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DUAL FUNCTION DURING DEVELOPMENT OF RAT SYMPATHETIC NEURONES IN CULTURE

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SUMMARY

Many sympathetic principal neurones of the superior cervical ganglion of the newborn rat are known to be plastic with respect to the choice between norepinephrine (NE) and acetylcholine (ACh) as transmitter; when the neurones are dissociated and placed in culture, a majority of them can be shifted from an initial, immature, adrenergic state to a cholinergic state by co-culture with a variety of non-neuronal cells or by medium conditioned by such cells.

To study this transition it has been helpful to grow single neurones, each in a microculture which also contains cardiac myocytes. The transmitter status of a neurone can be assayed by recording its effect on the myocytes (adrenergic excitation, cholinergic inhibition or dual function); then a fine structural assay of the neurone based on the appearance of the synaptic vesicles can be made and correlated directly with the physiology. In this paper we report the following findings on principal neurones developing in such microcultures. (i) During the transition period, a majority of the neurones were dual in function and in vesicular appearance. (ii) The physiological effects and vesicular appearance varied from mainly adrenergic to mainly cholinergic. (iii) In preliminary attempts to follow the transition by recording at least twice from the same microculture, partial transitions were observed, always in the direction adrenergic-to-cholinergic. (iv) The transitions were not synchronous or fixed in time course even in pairs of neurones grown side by side in the same microculture.

INTRODUCTION

Normal function of an adult nervous system depends on embryological mechanisms which ensure the appropriate connexion of neurones with their target cells. It also depends on the establishment, at chemical synapses, of a match between the transmitter expressed by the presynaptic cell and the receptor(s) expressed by the postsynaptic cell. This paper is concerned with the control of the choice of transmitter in a class of nerve cells which are known to express, collectively, more than one transmitter in adult mammals: the much-studied sympathetic principal neurone. Some of these neurones secrete norepinephrine (NE) (to control cardiac muscle, smooth muscle, gland cells or brown fat) while others secrete acetylcholine (ACh) (to control certain sweat glands or blood vessels). Moreover, Hökfelt and his collaborators have recently reported (for references see Hökfelt *et al.* 1980) that some adrenergic sympathetic neurones contain somatostatin-like or enkephalin-like

immunoreactivity and some cholinergic sympathetic neurones contain VIP-like immunoreactivity (VIP: vasoactive intestinal peptide). Hökfelt and his colleagues have questioned whether these adult neurones secrete both the amine and the peptide. So far as is known, all sympathetic principal neurones are derived from a common source, the neural crest. What developmental mechanisms ensure that each such neurone ultimately adopts a transmitter or transmitters appropriate to its target cells?

This question is also of interest in other classes of nerve cells which apparently express, within the class, more than one transmitter (e.g. primary sensory neurones, enteric neurones, certain types of retinal neurones and preganglionic sympathetic neurones). The latter were, until recently, thought to be exclusively cholinergic, but Jan, Jan & Kuffler (1979) have shown that some of these neurones in frogs secrete a peptide similar or identical to luteinizing-hormone-releasing-hormone (LHRH).

Since the early 1970s the control of transmitter choice in derivatives of the neural crest has been investigated *in vivo* and in culture. An unexpected finding has been that the choice can be influenced by a variety of non-neuronal cells and that certain neurones remain plastic with respect to transmitter choice until relatively late in development. Clear evidence, *in vivo*, for adrenergic-cholinergic plasticity in populations of bird neural crest derivatives, even after migration to ganglia, has been obtained in interspecific-transplant experiments by Le Douarin and her colleagues (e.g. Le Douarin *et al.* 1978). Evidence for peptide-peptide plasticity in populations of cultured chick sensory neurones was reported by Mudge (1979). The most intensively investigated example of control of transmitter choice is the adrenergic-cholinergic plasticity displayed by individual rat sympathetic principal neurones obtained from the perinatal superior cervical ganglion (SCG). This work has recently been reviewed by Patterson (1978), O'Lague, Potter & Furshpan (1978) and Bunge (1979); one aspect is the subject of this paper.

In the SCG of the adult rat, most of the principal neurones are adrenergic, but a minority is apparently cholinergic: about 5% of the neurones exhibit heavy staining for acetylcholinesterase and no detectable catecholamine fluorescence (Yamauchi, Lever & Kemp, 1973), and in decentralized ganglia a small number of synapses of cholinergic appearance persists (e.g. Östberg *et al.* 1976). In this ganglion, the principal neurones are immature at birth; they acquire adult adrenergic metabolism and incoming and outgoing cellular connexions in the first 3–4 postnatal weeks. The normal course of differentiation of the putative cholinergic principal neurones has not yet been reported. The control of the choice between NE and ACh in principal neurones of the SCG has been extensively studied in 'mass' cultures which contain several thousand neurones dissociated from the immature neonatal ganglia. Several important findings on such cultures are given here briefly (references are given in the reviews article cited in the preceding paragraph).

If the neurones are grown in the virtual absence of any other type of cell, they display a variety of adrenergic properties. Electron microscopy shows that during the first week in culture the neurons have small granular vesicles, and biochemical assays show that the ability to synthesize and store NE differentiates strongly in the next 2–3 weeks, as *in vivo*. The neurones secrete NE, when depolarized, by a Ca^{2+}-dependent mechanism. They form synapses of adrenergic appearance on each other

presence of small, granular vesicles). These synapses have little effect on the resting postsynaptic membrane potential; the resting potential of the neurones in culture is relatively insensitive to NE, as in the SCG of the adult rat (Horn & McAfee, 1980).

