 51). These findings cannot be explained by a selective survival of those cholinergic sympathetic neurons reported to exist in some sympathetic ganglia (1, 5, 61, 68) because the neurons are essentially postmitotic in culture and their numbers are stable from the first week in

vitro. There is also a gradual shift in synaptic vesicle cytochemistry in virtually all the cells examined (reference 40; see also discussion in reference 54). After 1 wk in vitro, synaptic vesicles are of the dense-core type, which is typical for adrenergic sympathetic neurons in vivo (59). During the ensuing weeks in culture, the vesicle population becomes increasingly agranular or clear, and after 8 wk in vitro synaptic profiles contain predominantly clear vesicles (40). Concomitant with these changes, and beginning at 2-3 wk in vitro, a dramatic increase is seen in choline acetyltransferase (CAT) activity and in the frequency and efficacy of cholinergic synaptic transmission between these sympathetic neurons.

Despite this shift to cholinergic mechanisms, these dissociated sympathetic neurons retain some properties characteristic of adrenergic neurons. There is a 50-fold increase in the ability of these cells to synthesize catecholamines (dopamine and norepinephrine) from L-tyrosine during their first 3 wk in vitro (44). When these neurons are grown in the presence of supporting cells under conditions known to allow expression of cholinergic transmission (38, 39), they are still able to take up exogenous norepinephrine (NE) with high affinity (54) and to release it upon depolarization with high K^+ or veratradine in a Ca^{++}-dependent manner (12, 54). This ability persists even up to 8 wk in culture (12).

The uptake of extracellular NE with a high-affinity transport mechanism is characteristic of adrenergic neurons both peripherally (3, 33, 34, 36) and centrally (4, 16, 29, 31, 66) and is thought to be the dominant mechanism for transmitter inactivation (3, 35). The amine uptake system is also used by guanethidine, a drug which is used clinically in the treatment of chronic hypertension (47, 55) and which has been shown to block the release of NE from peripheral adrenergic terminals (14, 15, 20, 21, 32, 45, 46). Although the mechanism of this blockade has yet to be elucidated, it is thought that the specificity of this blockade (i.e., confined to adrenergic neurons) is due to the use of the amine pump by guanethidine to gain entry into the cell (15, 32, 45, 46, 62). It has also been shown that, in sufficiently high doses, guanethidine will destroy the sympathetic nervous system in rats (11, 38). However, a small percentage of the principal neurons survive the drug treatment (2, 11). In cultures of chick and rat superior cervical ganglion explants, guanethidine also proved to be toxic to virtually all the cells (37).

The purpose of the experiment reported here was to determine whether any changes in the kinetics of the amine uptake system accompanied the switch to cholinergic transmitter mechanism by these cultures. This study was prompted by the observation that the susceptibility of these neurons to guanethidine cytotoxicity decreases as a function of age in vitro (65).

MATERIALS AND METHODS

Preparation of Cultures

Cultures of dissociated rat SCGN were established as previously described (40), with some modifications. Briefly, ganglia were removed from 21-day-old embryos, stripped mechanically of their connective tissue capsule, and incubated for 30-45 min in Hanks' Ca^{++}-Mg^{++}-free balanced salt solution (Grand Island Biological Co. [Gibco], Grand Island, N. Y.) with 0.25% trypsin (Sigma Chemical Co., St. Louis, Mo.; Worthington Biochemical Corp., Freehold, N. J.) at 35°C. After extensive rinsing in L-15 media (Gibco), ganglia were triturated in standard media (see Table I A), filtered through a Nitex filter (Tetko Inc., Elmsford, N. Y.; 15 μm pore size), and plated onto collagen-coated Aclar dishes (25 mm diam) (7). Cultures were maintained at 35°C and pH 7.3 in a CO_2 atmosphere. Medium was changed three times a week. Most of the experiments were done on SCGN cultures free of supporting cells (fibroblasts and Schwann cells). This was achieved by treating the cultures from days 3-6 in vitro with media containing fluorodeoxyuridine (Table I B). This brief treatment was usually sufficient to destroy all the supporting cells, as judged by visual inspection with phase-contrast microscopy.

