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CELL INTERACTIONS IN NERVE AND MUSCLE CELL CULTURES

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The neurotransmitter synthesized by a given class of neurones is subject to modification and, indeed, a qualitative switchover in transmitter biochemistry recently has been demonstrated (Furshpan, Potter & Landis, 1980; Walicke, Campenot & Patterson, 1977). In conjunction with the specification of transmitter biosynthesis that becomes established in a given neurone, a complementary specification of appropriate receptor production is required in any cell functionally post-synaptic to that neurone. An additional requirement of peculiar force in the nervous system has to do with the spatial organization of the receptors in the surface membrane of the post-synaptic cell once the receptors are synthesized. Inappropriately distributed receptors are useless receptors. The perfect registration of a variety of types of presynaptic release sites with high post-synaptic concentrations of appropriate receptors constitutes one of the outstanding features of nervous-system organization that must be accounted for. We report some experiments directed toward understanding the cell biology of regulation of receptor distribution over the surface membrane of muscle cells.

Functional synaptic connexions are formed quite early in development and the stability and maturation of synaptic networks is contingent on a number of factors. One interesting contingency is that related to the functional activity of developing networks. Do only those networks survive and mature which are activated by stimuli impinging from the environment? (Wiesel & Hubel, 1963). Put more simply, are action potentials and synaptic activity essential for neuronal maturation? We address this question in cell culture systems from the mammalian central nervous system.

I. NEURONAL REGULATION OF MUSCLE ACETYLCHOLINE RECEPTOR TOPOGRAPHY AND STABILITY

The neuromuscular junction has received a large proportion of the research on the development of post-synaptic specializations responsible for synaptic transmission. A number of features distinguish junctional from extrajunctional acetylcholinc receptors (AChR). Two of these distinguishing characteristics would seem to be particularly relevant to mechanisms of synapse formation:

(1) *Topographic distribution of the AChR.*

The distribution of AChR on innervated muscle cells is non-random (for reviews, see Changeux, 1979; Fambrough, 1979). The concentration of AChR at the tips of

the subsynaptic folds is estimated to be at least two orders of magnitude higher than the concentration of the AChR located elsewhere on the muscle membrane. Since the insertion of newly synthesized AChR into the membrane is a largely topographically random process, developing myotubes have randomly dispersed AChR which, at approximately the time of innervation, begin to concentrate near the tips of ingrowing neurites. There are at least three features of the topographic differentiation of muscle cells which require a molecular explanation:

(*a*) *The aggregation of AChR.* What are the forces which produce the non-random distribution of AChR in the adult innervated muscle cell?

(*b*) *The localization of AChR at endplates.* What factors determine the juxtaposition of the AChR aggregates with the release sites in the nerve endings of spinal cord motorneurones?

(*c*) *The lateral stabilization of the AChR.* What are the lateral constraints on the mobility of the muscle cell AChR?

(2) *Metabolic stability of the junctional AChR*

The total number of AChR on the muscle cell membrane results from the interplay of the processes of acetylcholine receptor synthesis and insertion into the membrane, on the one hand, and the rate of AChR degradation, on the other hand. A large literature has developed on the interplay and importance of these two aspects and the extent to which each is regulated by neuronal innervation. It is clear that innervation of normal muscle decreases the rate of synthesis of the muscle AChR. In that direct electrical stimulation can, in large part, mimic the effect of innervation on AChR synthesis, a major neuronal control mechanism is the presynaptic release of acetylcholine and its activation of muscle, involving ionic currents or some other aspect of excitation–contraction coupling in muscle cells. The extent to which the effects of released acetylcholine can explain the change in AChR synthesis with innervation, or whether some additional neuronal factor is required, remains an active area of research. The neuronal control of AChR degradation is less well understood. It is known that the rate of degradation of junctional AChR is considerably slower than that of the extra-junctional AChR, and that junctional AChR retain this metabolic stability following denervation.