In contrast, if the neurones are co-cultured with a variety of non-neuronal cells (including cells from the SCG, skeletal muscle, the heart, primary fibroblasts or serially-propagated C_6 glioma cells) cholinergic properties are expressed as well. This effect can be exerted by medium conditioned by non-neuronal cells grown in separate dishes (conditioned medium: CM); the identity of the active agent(s) is not yet known. Such mass cultures synthesize and store ACh, in addition to NE, and synapses of cholinergic appearance (absence of small, granular vesicles) are present on the neuronal cell bodies and dendrites. These synapses are excitatory, and the receptors are pharmacologically similar to nicotinic receptors in adult ganglia. In mixed cultures the neurones also form functional nicotinic junctions with skeletal myotubes and muscarinic junctions with cardiac myocytes.

It seems of interest that at each of the functional junctions (principal neurone-principal neurone; neurone-myotube; neurone-cardiac myocyte) the cultured target cell expresses an acetylcholine receptor similar, in the limited tests applied, to the receptor expressed *in vivo* at their normal junctions with preganglionic, somatic motor or parasympathetic neurones, respectively. Further, at the morphologically-adrenergic, neurone-neurone synapses there is resting insensitivity to NE, as *in vivo*. Thus, expression of these receptors appears not to be influenced by the presynaptic neurone or its transmitter choice but to be an independent property of the target cells, under these culture conditions.

Do the target cells influence the presynaptic choice of transmitter? The fact that cholinergic properties are induced by cells derived from skeletal muscle and the heart (two tissues which normally receive a cholinergic innervation) raises the question as to whether the effect is designed to ensure such an innervation. While this is possible, some facts do not fit comfortably with this view. For example, primary fibroblasts (and several other types of cells which do not receive a conventional innervation), exert this effect. Moreover, it is not yet known whether the effect of cells from skeletal and cardiac muscle originates in the myocytes or the accompanying fibroblasts or both. Further, it is not clear why the cardiac cells induce cholinergic properties in developing sympathetic neurones, the class of nerve cells which normally provides the adrenergic innervation of the heart.

The effect of the non-neuronal cells is graded in the sense that the larger the number of non-neuronal cells co-cultured with the neurones or the higher the proportion of conditioned medium fed to the neurones, the greater the synthesis of ACh relative to NE, the higher the incidence of synapses of cholinergic appearance and the higher the incidence of cholinergic interaction between pairs of neurones picked at random. Moreover, in culture conditions that bias transmitter choice most strongly there is a reciprocity in the expression of the two transmitter states, in the population of neurones; thus, when the neurones are grown in strongly 'adrenergic' conditions (e.g. L-15 Air medium or L-15 CO_2 medium which contains 20 mM-K^+) for about 4 weeks, they differentiate sharply in the adrenergic direction, and cholinergic properties are generally not detectable, but when the neurones are grown

in strongly 'cholinergic' conditions (e.g. 62% CM) for a similar period, they differentiate sharply in the cholinergic direction, and an initial adrenergic metabolism gradually disappears.

Non-neuronal cells affect transmitter choice, in these culture conditions, but not the number of neurones that survive or the rate of growth of the neurones. In these respects, the effect of the non-neuronal cells on the neurones is sharply different from that of Nerve Growth Factor which affects survival and growth, but does not influence transmitter choice and is equally active in promoting the differentiation of the two transmitter states. Since non-neuronal cells affect transmitter expression in mass cultures without affecting neuronal survival or growth, the clear implication is that at least a majority of the neurones placed in culture on the day of birth are still plastic with respect to the adrenergic-cholinergic choice and can be shifted from one state to the other.

In the work described above, cultures which contained several thousand neurones were studied. For several reasons it has been of interest to examine the transmitter status of single neurones at various times during their development. This has been done by growing the neurones in 'microcultures' which contain only a single neurone (or in some cases, several neurones). The sensitivity of current biochemical assays for synthesis and storage of ACh and NE has limited these methods to study of single neurones after they have grown to a large size, rather late in development (4–5 weeks). At this stage Reichardt & Patterson (1977) found that almost all single neurones expressed detectably only one of the two conditions with respect to synthesis and storage of transmitter: adrenergic under the most 'adrenergic' conditions and cholinergic, with increasing probability, as the conditions were made increasingly 'cholinergic'. This result led to the suggestion that the mechanism which controls transmitter choice operates in a flip-flop manner and the final status is exclusive. Furshpan *et al.* (1976) and Landis (1976) used a modified microculture procedure to study single neurones physiologically and morphologically in the period 13–18 days, when the neurones undergo the hypothesized transition from immature-and-adrenergic in the first week to cholinergic at later times. The neurones were grown on cardiac myocytes. To assay the transmitter status of a neurone, it was stimulated while recording electrically from the myocytes. In the transition period, some neurones were found to produce adrenergic excitation of the myocytes, other neurones cholinergic inhibition and still others a mixed cholinergic and adrenergic effect (dual-function). Following the physiological assay, the microcultures were fixed for electron microscopy. In cultures which contained only a single neurone, a direct correlation could be made between the function and fine structure of the junctions made by this neurone. In cultures fixed with $KMnO_4$, functionally-adrenergic junctions were found to contain predominantly small, granular vesicles, functionally-cholinergic junctions were found to contain only 'empty' vesicles and dual-function junctions were found to contain a mixture of the two types of vesicles (up to 30% granular).

The existence of dual-function neurones is consistent with adrenergic-cholinergic plasticity and clearly inconsistent with the possibility that each neurone in the SCG at birth has been given instructions which permit expression of only one transmitter. The existence of dual-function neurones implies that during the transition in trans

itter status the neurones began to incoporate cholinergic functions before the renergic functions, expressed at the outset, have been phased out. The fact that most thin sections through synapses and varicosities of dual-function neurones contain a mixture of the two types of synaptic vesicles suggests that cholinergic functions are added to existing adrenergic endings, and that the individual endings are dual in function.