Cytotoxicity Experiments

Experimental cultures were fed with standard medium (Table I A) containing 100 μM guanethidine sulfate (Ciba-Geigy Corp., Summit, N. J.) and maintained at (approx.) pH 8.0 (35) for 48 h. Lower concentrations of guanethidine required a longer exposure of the cells to the drug. Although control cultures tolerated the alkalinity well for 48 h, some deterioration occurred if the cells were maintained at the high pH for longer periods of time. Control cultures were similarly treated, but without the drug. At the end of the incubation period, the cultures were returned to standard medium at pH 7.3. To assess the toxic effects of guanethidine, cell numbers were determined before treatment and usually 1 wk or more after cessation of guanethidine treatment. The criteria for scoring a cell as viable by light microscopy (×160) after exposure to the drug were: (*a*) smooth plasma membrane; (*b*) visible nucleus and nucleolus; and (*c*) unfragmented process (see Results). Although these criteria are somewhat subjective, they usually

TABLE I

	Media		Solutions for transport study		
	Standard medium A	Antimitotic medium B	Prerinse C	Radioactive media D	Postrinse E
MEM	65% (vol/vol)	78% (vol/vol)	100%	100%	100%
Human placental serum	25% (vol/vol)	10% (vol/vol)	–	–	–
Chick embryo extract	–	–	–	–	–
Glucose	600 mg% (wt/vol)	600 mg% (wt/vol)	600 mg% (wt/vol)	600 mg% (wt/vol)	600 mg% (wt/vol)
Glutamine	1.4 mM	1.4 mM	–	–	
NGF	25-50 U/ml	25-50 U/ml	–	–	
HEPES*	–	–	25 mM	25 mM	25 mM
EGTA‡	–	–	4 mM	4 mM	4 mM
$MgCl_2$	–	–	5 mM	5 mM	5 mM
Pargyline§	–	–	10 μM	10 μM	10 μM
Ascorbic acid	–	–	1.2 mM	1.2 mM	1.2 mM
Fluorodeoxyuridine	–	10 μM	–	–	–
Uridine	–	10 μM	–	–	–
[^{3}H]NE	–	–	–	‖	–
[^{3}H]Guanethidine	–	–	–	‖	–
Cold NE§	–	–	–	–	25 μM
Cold guanethidine¶	–	–	–	–	200 μM

* Sigma Chemical Co.

‡ Ethylene glycol-bis(β-aminoethyl ether) N,N'-tetra acetic acid (Eastman Organic Chemicals Div., Eastman, Kodak Co. (Rochester, N. Y.).

§ Not used for [^{3}H]guanethidine uptake.

‖ See figure legends for concentrations.

¶ Not used for [^{3}H]NE uptake.

proved adequate for our purposes, due to the striking difference in appearance between the effected cells and either those that remained viable or the control cells (Fig. 2).

Transport Experiments

The uptake and accumulation of [^{3}H]NE (3.8–5.9 Ci/mmol; New England Nuclear, Boston, Mass.) and [^{3}H]guanethidine (0.12 Ci/mmol; kindly provided by Dr. A. M. White, Ciba, Horsham, England) was studied as a function of age in vitro and drug concentration. The purity of the [^{3}H]guanethidine was confirmed by paper chromatography. Cultures were preincubated with several changes of prerinse solution (see Table I C) for 20–30 minutes at 30°–35°C. Preliminary studies had indicated that [^{3}H]guanethidine uptake was linear for 45 min. Therefore, the radioactive incubation solution (Table I D) was left on the cultures for 15 min, to insure constant rates of uptake, after which they were removed into a 5°C cold room and rinsed repeatedly with ice-cold postrinse (Table I E) contianing an excess of nonradioactive carrier. This was done to remove nonspecific extracellular binding of label. Control studies showed that uptake of [^{3}H]NE and [^{3}H]guanethidine was inhibited 99 and 97%, respectively, by incubation in the cold (5°C). The pH of the solutions was 7.2 for [^{3}H]NE and 8.0 for [^{3}H]guanethidine uptake. Initial studies indicated no difference in the uptake of [^{3}H]guanethidine between pH 7.2 and 8.0; therefore, the latter pH was used to parallel the cytological experiment. Cultures were subsequently dried, solubilized, and counted in a Beckman LS-235 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Counting efficiencies (25–35%) were determined by the external standard ratios method, and all counts were corrected accordingly. Samples were run in triplicate (except where noted), and uptake values are given as mean ± SE on a per neuron basis. For the kinetic data, the uptake of the two compounds was determined as a function of drug concentration. Double-reciprocal plots of uptake vs. substrate concentration were drawn and a linear regression analysis of the points gave the values for K_m and V_{max}.