We will here discuss that portion of our work which relates to the two general phenomena outlined above. We have found that some neuronal cells produce factors which alter the topography and metabolic stability of the AChR of cultured myotubes. We have partially characterized one neuronal factor, and have evidence that neuronal factors modulate the state of aggregation and the stability of the AChR of myotubes by altering the relationship of AChR to the myotube cytoskeleton.

(A) *AChR aggregation factor*

The earliest topographic mapping of the AChR on cultured myotubes determined that in aneural cultures, AChR was found to be diffusely distributed over the entire myotube membrane, but that in addition, there were AChR aggregates, of some microns in diameter, at which the concentration of AChR was at least 10 times higher than in the areas of diffuse distribution (Fischbach & Cohen, 1973; Vogel, Sytkowski & Nirenberg, 1972; Sytkowski *et al.* 1973). This topographic inhomogeneity of th

AChR raised the question as to whether in synaptic development, the juxtaposition of pre- and post-synaptic elements was produced by the innervating nerve making preferential contact with the existing aggregations of AChR or whether the neurone induced the local accumulation of AChR in the vicinity of its contact with the myotube. At the time these mapping studies were being carried out, it was reported that cells of the continuous neuronal cell line ClA, when cultured with myotubes of the continuous muscle cell line L6, produced local accumulations of AChR sensitivity in areas of contact (Harris *et al.* 1971; Steinbach *et al.* 1973). These original studies demonstrated two important features of this receptor localization: it is not dependent on acetylcholine released from the presynaptic cell and occurs even when the AChR binding sites are occupied by cholinergic ligands. Subsequent studies have confirmed this original observation using pre- and post-synaptic cells derived from embryonic tissues, and have demonstrated that the modulation of AChR topography at least accompanies, even if it is not essential for the formation of functional cholinergic synapses (Fischbach *et al.* 1978). Elegant studies employing the fluorescent labelling of the AChR demonstrated in addition that when cultured myotubes are innervated by neurones, the outgrowing neurites could produce local aggregations of AChR where they touched the myotube, by initiating the motion of AChR in the plane of the myotube membrane (Anderson & Cohen, 1977; Anderson, Cohen & Zorychta, 1977). Because this polarized redistribution of some of the myotube AChR occurs within hours of its contact by a neurite, the phenomenon is an interesting point of departure from which to study the early steps in neurone muscle recognition, and early events in myotube membrane differentiation.

The presynaptic neurones used in our studies are from a continuous cholinergic neuronal cell line NG108-15, which form functional cholinergic synapses when co-cultured with myotubes derived from embryonic tissue (Nelson, Christian & Nirenberg, 1976), or a continuous myogenic cell line (Christian *et al.* 1977). When co-cultured for some days with myotubes, NG108-15 cells increase by three- to four-fold the number of AChR aggregates found on myotubes cultured alone (Christian *et al.* 1978). It was found that tissue culture medium in contact with the NG108-15 cells alone (here called neuronal conditioned medium, or NCM), when added to muscle cell cultures, produces within 1 day the same increase in the number of myotube AChR aggregates as found in co-cultures of the two cell types. More recently, it has been found that concentrated NCM produces a fourfold increase in AChR aggregates within 2 h (Prives *et al.* 1980). After a 1-day treatment with NCM, the total number of myotube AChR is increased less than 20%. The production of AChR aggregates by NCM is unaffected during this time period by the presence of cycloheximide. Thus, although the total level of AChR is slightly affected, probably due to the effect of NCM on the rate of degradation of AChR (see below), NCM induced AChR aggregation is not dependent on the ongoing synthesis and insertion of AChR into the myotube membrane, nor is it dependent on the synthesis of additional proteins during the course of its action. Moreover, the neuronal induced increase in AChR aggregates is the same if the AChR are labelled before or after NCM is added to the myotube cultures. Therefore, NCM produces an increased number of AChR aggregates by inducing the topographic redistribution of AChR in the plane of the myotube membrane.