Microcultures which contain a single neurone offer important advantages for study of developing transmitter functions, in addition to permitting a direct correlation of structure and function in the same neurone. In contrast to the situation in mass cultures, the processes of an isolated nerve cell are confined to a relatively small group of target cells; thus the target cells innervated by that neurone are clearly defined. Furthermore, a relatively high density of endings is made on the target cells; since these endings act in parallel, weak synaptic effects on the target cells are more likely to be detected. In addition, it is possible to record repeatedly from the same neurone over a period of many days, without ambiguity about its identity.

Dual function is a novelty in neuronal cell biology. Recent evidence that peptides are present in adrenergic and cholinergic sympathetic neurones of adult rats raises the possibility that dual function occurs more widely than simply during development in culture (see Hökfelt *et al.* 1980). Many questions remain about dual-function neurones. In this chapter we report the use of microculture procedures to investigate the variety of dual-function states in developing sympathetic principal neurones and initial attempts to follow the changes in this state, over time, in single neurones.

METHODS

Microcultures were prepared by the procedure described previously (Furshpan *et al.* 1976). Briefly, small islands to which cells could adhere were produced on an otherwise non-wetting plastic surface by drying tiny droplets of dissolved collagen about 0·5 mm in diameter. These islands were arranged in an orderly 5-by-5 array; after physiological study the position of a microculture in the array was recorded, and the same microculture could then be studied later, either physiologically or morphologically. Dissociated cells from whole neonatal rat hearts were added to the dish; they adhered to the collagen dots to form islands of confluent fibroblasts and beating myocytes. Neurones were added a few days later at a density such that some islands received only one neurone, others a small number.

The transmitter status of such a neurone was tested by impaling the neuronal cell body and the cardiac myocytes with microelectrodes. The neurone was stimulated by brief current pulses passed through the recording microelectrode, and the effect of neuronal activity was recorded simultaneously in the neurone itself and in the myocytes. Excitation of the neurone at synapses made on itself ('autapses'; cf. van der Loos & Glaser, 1972) was shown to be cholinergic by block with 50–1000 μM-hexamethonium. Inhibition of the myocytes (hyperpolarization and reduced frequency of action potentials) was shown to be cholinergic by block with $1–4 \times 10^{-7}$ M-atropine sulphate. Excitation of the myocytes (depolarization and increased frequency of action potentials) was shown to be adrenergic by block with 10^{-6} M-DL-proprano-.HCl, $2–5 \times 10^{-7}$ M-DL-alprenolol.HCl or $2–5 \times 10^{-5}$ M-DL-sotalol.HCl. These

blocking drugs were added to a steady stream of perfusion fluid; they could usually be washed in and out of the culture dish while recording continuously from the cells. Pharmacological tests of transmitter status often took longer than an hour. In many experiments several microcultures in the same dish were studied at a single session.

At the end of a recording session, the culture dish was either returned to the incubator for re-assay at a later time (in this case, sterile precautions were taken during the session) or fixed for electron microscopy. Glutaraldehyde followed by osmium was used to preserve general fine structure; potassium permanganate to reveal storage of NE in synaptic vesicles.

The composition of the perfusion fluid used in the physiological assays was usually as follows: L-15 medium 10% (v/v); NaCl 140 mM; KCl 5·4 mM; $CaCl_2$ 2·8 mM; $MgCl_2$ 0·18 mM; $NaHCO_3$ about 5 mM; NaH_2PO_4 0·56 mM; glucose 6·6 mM; choline chloride 0·07 mM; phenol red 0·03 mM; glutamine 2 mM; fetal calf serum 1–2% (v/v).

RESULTS

Fig. 1*a* is a phase micrograph of a microculture which contains only one neurone and cardiac myocytes. When such microcultures were studied during the 2nd to 7th weeks, a minority of the solitary neurones produced an apparently purely adrenergic effect on the myocytes, completely blocked by about 1 μM-propranolol or other β-adrenergic blockers; the synapses and varicosities of these neurones invariably contained numerous small, granular vesicles when fixed in $KMnO_4$ (cf. Furshpan *et al.* 1976; Landis, 1976). Another minority of the neurones produced an apparently purely cholinergic effect on the myocytes, completely blocked by about 10^{-7} M-atropine sulphate; the synapses and varicosities of the neurones contained only 'empty' synaptic vesicles. However, the majority of the neurones exhibited dual-function: cholinergic inhibition of the myocytes was followed by adrenergic excitation. The terminals of these neurones contained a mixture of 'empty' and granular synaptic vesicles, in a ratio never less than 2 to 1.

An example of dual function is shown in Fig. 2. When the neurone was stimulated with current passed through the recording microelectrode (Fig. 2*a*), the action potential was followed by an excitatory postsynaptic potential (e.p.s.p.; arrow in Fig. 2*a*) which arose at synapses made by the neurone on itself (autapses). In the case illustrated in Fig. 2, this e.p.s.p. was superimposed on a long-lasting after-hyperpolarization (duration about 70 ms). In many microcultures of comparable age or older such autaptic e.p.s.p.s were larger and sometimes led to multiple discharges (cf. Figs. 3*b* and 4*a*, *d*). The e.p.s.p.s were usually completely blocked by 0·05–1mM-hexamethonium chloride (not shown) and were clearly cholinergic. No electrical effect of secretion of NE was observed at autapses; the resting membrane potential of the cultured neurones is relatively insensitive to NE.