Electrophysiological Experiments

The development of synaptic interactions between the SCGN was assayed in these cultures with physiological techniques, as previously described (41). Recordings were obtained simultaneously from two neurons with a pair of intracellular electrodes. These cells were then

successively stimulated through the recording electrode to determine whether synaptic connections existed between the pair of cells. When synaptic potentials were seen, the culture was usually perfused with hexamethonium (bromide salt; Sigma Chemical Co.), a nicotinic receptor antagonist, to establish whether cholinergic transmission was present. A blocked synaptic potential had to recover upon return to control medium before it was considered a positive pharmacological observation.

Autoradiography

A combined physiology and autoradiography experiment was used as an additional test of whether a neuron could express cholinergic transmission together with a persistent amine uptake system. Cholinergic driver cells were identified as described above, and their positions were noted on a scored culture dish. The culture was then incubated for 30 min at room temperature with 11 μCi [^{3}H]NE in *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-buffered minimum essential medium (MEM; pH 7.4) at a concentration of 0.78 μM and diluted with 1.56 μM cold NE. After loading, cultures were rinsed repeatedly with HEPES-buffered MEM for a further 90 min and fixed overnight in Karnovsky's two-step aldehyde procedure (57) at 4°C. The culture was then washed with 0.1 M cacodylate, dried, and coated with Kodak NTB2 emulsion. After exposure for 1 wk at 0°C, the autoradiographs were developed with Kodak D-19, and counterstained with thionin.

RESULTS

Transport Experiment

The accumulation of [^{3}H]NE and [^{3}H]-guanethidine by supporting cell-free cultures of SCGN increased by three- and eight-fold, respectively, between 1^1/2 and 7^1/2 wk in vitro (Fig. 1). The 40-fold greater uptake of guanethidine compared to NE (cf. ordinate scales in Fig. 1) is due in large part to the difference in concentration between the two drugs during incubation (100 and 0.2 μM, respectively), although this is not the only explanation (see kinetics, below). Incubation in the cold inhibited the uptake of both drugs by >95%, indicating that the transport is probably not a simple diffusion process. Transport was also decreased by desmethylimipramine (5 μM; Geigy Pharmaceuticals, Ardsley, N. Y.), a potent antagonist of the amine uptake system (3, 33); NE uptake was 0.34% of control and guanethidine uptake was 35% of control. The difference in the degree of inhibition for the uptake of the two drugs again probably results from concentration differences in the incubation solutions (0.2 μM for NE, 100 μM for guanethidine). These data, taken together, further indicate (see Introduction) that guanethidine uses the amine uptake pump to get into these cells.

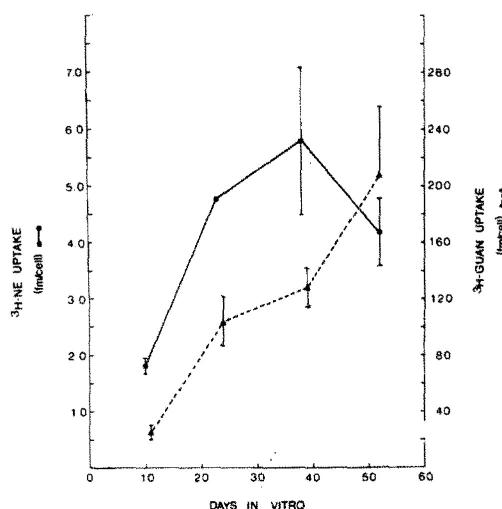

FIGURE 1 Uptake of [^{3}H]NE and [^{3}H]guanethidine as a function of age in vitro. Cultures were treated as described in Materials and Methods. In all cases, the drug concentrations were 0.2 and 100 μM for [^{3}H]NE and [^{3}H]guanethidine, respectively. Each point represents the mean ± SE of three cultures. The point without error bars represents the average of two cultures.

These findings also demonstrated that the uptake of both NE and guanethidine into SCGN increased as a function of age in vitro, up to 7 wk. At this age in vitro, several studies have demonstrated that from 5 to 70% of the neurons interconnect with cholinergically mediated synapses (40, 41, 51). As will be shown in the Physiology and Autoradiography section, SCGN grown without supporting cells also display a considerable degree of cholinergic interaction. Thus, although it appeared that these cells were switching from adrenergic to cholinergic neurotransmission, at least one feature characteristic of adrenergic neurons, the amine pump, was retained by the neurons for at least 2 mo in vitro.