The morphology of NCM induced AChR aggregates differs somewhat from the appearance of the endogenous aggregates found on cultured myotubes in the absence of neuronal influence. The endogenous aggregates are often quite large, and located on the surface of the myotube next to the surface of the culture dish, and are often at the points of contact of the myotube with the culture dish. The endogenous AChR aggregates often have a striped appearance, suggestive of the distribution of integral membrane proteins on other cell types which are associated with cytoplasmic stress fibres. When 7-day-old rat muscle cultures are treated for 1 day with NCM, the induced AChR aggregates are found on the top surface or on the edges of myotubes, and appear less well organized than endogenous AChR aggregates.

Although a wide variety of cell types have not been examined, AChR aggregation activity has to date only been detected in medium conditioned by neuronal cells. The NG108-15 cell is a somatic cell hybrid of a N18TG-2 neuroblastoma and a C6BU-1 glioma cell. Medium conditioned by cells of the neuroblastoma cell line have as high a titre of AChR aggregation activity as hybrid cells, whereas medium conditioned by cells of the glioma parent clone (here called Glial Conditioned Medium or GCM), have no detectable aggregation activity. Medium conditioned by cells of other non-neuronal cell lines are without AChR aggregation activity.

AChR aggregation activity can be extracted from the soluble cytoplasmic fraction of NG108-15 cells, but not from C6BU-1 glioma cells (Bauer *et al.* 1979; Bauer *et al.* in press). AChR aggregation activity with a high specific activity is extracted from embryonic rat brain but no detectable activity is found in adult rat brain. No activity is found in liver tissue. Therefore, of the cell types and tissues studied, it is tentatively concluded that only developing neurones contain and release AChR aggregation activity.

A partial physical and biochemical characterization of the AChR aggregation factor indicates that it is a large protein. It is heat-labile and degraded by proteolytic enzymes. Activity of conditioned medium is retained by dialysis membranes and ultrafiltration membranes having a nominal molecular weight exclusion of 100000 daltons. AChR aggregation activity elutes from various molecular sizing columns generally as one peak in the range above 150000 daltons. It is of some interest that on molecular sizing columns, fractions in the molecular weight range below 100000 daltons have occasionally shown an inhibitory effect on the number of AChR aggregates. Isoelectric focusing of NCM yields one peak of AChR aggregation activity with an isoelectric point of approximately 4·5. Similar molecular sizing and isoelectric focusing experiments using NG108-15 cell cytoplasmic extracts, or extracts of embryonic rat brain yield an AChR aggregation factor with similar molecular properties. (Bauer *et al.* 1979; Bauer *et al.* in press).

This factor has been partially purified on the basis of its ability to quickly induce the topographic redistribution of AChR on cultured rat muscle cells. It remains to be determined what effect this protein has on other aspects of muscle cell membrane topography and myotube differentiation. We find its mode of action and physical properties sufficiently different than other reported neuronal factors to warrant the conclusion that nerve cells produce a number of different factors having inductive effects on muscle cells. To date, the best characterized factor produced by neuronal tissue which has an effect on muscle cells is a protein of approximately 85000 daltons extracted from nerve or brain, which has a long-term effect on the morphology and

protein synthesis of cultured chick myotubes (Markelonis & Oh, 1979). A peptide, released into the medium by cultured neurones and also extractable from nerve or brain, has a long-term effect on the total number of AChR in cultured chick myotubes, as well as increasing the number of AChR aggregates (Jessel, Siegel & Fischbach, 1979) A peptide of similar size, extractable from mouse spinal cord or motor nerve, reverses the loss of TTX sensitivity of denervated muscle cells (Kuromi, Gonoi & Hasegawa, 1979). Both on the basis of its physical properties and the nature and time course of its effect on muscle cells, it is highly probable that the AChR aggregation factor is a different molecule than these other factors. The one factor to which the AChR aggregation factor may be akin is a protein of molecular weight greater than 100000 daltons, extracted from nerve or brain, which increases the total number of AChR receptors as well as inducing the aggregation of AChR receptors on cultured L6 myotubes (Podleski *et al.* 1978). The role of these factors in synaptogenesis remains to be determined.