Fig. 2*b* shows the effect of the neurone on the cardiac myocytes, in normal, 'control' perfusion fluid. In this microculture, the myocytes did not beat spontaneously in control fluid, as was true of some other microcultues. The first downward deflexion of the lower beam was the after-hyperpolarization of a neuronal impulse (the spike was not visible with this exposure); this single impulse elicited a hyperpolarization of about 11 mV in the quiescent myocytes. A subsequent brief train

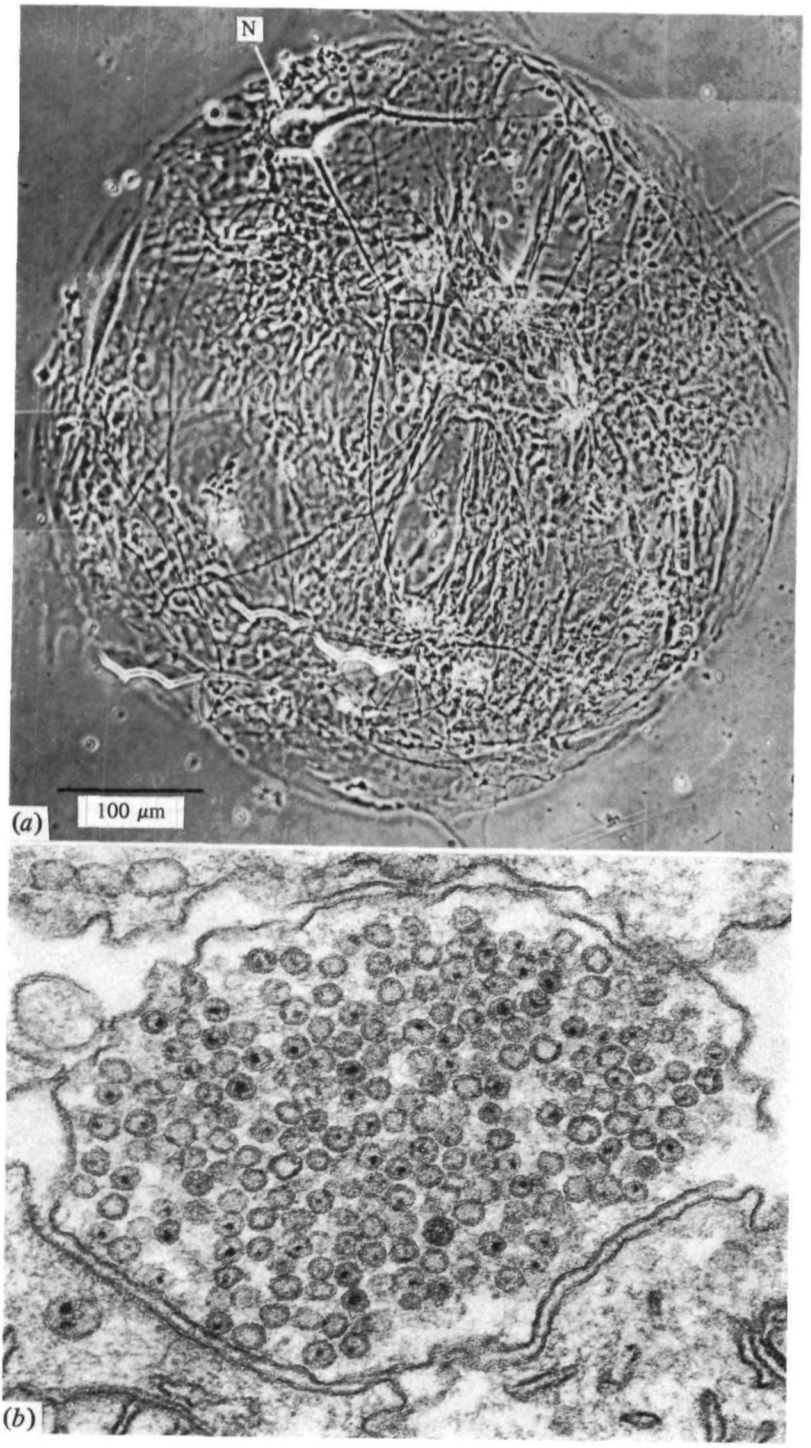

Fig. 1 *a*). Phase contrast micrograph of a living microculture containing a single sympathetic principal neurone (N; arrow) and cardiac myocytes which are not individually resolvable. Nineteen days in culture. Scale: 100 μm. (*b*) Electron micrograph of an autapse in a microculture. Before permanganate fixation, the transmitter status of the neurone in this microculture was examined; records from this experiment are shown in Fig. 2. After recording, the microculture was incubated in 10^{-5} M-5-hydroxydopamine. Approximately half the synaptic vesicles contain granular precipitate. × 75 000.