It seemed important, therefore, to ascertain quantitatively whether the properties of the uptake pump changed with age in culture. Consequently, the kinetic parameters, K_m and V_{max}, were determined for NE and guanethidine transport at various times in vitro. Table II shows the result of a linear regression analysis of double reciprocal plots obtained from the uptake data.

TABLE II

*Kinetics of [^{3}H]NE and [^{3}H]Guanethidine Uptake as a Function of Age in Culture**

Days in vitro	NE				Guanethidine			
	K_m	V_{max}	R‡	P	K_m	V_{max}	R‡	P
	μM	*fmol/cell/15'*			μM	*fmol/cell/15'*		
14	0.91	38.1	0.995	0.005	0.43	61.6	0.990	0.005
47	1.5	150	0.998	0.05	1.03	222	0.920	0.12

* Cultures were treated as described in Materials and Methods. Various concentrations of [^{3}H]NE (0.1, 0.2, 0.5, 2.0 μM at 13 days *in vitro;* 0.2, 0.5, 2.0 μM at 46 div) and [^{3}H]guanethidine (0.5, 0.67, 1.0, 5.0 μM at 14 div; 0.5, 1.0, 5.0 μM at 48 div) were used in the incubation medium. Data were graphed in double reciprocal form (one/uptake vs. one/substrate concentration) and analyzed for K_m and V_{max} with a linear regression program on a desk calculator.
‡ Correlation coefficient of linear regression.

The values for K_m, which is a measure of the apparent affinity of the substrate for the transport molecule (strictly speaking, it cannot be considered an affinity constant since equilibrium conditions cannot be assumed), are similar for both drugs: 0.9 μM for NE and 0.43 μM for guanethidine at 2 wk in vitro. Considering the variability inherent in the tissue culture system and because the low specific activity of the [^{3}H]guanethidine makes measurements at low concentrations inaccurate, the K_m's are essentially the same. Moreover, these values change very little over the 5-wk interval between data points. Although the V_{max} for guanethidine is almost twice that of NE, both values increase approx. fourfold over the time-course studied. Similar values for K_m and V_{max} for NE uptake have been reported for cultures of rat SCGN by Patterson et al. (54).

Cytotoxicity Experiments

Previous studies of guanethidine toxicity on cultured explants of chick and rat SCG demonstrated a requirement for alkaline conditions (pH 7.8–8.0) in order for toxic effects to be observed (37). Preliminary work on dissociated rat SCGN replicated this finding, but transport experiments at pH 7.2 and 8.0 showed no difference in the net uptake of [^{3}H]guanethidine. Although no explanation for this high pH condition is currently available, all subsequent incubations with guanethidine (or control) were done at the elevated pH.

Neurons treated at 1–2 wk in vitro showed changes in their light microscope appearance in <2 days. Processes took on a beaded discontinuous appearance (or "blebbed"; see reference 23), the somal cytoplasm became granular and vacuolated, and the plasma membrane became irregular in its contours. These changes continued after guanethidine removal until fragmentation of the processes and disintegration of the cell body signaled neuronal demise (Fig. 2*b*). Control cultures displayed normal neuronal morphology, demonstrating that the 48 h at pH 8.0 or above was not deleterious to the appearance of the cells (Fig. 2*a*). Cultures treated at later in vitro stages manifested similar cytologic changes, but these were delayed in onset.

Cell counts of the young cultures at various times after cessation of drug treatment showed a rapid decline in the number of viable neurons until <10% of the cells remained, while control cultures showed no change in cell number. In older cultures, fewer cells were destroyed (Table III) by the treatment regimen, although most of the cells were affected to some degree. This result indicated that the older neurons were more resistant to the effects of guanethidine. It should be noted, however, that the percentage of cells surviving the treatment was subject to wide variation (Table III). Part of this variability may be due to the need for alkaline pH in order for guanethidine to be toxic (see above); cultures incubated with guanethidine had a tendency to become acidic, whether the medium had a bicarbonate or HEPES buffer system. Those cultures in which acidity became a problem suffered less severe cell destruction than those which remained at the alkaline pH for the duration fo the incubation period. This problem was most evident in cultures of high cell density (>3,500). This does not completely rule out the possibility that the variability in cell survival is a real phenomenon. Partly because of this variability, no consistent sparing of a subpopulation of cholinergic sympathetic neurons could be detected.