(B) *Mode of action of NCM: constraints on AChR lateral mobility*

The non-random distribution of the myotube AChR, even before treatment with NCM, suggests that the myotube has an endogenous system which constrains the lateral mobility of the AChR. This mobility can be assessed by labelling myotube AChR with fluorescent α-bungarotoxin (α-btx), irreversibly bleaching the fluorescent probe in a small spot of membrane and measuring the rate of recovery of fluorescence as unbleached toxin-AChR complexes diffuse into the bleached spot. The method yields the fraction of the total AChR which are mobile in the membrane, as well as a two-dimensional diffusion constant for the mobile receptors. The entire population of AChR in the endogenous AChR aggregates of cultured myotubes is found to be immobile, as is the junctional AChR at the neuromuscular junction (Axelrod *et al.* 1976). In addition, approximately 40% of the diffusely and apparently randomly distributed AChR on cultured myotubes is also found to be laterally immobile. The mobility of the mobile fraction of diffuse AChR is approximately 1×10^{-10}, $cm^2\ sec^{-1}$, at least one order of magnitude lower than diffusion constants for the lipids of the cell membrane. This disparity between the lipid and the integral membrane protein mobilities in the plasma membrane is common to other receptors in various types of cells (Nicolson *et al.* 1977). A number of proposed mechanisms can account for this lateral immobility as well as for the non-random distribution of receptors (Nicolson, 1976). These include the direct protein–protein interactions of integral membrane proteins, the interaction with peripheral membrane proteins or the interaction with membrane associated proteins, most probably involving cell cytoskeletal elements. In the case of the myotube AChR there are two degrees of restraint: one which gives the mobile AChR a lower diffusion constant than the lipids, and at least one other which immobilizes all the aggregated AChR and a portion of the diffusely distributed AChR.

Because NCM induces the aggregation of a portion of the diffusely distributed AChR, it was of interest to determine what effect this material had on the lateral mobility of the diffusely distributed AChR. Although glial conditioned medium had no effect on the mobile fraction or diffusion constant of cultured myotube AChR, within 8 h of the addition of NCM, the mobile fraction of diffusely distributed AChR

was reduced from 0·6 to approximately 0·35 (Axelrod *et al.*, submitted for publication). The diffusion constant of the remaining mobile fraction was not different from control values. Thus, during the time that NCM is shifting a population of diffuse AChR into aggregates, it also immobilizes up to 50% of the mobile AChR in diffuse areas. The AChR of rat myotubes can thus exist in two states with respect to both aggregation and lateral mobility, and NCM treatment initiates global dynamic state transitions.

Another parameter by which junctional and extrajunctional AChR differ is the rate of degradation; extra-junctional AChR have a half-life of approximately 24 h, whereas the junctional AChR are degraded more slowly. It was of interest, therefore, to measure the rate of degradation of AChR on rat myotubes, and to determine whether two populations of AChR could be distinguished by differing rates of degradation. As with other degradation studies on cultured myotubes (see Fambrough, 1979), the rate of AChR degradation on rat myotubes, observed for as long as 36 h, was adequately fit by one exponential having a half-time of approximately 22 h (Hasegawa, Bauer & Christian, in preparation). As observed in cultured chick myotubes (Prives *et al.* 1979), the glycoprotein cross-linker Concanavalin-A increased the half-time approximately two-fold, whereas anti-AChR antibodies decreased the half-time approximately two-fold. In addition, it was found that NCM decreases the rate of AChR degradation. Rat myotube cultures were treated for 24 h with NCM, labelled with radioactive α-btx and the rate of release of radioactivity from the myotube cultures was followed during continued treatment with NCM. The rate of AChR degradation was again adequately fit by one exponential, but with a half-time that was 40% greater than found in myotube cultures not treated with NCM. Additional experimentation is required to determine whether the aggregated receptors (or laterally immobile AChR) are degraded at a different rate than the diffuse AChR, or whether NCM down-regulates a non-specific mechanism which internalizes and degrades all AChR.