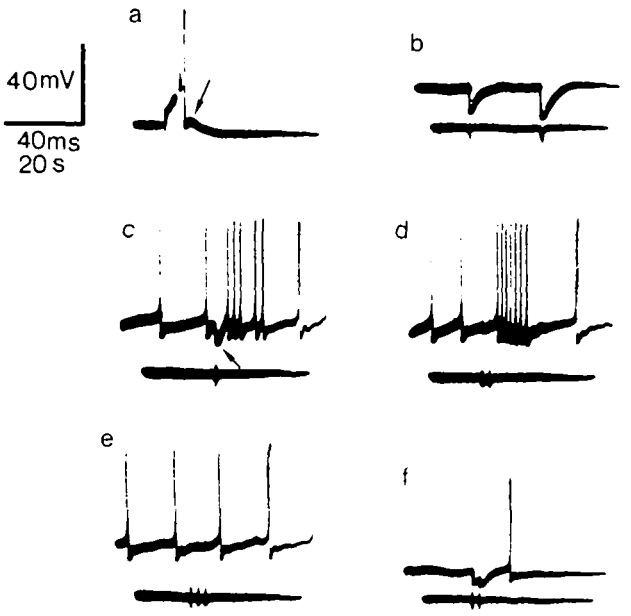

Fig. 2. An example of dual function; neurone 18 days in culture. (*a*) When the neurone was stimulated by a current pulse (not shown) passed through the recording microelectrode, a small autaptic e.p.s.p. (arrow) followed the evoked impulse. (*b*) In this and the remaining parts of the figure, the upper trace was recorded from the cardiac myocytes, and the lower trace from the neurone. The neurone was stimulated once and then with a brief train (30/s; downward deflexions of the lower trace). The second hyperpolarization of the myocytes was followed by a small slow depolarization. (*c*) The culture was perfused with atropine sulphate (10^{-7} M) for about 2 min, approximately the wash-in time of the perfusion system. The myocytes became spontaneously active (impulses on the upper trace). Now a train of neuronal impulses produced a hyperpolarization of the myocytes (arrow) followed by a rather weak excitation. (*d*) The sweep which immediately followed (*c*); the atropine now completely blocked the inhibitory effect of two brief trains of neuronal impulses, and the residual effect was excitatory. (*e*) About 10 min after start of perfusion with a mixture of atropine sulphate (10^{-7} M) and DL-propranolol.HCl (10^{-6} M); three brief trains of neuronal impulses produced virtually no effect on the myocytes. (*f*) After 20 min of perfusion with drug-free fluid, the neurone again exerted a dual effect on the myocytes. In this and subsequent figures, some cardiac action potentials were retouched for clarity. Calibrations: 40 mV, 40 ms for (*a*); 40 mV, 20 s for (*b*)–(*f*).

neuronal impulses on the same sweep produced a larger hyperpolarization followed by a slight depolarization of the myocytes. To verify that the hyperpolarization was produced by secretion of ACh, the muscarinic blocker atropine sulphate (10^{-7} M), was now added to the perfusion fluid. The drug slightly depolarized the myocytes (a common observation in these experiments), and the myocytes became spontaneously active at a low frequency. Shortly after atropine was added (Fig. 2*c*) a brief train of neuronal impulses elicited a somewhat reduced hyperpolarization of the myocytes (arrow) followed by an increased frequency of firing. About 50 s later (Fig. 2*d*), two short trains of neuronal impulses no longer hyperpolarized the myocytes; the effect was now excitatory and remained so thereafter in the presence of atropine. To verify that the excitation was produced by secretion of NE, the β-blocker propranolol (1 μM) was added to the atropine in the perfusion fluid; 10 min later (Fig. 2*e*), three brief trains of neuronal impulses produced virtually no effect on the myocytes. To remove the two blocking drugs the culture was now perfused with control fluid.

After 20 min (Fig. 2*f*), the myocytes had become quiescent, and two trains of neuronal impulses elicited a hyperpolarization followed by a single cardiac impulse; that dual function was at least partially restored.

At the end of the physiological assay, this microculture was incubated for 30 min in 10 μM-5-hydroxydopamine and then fixed in $KMnO_4$, to determine the proportion of the synaptic vesicles capable of storing this analogue of NE (cf. Tranzer & Thoenen, 1967). Fig. 1*b* shows an autapse in this microculture; 50% of the small synaptic vesicles contain dense cores, a property of adrenergic vesicles *in vivo*. Presumably, many of the clear vesicles were cholinergic. This dual fine structure, typical of junctions in such microcultures, suggests that both NE and ACh were secreted at this autapse. Although the cholinergic effect on the myocytes always preceded the adrenergic effect, the two transmitters may have been secreted simultaneously, for when a mixture of ACh and NE is applied locally to the myocytes ('puffed' from a broken micropipette), the cholinergic effect precedes the adrenergic effect.

We have recorded from over 100 dual-function neurones in culture conditions similar to that of Fig. 2, ranging in culture age from 9 to 45 days. Neurones younger than about 12 days usually produced weaker effects on themselves and on the myocytes than older neurones, doubtless at least in part because, as shown by electron microscopy, fewer junctions had been formed. In a population undergoing a transition from adrenergic to cholinergic status, the relative strength of the two effects on the myocytes might be expected to vary from predominantly adrenergic to predominantly cholinergic and in fact such variation was observed. An example of dual-function which appeared relatively more cholinergic than that of Fig. 2 is shown in Fig. 3. A single impulse in this neurone produced a pronounced hyperpolarization of the myocytes, and during occasional periods when this neurone became spontaneously active (a rare occurrence in the microcultures) the spontaneous activity of the myocytes was blocked, as shown in Fig 3a_1 and a_2. Two consecutive sweeps (duration 50 s) are shown in Fig. 3a_1 and a_2; in each, an episode of spontaneous neuronal impulses (small deflexions of lower traces) suppressed the myocyte activity. The neurone had a pronounced cholinergic autaptic effect on itself (Fig. 3*b*); after the first evoked impulse, a second impulse arose from a large e.p.s.p. In control solution, the effect of a train of impulses in the neurone was a long-lasting inhibition of the myocytes (Fig. 3*c*), followed by resumption of impulses at about the resting frequency (not shown; as in Fig. 3*f*). However, when atropine sulphate (10^{-7} M) was added to the perfusion fluid, and the cholinergic effect on the myocytes was blocked Fig. 3*d*), a previously-hidden excitatory effect on the myocytes was revealed. The addition of 10^{-6} M-propranolol to the perfusion fluid blocked this adrenergic effect (Fig. 3*e*). Perfusion with control solution for about 45 min restored the initial condition (Fig. 3*f*). Thus, ACh was secreted in an amount sufficient to make this neurone effectively mono-functional in control fluid, in spite of the secretion of NE. In correspondence with this predominantly-cholinergic function, the fine structure of the junctions made by this neurone was predominantly cholinergic (not shown; small granular vesicles comprised only less than 1% of the vesicle population after fixation in $KMnO_4$; no prior incubation in 5-hydroxydopamine).