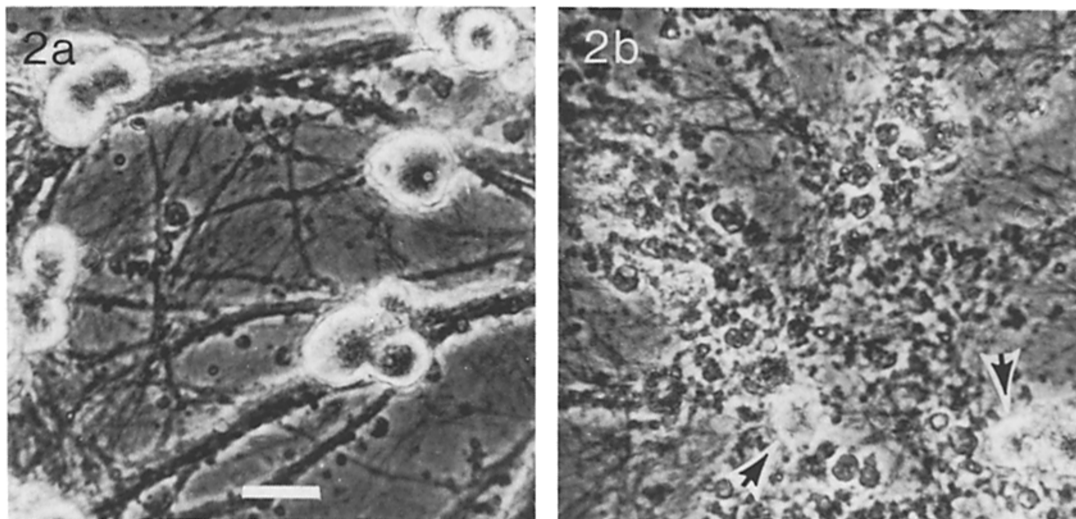

FIGURE 2 These phase-contrast photomicrographs of living cultures were taken ~7-10 days after guanethidine treatment of 1-wk-old cultures. (*a*) Control culture, incubated at pH 8.0 without guanethidine. Processes remain intact and the neuronal somas round, with a few nuclei and nucleoli visible. Most of the neurons in clusters are out of the plane of focus. Supporting cells, which are more sensitive to the high pH than the neurons (E. Wakshull, unpublished observations), account for the debris in this field. (*b*) This culture was treated with 100 μm guanethidine at pH 8.0, as described in Materials and Methods. Destruction of the neurons is complete at this stage, with only fragmented debris left to identify where cell bodies (arrows) and processes had been. Bar (for *a* and *b*) 30 μm. × 350.

TABLE III
Effect of In Vitro Age on Guanethidine Toxicity

Culture	Treatment*	Days in vitro‡	Survival§
			%
1	Control	12	104§
2	100 μM guanethidine	12	0
3	100 μM guanethidine	12	0
4	100 μM guanethidine	26	46
5	100 μM guanethidine	26	16
6	100 μM guanethidine	41	59
7	100 μM guanethidine	41	37

* Incubation at approx. pH 8.0.
‡ Age at the time of treatment.
§ The fact that this number is not 100% reflects the counting variability, which was never >10% between two observers counting the same culture, or one observer counting the same culture twice.

Physiology and Autoradiography

From their observations in the physiology of SCGN cultures, O'Lague, et al. (51) concluded that few, if any, synaptic interactions could be found in cultures free of supporting cells. Because the uptake experiments were done on such cultures (to eliminate complications introduced by the presence of non-neuronal cells), several cultures sisters to those used for uptake and other cultures taken randomly from various culture series grown without supporting cells were examined physiologically for the presence of synaptic interactions. The findings are summarized in Table IV. As can be seen, a substantial number of "driver" neurons could be found, although in some cultures no synapses were found even at long in vitro times. The time-course of development of synaptic interactions in these "pure" neuronal cultures was similar to that found in cultures grown with non-neuronal cells (cf. series 533 with data in reference 40). Thus, the presence of non-neuronal cells per se is not necessary for the expression of cholinergic synaptic transmission.

