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Rat sympathetic neurons and cardiac myocytes developing in microcultures: Correlation of the fine structure of endings with neurotransmitter function in single neurons

(autapses/transmitter differentiation/adrenergic synapses/cholinergic synapses)

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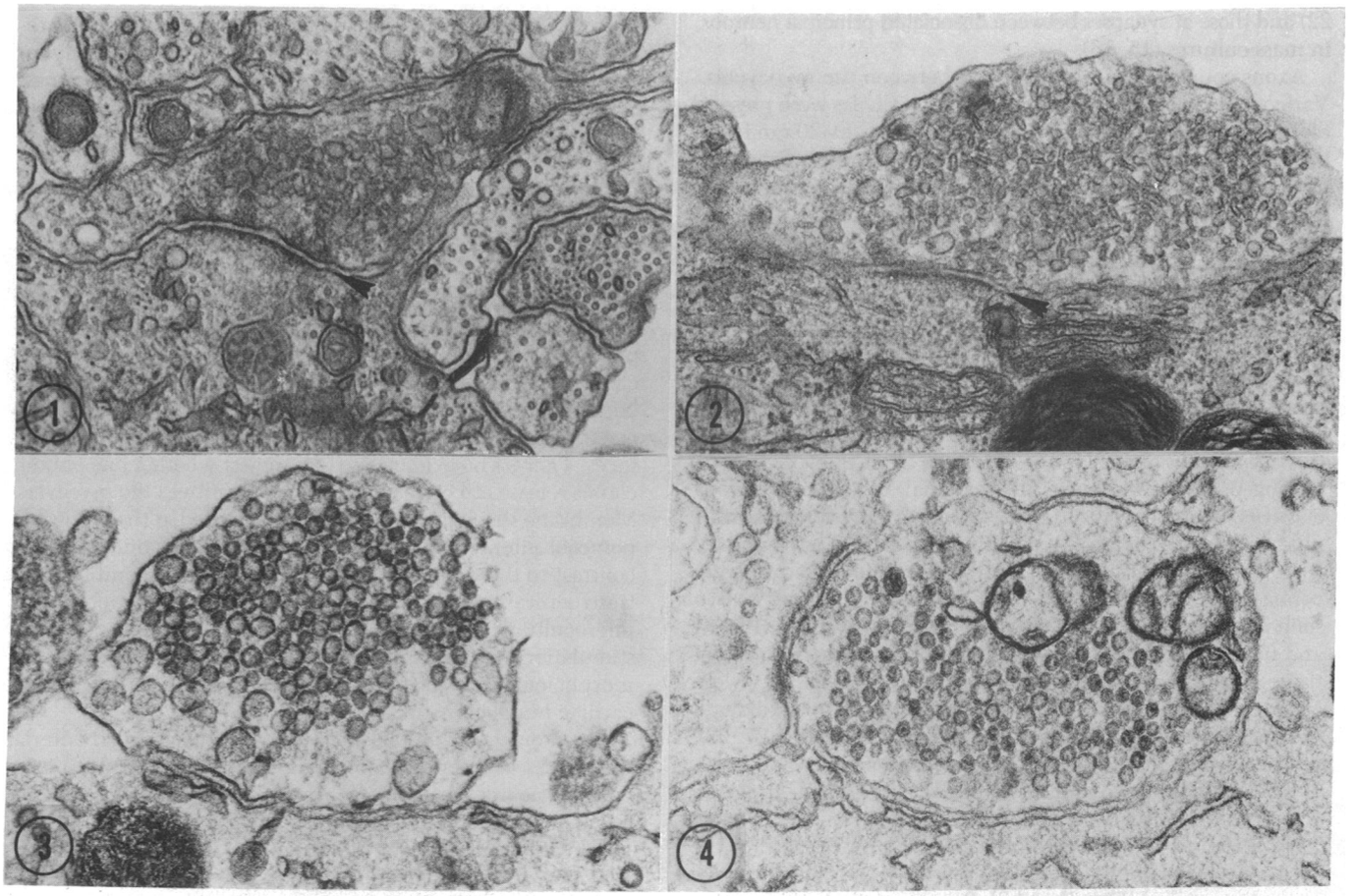
ABSTRACT **Microcultures containing single sympathetic principal neurons and small numbers of dissociated heart myocytes were prepared from newborn rats. After the transmitter properties of the neuron were studied by electrophysiological experiments, the microculture was examined with the electron microscope. Single neurons of either putative cholinergic or putative adrenergic character made morphological synapses on themselves (autapses), although only cholinergic autapses were detected electrophysiologically. Numerous axonal varicosities were present adjacent to the myocytes but no synaptic specializations were evident. After permanganate fixation to localize endogenous norepinephrine, the endings of neurons which appeared to secrete catecholamines contained many small granular vesicles, while the endings of neurons which appeared to secrete acetylcholine contained none. The endings of neurons which apparently secreted both catecholamines and acetylcholine contained only occasional small granular vesicles.**