While variations in dual function, like those illustrated in Figs. 2 and 3, might be expected of neurones in transition, interpretation of such tests of neuronal sta

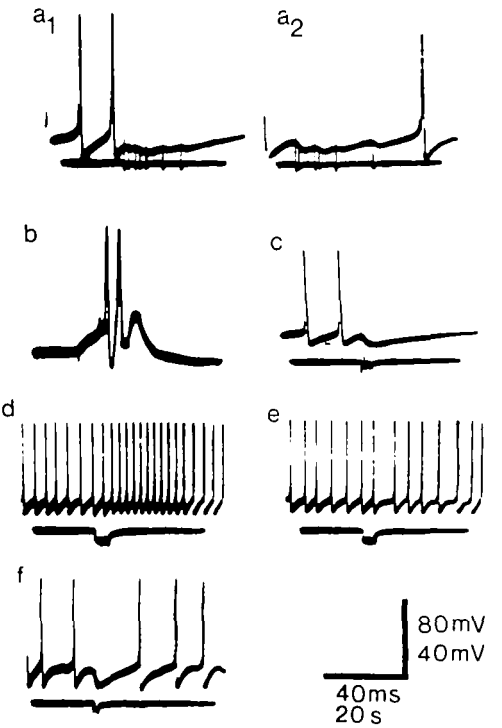

Fig. 3. Relatively-more-cholinergic dual function; neurone 24 days in culture. (a_1 and a_2) Two successive sweeps which show spontaneous impulses in the neurone (deflexions of the lower trace) and their effect on the activity of the myocytes (upper trace). Each neuronal impulse was accompanied by a hyperpolarization of the myocytes. (*b*) An evoked impulse in the neurone was followed by a large autaptic e.p.s.p. which produced a second impulse. (*c*) A brief train of impulses in the neurone (10/s) produced a prolonged inhibition of the myocytes; the myocytes began to beat again on the next sweep at about the initial frequency (not shown, but similar to effect in (*f*). (*d*) Atropine sulphate (10^{-7} M) blocked the inhibitory effect and unmasked an excitatory effect of nerve stimulation. (*e*) The simultaneous presence of atropine sulphate (2×10^{-7} M) and DL-propranolol.HCl (10^{-8} M) rendered neuronal activity almost completely ineffectual against the myocytes. (*f*) Return to drug-free fluid for about 45 min restored the initial, mono-functional effect of neuronal activity. Calibrations: 40 mV, 20 s for (a_1) and (a_2); 40 mV, 40 ms for (*b*) and (*f*); 80 mV, 20 s for (*c*)–(*e*).

is complicated by the fact that the effect of neuronal activity on the myocytes depended not only on the amounts of NE and ACh secreted by the neurone but also on the postsynaptic sensitivity of the myocytes to the two transmitters. Did the microcultures of Figs. 2 and 3 differ, not in the relative amounts of NE and ACh secreted by the two neurones but in the relative sensitivity of the myocytes to NE and ACh? No final conclusion can be drawn in these two cases, because no independent tests were made of the responsiveness of the myocytes to the two transmitters. However, for the following reasons we assume that differences in neuronal effects on the myocytes were attributable mainly to differences in transmitter status: (i) as mentioned above, differences in neuronal effect were correlated with differences in neuronal fine structure (proportion of small granular vesicles); (ii) differences in neuronal effect were seen in adjoining microcultures, which received myocytes from the same cell suspension, and in pairs of neurones grown in the same microculture assayed on the same population of myocytes (this point is illustrated below).

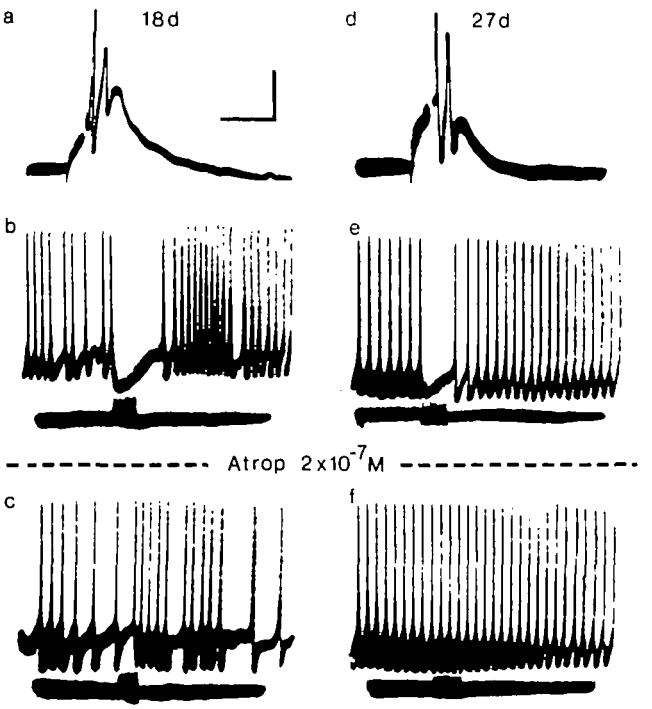

Fig. 4. Repeated assay of transmitter status in a neurone: (*a*), (*b*) and (*c*) on day 18; (*d*), (*e*) and (*f*) on day 27. (*a*) and (*d*) The neurone produced a pronounced autaptic excitatory effect on itself on both days. (*b*) and (*e*) A train of impulses in the neurone (10/s) produced a dual effect on the myocytes on day 18 but not on day 27. (*c*) and (*f*) Atropine sulphate (2×10^{-7} M) blocked the inhibitory effect on the myocytes on both days; a residual excitatory effect was present on day 18 but not on day 27. Thus on day 27 this neurone was mono-functional and apparently secreted only ACh. Calibrations: 20 ms, 20 mV for (*a*, *d*); 10 s, 10 mV for (*b*, *c*); 10 s, 20 mV for (*e*, *f*).