From the two cultures which were examined physiologically (Table IV, culture nos. 442-20, 21) and processed for autoradiography, a total of 55 cells were penetrated, and nine driver cells were found. Although not all of these synaptic potentials were investigated pharmacologically, we believe it probable that all are cholinergic

TABLE IV
Synaptic Transmission in SCG Cultures Free of Supporting Cells

Culture*	A Neurons recorded	B Synapses	B/A × 100	Age
			% interaction	*days in vitro*
350-16	9	0	0	69
366-25	3	0	0	47
384-16	25	0	0	51
410E-3	8	2	25	43
442-20‡	18	4	22	24
442-21‡	37	5	14	28
533-20	12	0	0	11
533-6	24	0	0	13
533-4	27	0	0	15
533-5	30	0	0	20
533-9	26	1	4	22
533-11	38	8	21	28
563-26c	13	0	0	49
677	22	10	45	34
722	20	1	5	38

* The first number indicates the culture series; the second number indicates a particular culture within the series; thus, all the 533 cultures were "sister" cultures. Note appearance of synapses vs. age in this series (cf. reference 42).
‡ Also processed for autoradiography.

because no other type of synaptic interactions has been desribed in an SCGN culture. Fig. 3*a* and *b* shows an autoradiograph of a culture previously used for physiology and prepared as described in Materials and Methods. Silver grains appear over every cell, indicating that all of the neurons were capable of taking up exogenous NE. Figure 3*c–f* shows recordings made from two cells in a culture sister to the one shown in Fig. 3*a*, and also processed for autoradiography. As shown in Fig. 3*d*, these neurons were synaptically interconnected. The synaptic potential was shown to be sensitive to hexamethionium (Fig. 3*e*). These cells, which had silver grains concentrated over them (but not so striking as in Fig. 3*a* and *b*), are therefore capable of taking up exogenous NE while using acetylcholine (ACh) as their neurotransmitter.

DISCUSSION

Two basic conclusions can be drawn from these results: First, the amine uptake system does not significantly change either in its affinity for substrate or in the density of the transport molecules in the surface membrane (see below), even though these neurons make cholinergic synapses with each other (40, 41, 50, 51). Second, dissociated sympathetic neurons in culture are destroyed by guanethidine treatment; this is consistent with both in vivo (2, 11, 17, 26, 27, 37–39) and in vitro (25, 28, 37) observations on rat sympathetic neurons. Despite some variability, as the neurons mature in culture they tend to become more resistant to the toxic effect of guanethidine (see Table III).

Destruction of the cells upon exposure to guanethidine involved progressive chromatolytic changes in the cytoplasm, nucleus, and cell membrane. No evidence of axon retraction was seen (cf. references 25 and 28), although in established dissociated cultures this would be difficult to detect. The variability in the percentage of cell survival after guanethidine treatment within and between different culture series precluded our attempts to show a subpopulation of (presumably) cholinergic neurons which might be resistant to the drug because of their cholinergicity. There was, however, a definite trend toward increased resistance to drug treatment with age in vitro (Table III), even though guanethidine uptake increased with age in culture (Fig. 1). This suggests that some (as yet unknown) metabolic change(s) within the neurons accounts for their resistance, rather than a lowering of intracellular guanethidine concentration. Since this decreased susceptibility has been described in vivo (39), where 90–95% of sympathetic cells are clearly adrenergic (68), it is unclear whether the switch to cholinergicity by the SCGN in vitro contributed to their altered sensitivity to guanethidine. One implication that comes out of these observations is that the neurons that survive guanethidine treatment in the animal (2, 11) may not be just the cholinergic principal sympathetic cells (see reference 25).

The finding that cholinergic synapses could be found without undue difficulty in SCGN cultures free of non-neuronal cells requires comment, since this observation contradicts previously published results (50, 51). Because both groups of investigators use the same starting material for the culture tissue (embryonic rat superior cervical ganglia), the cause of the discrepancy is most likely the culture conditions and, more specifically, the culture medium. Table IA shows the standard maintenance feed used in the present study; high levels of human placental serum and