If sympathetic neurons, dissociated from the superior cervical ganglia of newborn rats, are grown in the absence of non-neuronal cells, then the cultures are predominantly adrenergic in character (1). However, if these same neurons are grown in the presence of non-neuronal cells or in medium conditioned by them, the cultures synthesize significant amounts of acetylcholine (AcCh) as well as catecholamines; if sufficiently high concentrations of conditioned medium are used, then the cultures become predominantly cholinergic (2–5). An interesting question is whether single neurons in these cultures display either exclusively adrenergic or cholinergic properties or whether single neurons possess mixed properties. In a preliminary series of experiments (6), 3-week-old cultures that synthesized predominantly catecholamines, predominantly AcCh, or significant amounts of both were obtained by using varying proportions of conditioned medium; the incidence of cholinergic synapse formation was estimated electrophysiologically and, varicosities were examined with the electron microscope in sister cultures fixed with permanganate to localize norepinephrine (NE) in small granular vesicles (SGV) (7). In predominantly adrenergic cultures, SGV were absent from only 1% of the endings, but as the amount of AcCh synthesized and the fraction of neurons shown to interact cholinergically increased, the proportion of terminals in the culture lacking SGV also increased. Because some terminals containing numerous SGV and resembling those in predominantly adrenergic cultures were also observed in mixed cultures, it seemed likely that the cultures contained at least two populations of neurons. On the other hand, several observations raised the possibility that some neurons were not exclusively adrenergic or cholinergic: (*i*) in some cultures, the proportion of terminals which contained no SGV and were presumably cholinergic was less than the proportion of neurons interacting cholinergically; (*ii*) some terminals with SGV contained relatively few SGV; and (*iii*) incubation with 5-hydroxydopamine (50HDA), a nontoxic NE congener, increased the proportion of terminals that contained SGV, and the results suggested that some cholinergic terminals could take up and store catecholamines in SGV. This possibility was also raised by Claude's observation that synaptic endings on neurons shown to receive cholinergic synapses possessed SGV after 50HDA incubation (8, 9). The properties of individual sympathetic neurons in culture are difficult to define in cultures containing thousands of neurons and their endings. Reichardt and Patterson (10) developed methods for growing single neurons in isolation, examined their neurotransmitter properties biochemically after a month in culture, and reported that single neurons make detectable amounts of only one transmitter although single cholinergic neurons can take up and store NE. In an accompanying paper (11), Furshpan, MacLeish, O'Lague, and Potter describe a second method for growing single neurons with heart myocytes in microcultures and report electrophysiological and pharmacological evidence for the presence of cholinergic, adrenergic, and dual-function neurons. This paper presents morphological observations made on the same microcultures.

Abbreviations: AcCh, acetylcholine; Ne, norepinephrine; SGV, small granular vesicles; 50HDA, 5-hydroxydopamine.