Serial assays of transmitter status in the same neurone

In the work described above, the transmitter status of the cultured neurones was assayed at various ages, in mass cultures or microcultures, and the transition from adrenergic to cholinergic status was deduced from the observations collectively. Repeated assays on the same neurone in a microculture would provide direct evidence for the adrenergic-cholinergic transition and permit investigation of several other questions. Our preliminary attempts to do this have often failed because the culture became contaminated during the recording session or because the neurone died between assays. However, in 16 cases we have recorded at least twice from the same neurone (maximum: four assays in a 32-day period).

No neurone had been followed throughout the whole postulated transition from purely adrenergic to purely cholinergic, but partial transitions were observed, always in the direction adrenergic-to-cholinergic. Three neurones, apparently purely adrenergic at the outset, were clearly dual in function when last assayed (the transitions occurred between days 11 and 23, days 13 and 24 and days 13 and 45, respectively). Two neurones, dual in function when first assayed were apparently purely cholinergic when last assayed. The first underwent the transition betwe[illegible]

days 13 and 45. The second case, illustrated in Fig. 4, underwent the transition between days 18 and 27. On day 18, the neurone exerted a pronounced autaptic effect on itself (Fig. 4*a*) and a dual effect on the cardiac myocytes (Fig. 4*b*). The initial inhibitory effect was was blocked by atropine sulphate (2×10^{-7} M) (Fig. 4*c*) and a rather weak excitatory effect remained; this was blocked by 5×10^{-7} M)-alprenolol (not shown). On day 27, this neurone was examined again. It still produced a pronounced autaptic effect on itself (Fig. 4d). The effect on the myocytes was somewhat weaker than in the first test, but it was apparently purely inhibitory (Fig. 4*e*). After block by atropine (2×10^{-7} M; Fig. 4*f*), no excitatory effect was visible. The neurone, clearly dual in function on day 18, now appeared to be mono-functional. The neurone was re-examined on day 38 and again appeared to be purely cholinergic (not shown).

In four of the remaining 11 cases in which neurones were examined at least twice, there was a change from dual function to relatively-more-cholinergic dual function. In the other cases there was no obvious change in dual function over periods of 2–15 days.

Heterogeneity of transmitter status

It was mentioned above that neurones on adjoining microcultures in the same dish or even in the same microculture often produced markedly different effects on the myocytes. An example of this heterogeneity is shown in Fig. 5. The cell bodies of neurones N_1 and N_2 lay side by side in a microculture 12 days old (not shown). From day 4 to day 12 the medium contained 20 mM-K^+ in an attempt to reduce the responsiveness of the neurones to cholinergic induction by the co-cultured heart cells (see Walicke, Campenot & Patterson, 1977). When stimulated by an intracellular pulse of current, neither neurone produced a detectable cholinergic effect on itself (Fig. 5*a*, *d*). However, activity in N_1 produced a small cholinergic e.p.s.p. in N_2 (not shown); the e.p.s.p. was completely eliminated by perfusion with 2×10^{-4} M-hexamethonium (C_6). N_2 produced no visible synaptic effect on N_1 (not shown). In the tests of the effects of N_1 and N_2 on the myocytes, 2×10^{-4} M-C_6 was present in the perfusion fluid to ensure that N_1 did not act via nicotinic synapses on N_2. Fig. 5*b* shows that a train of impulses in N_1 (deflexion on lower trace) produced a hyperpolarization of the myocytes followed by an increased frequency of action potentials (upper trace). When 10^{-7} M-atropine sulphate (Atrop) was added to the perfusion fluid the hyperpolarization was eliminated, and the excitation remained. Thus, N_1 was dual in function. When N_2 was stimulated repetitively (Fig. 5*e*; deflexion on lower trace), the effect on the myocytes was apparently purely excitatory (upper trace of Fig. 5*e*); addition of 3×10^{-7} M-alprenolol (Alpren) to the perfusion fluid reduced the excitatory effect (Fig. 5*f*) without unmasking a cholinergic hyperpolarization. Thus, N_2 appeared to be mono-functional and adrenergic. In spite of the similarity of their culture environment, these two neurones were distinctly different in function when assayed on the same population of myocytes.

In another case in which 2 neurones in the same microculture were assayed 4 times, one of the neurones progressed from dual function to apparently purely cholinergic while the other neurone progressed from apparently purely adrenergic to dual function. As late as 45 days after being placed in culture, the neurones were distinctly different in transmitter status.