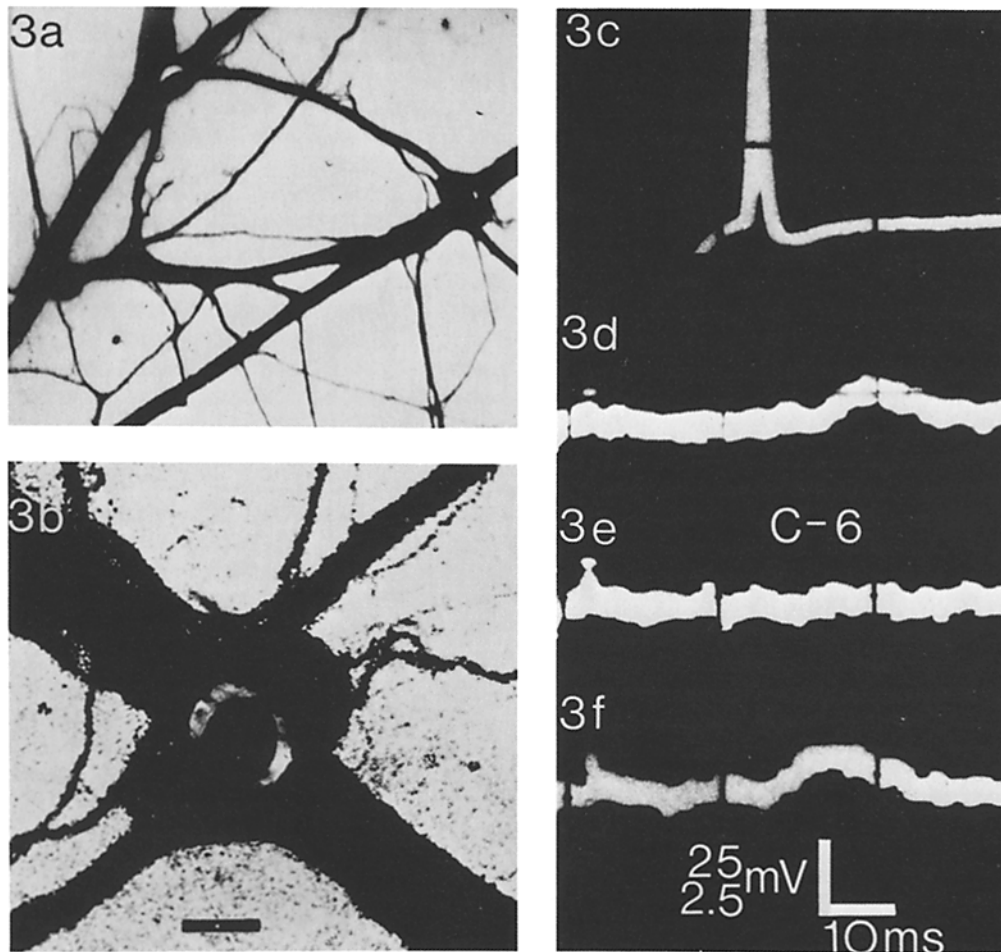

FIGURE 3 Autoradiographs of cultures incubated with [^{3}H]NE after physiological identification of cholinergic driver neurons. Cultures were incubated with [^{3}H]NE and processed for autoradiography as described in Materials and Methods. Intracellular recordings (*c–f*) were obtained as described in Materials and Methods. (*a*) Low-power autoradiograph of supporting cell-free SCGN culture. Every neuron, as well as all the processes visible in the culture, were virtually opaque with silver grains, as shown in this field. (*b*) A higher-power view of a single neuron is this same culture shows the grains more clearly, particularly over the finer neurites. (*c*) Action potential, elicited by anode break excitation, in driver neuron. Break in baseline indicates initiation of current injection. (*d–f*) "Receiver" neuron. Initial small downward and upward deflection on the extreme left of each recording trace indicates stimulus artifact. Stimulation of neuron in Fig. 3*c* produced a synaptic potential in the receiver cell, which was blocked by perfusion with 10^{-4} M hexamethonium (*C-6*) (*e*). (*f*) Return to control solutions allows the recovery of the synaptic potential. The neurons in Fig. 3*c–f* taken from a culture sister to that shown in Fig. 3*a* and *b* also had silver grains accumulated over them, but had too high a background for photographic purposes. Bar (for *a*) 100 μm; (for *b*) 25 μm. (*c*) 25 mV, 10 ms. (*d–f*) 2.5 mV, 10 ms. (*a*) × 105; (*b*) × 420.

9-day old chick embryo extract (25 and 10% by volume, respectively), are present. These ingredients were not used by O'Lague et al. (50, 51). Recent evidence from our laboratory (reference 60 and D. Ross, unpublished observations) indicates that these two components of the media act to increase CAT levels in cultured SCGN in much the same way as media from non-neuronal cell cultures (conditioned media; 53). Thus, it seems probable that the same factor or factor(s) similar

to that found in conditioned media, which has a substantial variety of cellular sources (53), is present in our media and acts to increase the ACh through increaseed CAT activity.