METHODS

One or two neurons dissociated from superior cervical ganglia of newborn rats were grown on small numbers of previously dissociated heart cells in microcultures as described in the preceding paper (11). After electrophysiological experiments which characterized a particular neuron as apparently cholinergic, adrenergic, or dual-function, the microculture was photographed, marked, and prepared for electron microscopy. Some microcultures were fixed *in situ* with 3% glutaraldehyde in 0.12 M phosphate buffer at pH 7.2, stained *en bloc* with uranyl acetate in acetate buffer, dehydrated with ethanol, and embedded in a thin wafer of Epon. Others were fixed with cold 4% potassium permanganate (7), stained *en bloc*, dehydrated, and embedded. Permanganate fixation demonstrates vesicular stores of NE and 50HDA, but not other monamines such as dopamine (12, 13). One microculture containing a single cholinergic neuron and one containing a single adrenergic neuron were incubated in 10 μM 50HDA (14) in perfusion medium (11) for 30 min prior to permanganate fixation. The marked microcultures were identified with phase microscopy, removed from the wafer, and mounted. Semi-serial thin sections were cut through the entire microculture either parallel or perpendicular to the culture plane, and were picked up on Formvar-coated slot grids. Those from aldehyde-fixed cultures were stained with lead citrate while those from permanganate-fixed



FIGS. 1–4. *Fig. 1.* An axonal process of a single cholinergic neuron makes a synapse (arrow) onto one of several dendrites in a bundle. Prominent membrane specializations are present, and the synaptic vesicles appear rounded. Culture age: 14 days; aldehyde-osmium fixation, ×38,000. *Fig. 2.* An axon of a single adrenergic neuron forms a synapse (arrow) onto its own soma. The synaptic vesicles appear more pleomorphic and flattened than at cholinergic autapses. Culture age: 10 days; aldehyde-osmium fixation, ×38,000. *Fig. 3.* A cholinergic varicosity adjacent to a dendrite in a microculture containing two cholinergic neurons possesses no SGV. Culture age: 19 days; permanganate fixation, ×38,000. *Fig. 4.* A synaptic ending of an adrenergic neuron has predominantly SGV. After permanganate fixation, vesicles appear rounded in both adrenergic and cholinergic endings. From a 21-day-old microculture containing two adrenergic neurons. Permanganate fixation, ×38,000.

cultures were examined without further staining. A useful parameter for comparing single neurons was the percentage of small synaptic vesicles with granular deposits after permanganate fixation. Five thin sections, separated by 2 μm, were scanned completely, and synaptic endings and varicosities were randomly chosen for photography. Small granular and clear vesicles in terminals with at least 25 vesicles were counted on prints at 38,000 times magnification; the number of SGV was divided by the total number of small vesicles, and the value multiplied by 100 to yield a percentage. This percentage is only an approximation, because any particular terminal was sampled in only one thin section which represents about 5–10% of the volume of the varicosity. A minimum of 25 terminals were sampled for each neuron.

RESULTS

The microcultures selected for study routinely contained a single neuron along with heart myocytes and fibroblasts, and were isolated from other microcultures in the dish. The fine structure of solitary neurons in microcultures resembled that of neurons in mass cultures (15, 16) and principal cells in the intact rat superior cervical ganglion (17–19). Neuronal somas were filled with cisternae of rough endoplasmic reticulum, Golgi bodies, and free polysomes; the nucleus possessed little heterochromatin. Dendrites contained numerous microtubules, mitochondria, and smooth endoplasmic reticulum and were often accompanied by thin axonal processes with microtubules and filaments in lucent axoplasm. The axons extended beyond the dendrites and reached the outskirts of the microculture. Myocytes in the underlying monolayer contained myofibrils and were joined by intercalated disks and gap junctions and so could be distinguished from fibroblasts.

Ultrastructural examination of aldehyde-osmium fixed microcultures disclosed the presence of synapses formed on single isolated neurons by their own axonal endings (autapses, see ref. 20). Autapses of neurons which putatively secreted AcCh occurred most frequently on dendritic evaginations and possessed predominantly rounded synaptic vesicles (Fig. 1). In contrast, autapses of neurons which putatively secreted catecholamines occurred more frequently on the soma and the synaptic vesicles appeared flattened or pleomorphic (Fig. 2). A similar difference in the shape of cholinergic and adrenergic vesicles was observed in cultures of spinal cord and sympathetic neurons by Bunge and co-workers (16, 21). Both classes of autapses had a 20 nm synaptic cleft and similar pre- and post-synaptic membrane specializations; the post-synaptic thickening was more prominent than the pre-synaptic one which consisted of faint tufts of fuzz. These membrane specializations resembled those at synapses formed by preganglionic cholinergic fibers on principal neurons in the intact superior cervical ganglion (17–19,

22) and those at synapses between dissociated principal neurons in mass cultures (15, 16).