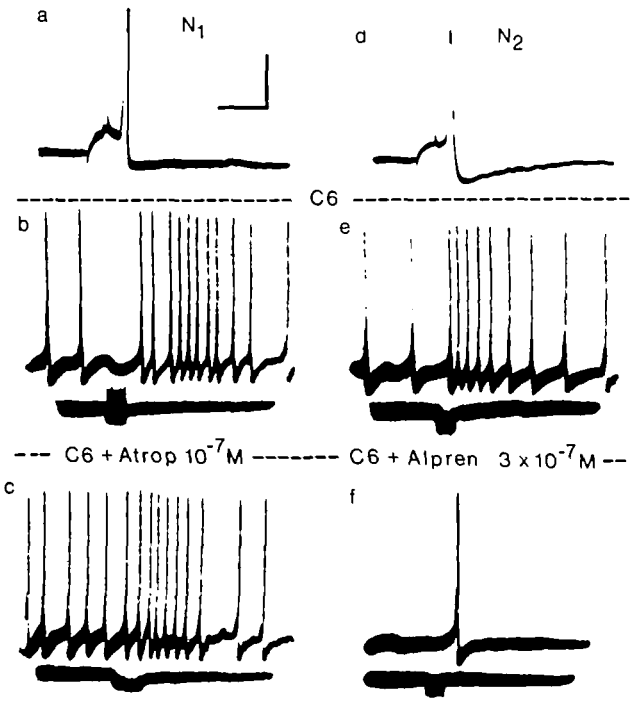

Fig. 5. The cell bodies of neurones N_1 and N_2 lay side by side in a microculture, 12-days old, fed with medium containing 20 mM-K^+ from day 4 to day 12. (*a*) and (*d*) Neither neurone produced an autaptic e.p.s.p. in itself, (*b*) and (*e*) After the culture was perfused with hexamethonium chloride (C_6; 2×10^{-4} M), N_1 produced a dual effect on the myocytes, but N_2 produced only an excitatory effect. (*c*) and (*f*) The inhibitory effect of N_1 on the myocytes was blocked by 10^{-7} M atropine sulphate, leaving an excitatory effect; the excitatory effect of N_2 was almost blocked by DL-alprenolol.HCl (3×10^{-7} M) without unmasking an inhibitory effect. Thus N_1 was dual in function at 12 days, and N_2 was mono-functional. Calibrations: 20 mV, 20 ms for (*a*) and (*d*); 20 mV, 10 s for (*b*) and (*c*); 20 mV and 4 s for (*e*) and (*f*).

There was additional heterogeneity among the neurones with respect to the relative strengths of their actions on themselves, at autapses, and on the cardiac myocytes. Most neurones which produced a pronounced cholinergic effect on the myocytes. also exerted a pronounced cholinergic effect on themselves, but not all neurones did so. The neurones of Figs. 2 and 3, 18 days and 24 days in culture respectively, both produced a large hyperpolarization of the myocytes with a single impulse (Figs. 2*b* and 3a_1, a_2) but the autaptic effect was much weaker in the first case than in the second (Fig. 2*a vs.* Fig. 3*b*).

DISCUSSION

A large body of indirect evidence reviewed on the *Introduction* to this paper led to the conclusion that many principal neurones of the rat SCG at birth can be shifted from an initial, immature, adrenergic condition to a cholinergic condition by non-neuronal cells. In this paper we report preliminary direct evidence for this plasticity in neurones whose transmitter status was followed over time in microcultures containing cardiac cells; all the observed changes in status were in the expected direction, adrenergic to cholinergic.

We also report marked heterogeneity in the *rate* of the transition in the 16 neurones followed over time. By itself, this observation is not conclusive because of the possibility of damage to the neurones by the repeated impalements. However, additional evidence for heterogeneity of the rate of transition comes from the pairs of neurones grown in the same microcultures and assayed only once; the neurones in such pairs often had different transmitter status even though they were grown and assayed on the same myocytes and not previously impaled. These observations raise the possibility that there is intrinsic hereogeneity among the neurones, with regard to this property. Some intrinsic heterogeneity might be expected from the fact that the neuroblasts of the rat SCG are produced by cell divisions scattered over at least a 7-day perinatal period (Hendry, 1977) and from the fact that the neurones are destined *in vivo* to innervate diverse target cells.

Is the *rate* of the transition also dependent on the intensity of the influence of the non-neuronal cells? Two pieces of evidence, from mass cultures, are consistent with this view. (i) Patterson & Chun (1977) grew mass cultures for 20 days in six different concentrations of medium previously conditions by confluent cardiac cells (CM) and found that the higher the concentration of CM, the greater the ACh synthesis and the lower the NE synthesis. In cultures fed the highest concentration of CM, 62%, NE metabolism was reduced to about 4% of the control value (cultures fed no CM). Since a majority of the neurones respond to CM, the graded status of the mass cultures at day 20 raises the possibility that individual neurones underwent the transition at different rates in the different concentrations of CM. (ii) Landis (1980) measured the loss of small, granular vesicles and rise in clear, possibly-cholinergic vesicles in mass cultures grown in 'cholinergic' conditions of two intensities; in a weakly 'cholinergic' medium (L-15 CO_2) and in 50% CM. She found that the proportion of small, granular vesicles was already lower at 7 days in 50% CM than in the absence of CM, and fell faster during the next 2 weeks. Thus the transition was more extensive at each time point in the more 'cholinergic' environment. These observations are strongly suggestive, but further work is required to establish whether the mechanism which controls transmitter choice has a variable time course.

A point of obvious interest is how long plasticity of transmitter choice persists after birth, *in vivo*. Wakshull, Johnson & Burton (1979) recently reported that some neurones dissociated from the rat SCG and placed in culture as late as 10 weeks after birth from cholinergic junctions with each other and with skeletal myotubes. It is not known whether these neurones were cholinergic when isolated from the ganglion, or dual in function, or adrenergic but still plastic; the physiological assay of these interesting cultured neurones would not have disclosed dual function. Among other important questions still to be investigated are the following: (i) whether non-neuronal cells affect transmitter choice by the same mechanism *in vivo* and *in vitro*; (ii) whether control of transmitter choice is the same in neurones that innervate such diverse tissues as cardiac muscle (responds oppositely to NE and ACh) and the nictitating membrane (responds similarly to NE and ACh); (iii) how expression of peptides (somatostatin-like, enkephalin-like or VIP-like) is controlled.

This paper began with the point that in normal development of chemical junctions a match is established between transmitter and receptor(s). A start has been made in investigating the control of transmitter choice, but the control of receptor choice is

largely unexplored. The strongest suggestion known to us that a novel innervation can alter the receptors expressed by a target cell *in vivo* is the report by Landmesser (1972) that neuromuscular junctions formed by vagal cholinergic axons in frog skeletal muscles are more sensitive to hexamethonium than normal neuromuscular junctions. Not all frog preganglionic axons are similarly effective (Grinnell & Rheuben, 1979). Thus, present understanding of the normal development of chemical synapses with respect to the match between transmitter and receptor is fragmentary.

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