In each of the cultures studied with the autoradiographic method, extensive labeling was found over all of the neurons and their processes. It is possible that the labeling is due to nonspecific binding of [^{3}H]NE to the culture. Two points render this objection untenable. First, one must propose a differential affinity for nonspecific binding to the neuronal membrane vs. the collagen substrate, since there was a significantly higher density of grains over the neurons as compared to background (see Fig. 3*a* and *b*). Second, previous studies (12) on the uptake of [^{3}H]NE into dorsal root ganglion cultures indicated a virtually complete washout of radioactivity after a postincubation rinse period comparable to that used in the present study. It is, however, still conceivable that there was nonspecific binding to the neuronal membranes. Another possibility is that microelectrode damage to the membrane of the driver cells allowed nonspecific leakage of label into these neurons. However, if one assumes that the random sampling of neuronal pairs accurately reflects the percentage of cholinergic driver cells in a culture (a percentage which, in fact, probably underestimates the number of driver cells; see reference 41 for discussion), then other cholinergic driver cells must be present (undamaged) in the culture. Given the uniformity of labeling (see above), this indicates that the [^{3}H]NE was also taken up by undamaged cholinergic neurons. However, the question of whether cholinergic sympathetic neurons in vivo have a specific amine uptake system remains unanswered.

As stated above, a uniform distribution of silver grains was seen over the somal and neuritic membrane. Theoretically, one might have expected to see localization of silver grains over regions specialized for transmitter release (i.e., synaptic terminal and varicosities along the neuritic processes). It is possible that [^{3}H]NE was taken up in the terminals and varicosities and subsequently transported throughout the cells, since iontophoretic intracellular injection of horseradish peroxidase will fill extensively the neuronal processes within 90 min (E. Wakshull, unpublished observations). Alternatively, evidence exists suggesting that NE is taken up and accumulated over most or all of the adrenergic neuronal surface (18, 24). A distinction between these possibilities cannot be made on the basis of the data presented here.

The present evidence supports the idea that these neurons have a high-affinity amine uptake system, and that guanethidine is transported into the cells via this system (Tables II and III). Both NE and guanethidine uptake are inhibited by cold temperature and desmethylimipramine. The values for the kinetic parameters, K_m and V_{max} (Table II), for NE are comparable to those reported in a similar culture system by other workers (53, 54), and the K_m is within the range of values reported for peripheral adrenergic terminals in acute preparations (35, 52). Although V_{max} for both NE and guanethidine increased fourfold over the time period studied, the phospholipid content of these neurons, which is a measure of total membrane area, also increased approximately fourfold (E. Wakshull, unpublished observations). If one considers V_{max} as being proportional to the density of transport molecules (64), these results suggest that the neurons maintain the amine pumps at a relatively constant density on their surface. This specific transport system, therefore, is maintained unchanged throughout the in vitro life of these neurons. This finding is contradictory to that reported by Hill and Hendry (30), who used catecholamine histofluorescence as a measure of the uptake system and found a decrease in fluorescence with age in vitro.

What function the amine uptake pump serves in these neurons in culture is not known, because it is clear that these cells are using ACh as a neurotransmitter (13, 40, 41, 51). Alternatively, the cells could be releasing both ACh and NE, as proposed by Burn and Rand (9) many years ago for in vivo sympathetic neurons (see also reference 8), but because the SCGN have no demonstrable adrenergic receptors (reference 49 and E. Wakshull, unpublished observations), any release of NE could not be detected by the physiological methods employed. In fact, it has been shown that these cells not only produce NE (44, 53) but can also, under appropriate conditions, release both ACh and NE simultaneously onto co-cultured atrial muscle cells (19). These cases are rare, however, and may represent an immature state of development (58). Recent evidence indicates that most sympathetic neurons in culture will choose one transmitter over the other (58). Although Patterson and Chun (53) have shown that cultures of dissociated SCGN will take up exogenous choline to produce ACh, it is not clear from their

studies whether the neurons have developed the high-affinity choline uptake system thought to be specific for recapturing and recycling choline for ACh synthesis (22, 23, 63, 66). Thus, while these normally adrenergic sympathetic neurons shift to "cholinergicity," the high-affinity amine uptake system (and, by inference, the protein(s) necessary for its continued function) is maintained. Other workers have reached a similar conclusion (58).

If one assumes that normally there is a regular turnover of membrane proteins, then the persistence of the amine pump in cholinergic neurons suggests that maintenance of the pump, which normally is associated with the transmitter released by a neuron, is not regulated by the same factors that determine the transmitter that a neuron will use (42, 43, 53; see also discussion in reference 58). These findings raise doubts about the use of uptake experiments to identify the neurotransmitter used by a neuron.

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