Axons coursed above, below, and between the myocytes. Varicosities containing numerous synaptic vesicles were present along the length of these axons and were seen 20–30 nm from the myocyte surface but also occurred at greater distances. Occasionally a varicosity appeared to indent the muscle cell surface. As *in vivo* (23, 24) no specializations have been observed in the myocytes or the varicosity at these appositions.

Permanganate fixation was used to localize vesicular stores of endogenous NE as SGV. Four classes of vesicle-containing axonal profiles or endings were present in the microcultures: those that participated in autapses, those that occurred adjacent to neuronal surfaces but did not possess membrane specializations, those that occurred adjacent to myocytes, and those that appeared isolated or in bundles of axons. For a given neuron, the proportions of SGV were similar in all of the vesicle-containing profiles examined. The terminals of solitary neurons classified a cholinergic, based on electrophysiological and pharmacological evidence, contained no SGV (Figs. 3 and 5). In contrast, the terminals of neurons classified as adrenergic contained numerous SGV; for example, the terminals of two such neurons contained averages of 70 and 75% SGV (Figs. 4 and 6), somewhat fewer than observed in terminals in target tissues *in vivo* such as the iris (25, 26). Only occasional SGV were observed in the perikarya of single adrenergic neurons although they are a frequent finding in the somas of adult (16, 27, 28), but not neonatal (29), rat principal cells *in vivo*.

The endings of two solitary neurons each of which appeared to secrete both AcCh and NE have also been examined after permanganate fixation. In both cases, about one third of the terminals seen contained one or a few SGV (1–4% of the small synaptic vesicles) (Figs. 7 and 8). Because a single thin section through each terminal containing only a small fraction of the vesicles present was sampled, it seems likely that there was at least one SGV in each varicosity. It is also possible, however, that dual-function neurons possess two classes of terminals.

One putative adrenergic single neuron and one putative cholinergic single neuron were incubated in 50HDA, prior to permanganate fixation, to examine the ability of the neuron to take up and store exogenous catecholamines. Almost exclusively SGV were present in the terminals of the adrenergic neuron. Approximately one third of the varicosities of the cholinergic neuron observed contained SGV and these contained only one or a few SGV (1–11% of the small vesicles) (Fig. 9). This suggests that these sympathetically derived single neurons which appear to secrete only AcCh exhibit the adrenergic property of taking up exogenous catecholamine and storing it in morphologically, as well as biochemically (10), detectable quantities, although they do this less well than adrenergic neurons. In contrast, parasympathetic cholinergic terminals in irides incubated with similar concentrations of 50HDA do not exhibit this adrenergic property (ref. 12; S. C. Landis, unpublished).

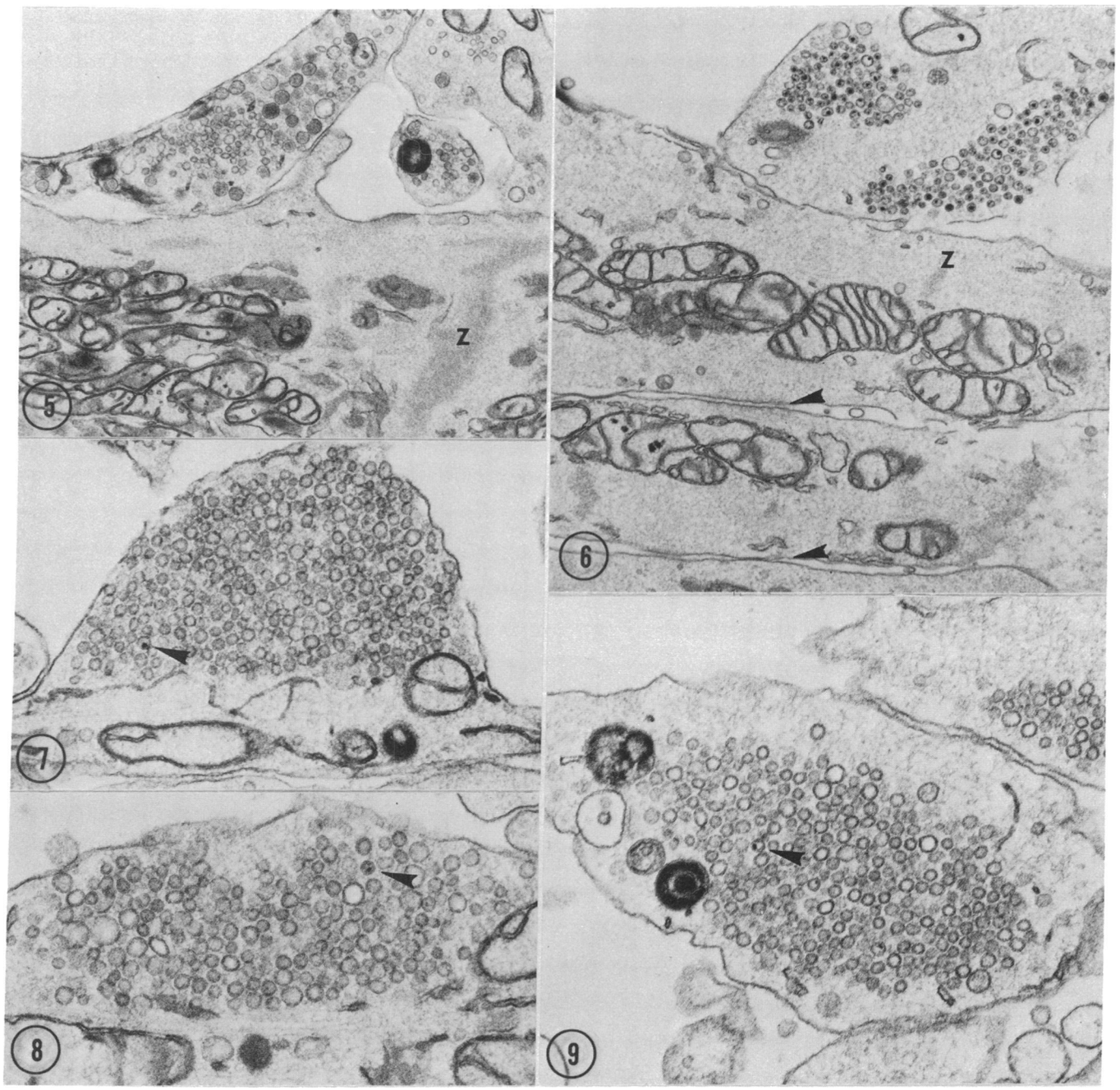
DISCUSSION

Single sympathetic neurons grown with previously dissociated heart cells in microcultures formed morphological synapses upon themselves. As described in the accompanying paper (11), post-synaptic potentials were observed at autapses on neurons that apparently secreted AcCh but not catecholamines. In mass cultures release of catecholamines occurs in the presence of depolarizing agents (3, 29, 30), and in the microcultures stimulation of the solitary putative adrenergic neurons excited heart myocytes (11). Because the autaptic endings contain many SGV, catecholamines are almost certainly released at these autapses. The most likely explanation for the lack of a post-synaptic response is that the cultured principal neurons are relatively insensitive to catecholamines, a property which was noted in mass cultures (9). The adrenergic autapses present in microculture thus provide an example of a morphological synapse that is apparently electrophysiologically silent.

Physiological evidence for chemical transmission between cultured sympathetic neurons and cardiac myocytes is presented in the accompanying paper (11). Cardiac neuromuscular junctions *in vivo* have been difficult to characterize morphologically. They have been described as either close (15–20 nm) or distant (100 nm or more), and as lacking specializations (23, 24). Similarly, ambiguous neuromuscular relationships were observed in the microcultures. Close appositions were present between axonal varicosities and myocytes, but varicosities also occurred at much greater distances from the muscle cell surfaces. There is no evidence yet to suggest whether one or both classes release effective amounts of transmitter onto myocytes. Membrane specializations were not observed at these possible points of interaction between the axon and the muscle cell in contrast to those present at neuronal autapses. Combined ultrastructural and electrophysiological reconstruction of the microcultures by using morphological markers and focal stimulation to define transmitter release sites may establish the morphological correlates of chemical transmission at neuron-cardiac muscle junctions.

Based on electrophysiological and pharmacological criteria, three classes of neurons appeared to be present in the microcultures; neurons that secreted AcCh, neurons that secreted NE, and neurons that secreted both (11). When microcultures were fixed with permanganate and examined with the electron microscope, the varicosities of neurons identified as cholinergic contained no SGV while those of neurons identified as adrenergic contained predominantly SGV. *In vivo*, the morphological dichotomy between cholinergic and adrenergic terminals has been well established in autonomically innervated tissues (12, 13, 18, 31, 32); following permanganate fixation, cholinergic endings contain only clear small synaptic vesicles while the presence of SGV is diagnostic for adrenergic endings. The ultrastructural cytochemistry of the endings found in microcultures containing a single cholinergic or a single adrenergic neuron corroborates the physiological determination of the neurotransmitter secreted. At least some of the apparently cholinergic neurons which do not secrete physiologically effective amounts of catecholamines can, however, take up and store catecholamines in SGV.

The endings of the two putative dual function neurons examined ultrastructurally contained only occasional SGV. *A priori*, one might have expected dual-function endings to contain numbers of SGV more nearly intermediate between those observed in cholinergic terminals and adrenergic terminals. There are several possible explanations for this apparent discrepancy. (*i*) The physiological recording and pharmacological manipulations used to demonstrate the secretion of both transmitters may have resulted in the depletion of NE from the synaptic vesicles. (*ii*) The electrophysiological responses of myocytes may reflect the release of NE from synaptic endings more sensitively than the SGV reflects its presence, so that apparently empty vesicles contain effective amounts of NE. (*iii*) The number of SGV present are sufficient to account for the excitation of the myocytes. *In vivo*, cardiac responses to accelerans nerve stimulation were obtained in guinea pigs after reserpine treatment that caused the loss of almost all granular material from synaptic vesicles in adrenergic terminals in the atrium (33). More putative dual-function neurons must be ex-



FIGS. 5–9. *Fig. 5.* Axonal varicosities come close to the muscle cell surface. Permanganate fixation does not preserve the myofibrils well and only the Z band (Z) is evident. From a 19-day-old microculture containing two cholinergic neurons; permanganate fixation, ×26,000. *Fig. 6.* Three myocytes are linked by intercalated disks and gap junctions (arrows). An adrenergic varicosity lies closely apposed to the muscle cell surface. From a 19-day-old microculture containing a single adrenergic neuron; 2 hr prior to fixation, this microculture was perfused for 6 min with 1.0 and 0.10 μM NE. Permanganate fixation, ×26,000. *Figs. 7* and *8.* Varicosities of a single neuron that apparently secreted both AcCh and NE contain almost exclusively clear vesicles, but a few SGV are present (arrows). Culture age: 14 days; permanganate fixation, ×38,000. *Fig. 9.* Occasional SGV are present in varicosities of a single cholinergic neuron after incubation with 50HDA. One SGV is indicated by an arrow. Culture age: 14 days; permanganate fixation, ×38,000.

amined to clarify this point. Even though uncertainties remain concerning the relative proportion of the two classes of vesicles, the presence of both small granular and clear vesicles in the varicosities of neurons which appear to secrete both AcCh and catecholamine is consistent with the physiological characterization of these neurons as dual-function.

The development of a method for growing single neurons in microcultures suitable for both microscopy and electrophysiological recording makes it possible to examine all the endings of an identified neuron, and correlate their fine structure with electrophysiologically defined neurotransmitter functions.

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