The global effects of NCM on myotube AChR aggregation, lateral mobility, and degradation rate can be explained by a number of proposed mechanisms which constrain the random diffusion of integral membrane proteins. One mechanism, which initiates the patching or capping of integral membrane proteins in a variety of cell types, is the direct crosslinking of integral membrane proteins by multivalent ligands, such as antibodies or lectins (dePetris, 1977). Since it is known that the AChR is a glycoprotein, it is possible that NCM contains a lectin with specificity for the AChR, and hence can be displaced by sugars. However, various sugars at concentrations as high as 50 mM have no effect on the NCM induced aggregation of AChR (Table 1). Glucosamine or galactosamine, at 50 mM, partially inhibited the NCM induced aggregation, but was without inhibitory effects at lower concentrations (Christian, Jacques, Bauer & Daniels, in preparation).

The direct crosslinking by a multivalent ligand found in NCM seems insufficient to explain the aggregation of AChR or the increase in metabolic stability of the AChR. Although cross-linking of myotube membrane glycoproteins (including AChR) by Concanavalin-A decreases the rate of AChR degradation, it does not induce the aggregation of AChR. The specific crosslinking of AChR by antibodies directed against the AChR does not induce receptor aggregation in rat myotubes, and increases the rate of degradation of AChR. There is thus no direct evidence to suggest that cross-linkag

Table 1. *Inhibition of acetylcholine receptor aggregation*

(Myotube cultures 7–10 days old were labelled with rhodamine α-btx and incubated in Dulbecco's Modified Eagles Medium, containing 2 mg/ml bovine serum albumin and 0·5 μg/ml tetrodotoxin (control), or control medium containing redissolved lyophilized NG108-15 cell conditioned medium equivalent to a 25-fold concentration of the initial conditioned medium (NG108-15 CM). Compounds at the indicated concentrations were dissolved in the control medium or NCM before the media were added to the myotube cultures. After 4 h at 37 °C the myotube cultures were fixed and the number of AChR aggregates per myotube was determined as previously described (Christian *et al.* 1978).)

		Aggregates/myotube	
Inhibitor	Concentration	Control	NG108-15 CM
—	—	1·09	1·93
Glu-NH_2	50×10^{-3}	1·13	1·31
Gal-NH_2	50×10^{-3}	0·72	1·16
Methylamine	5×10^{-2}	0·76	0·89

Sugars with no effect at 50×10^{-3} M: Mannose, N-Ac-glucosamine, N-Ac-galactosamine, methyl galactose, fucose, melibiose, cellobiose, galactose, lactose.

by multivalent ligand initiates the events which produce AChR immobilization and aggregation.

There is reason to think that the patches of aggregated AChR are not held together by multivalent cross-linkers. First, large AChR patches have a microstructure composed of associated clusters of AChR, and multivalent ligands cannot account for this loose association, nor the polarized motion of AChR clusters in the membrane which produced it. Secondly, in myotubes treated with the metabolic inhibitor sodium azide, the AChR diffuse away from aggregates, which reform when the inhibitor is removed (Bloch, 1979). Such reversal and reformation of AChR aggregates cannot be explained by the displacement of a multivalent ligand.

Another mechanism which more probably accounts for both the AChR immobility and aggregation is the trans-membrane control of the receptor by cytoplasmic elements. We tested a number of agents which have their primary effects on the cell cytoskeleton for their efficacy in inhibiting the AChR aggregation induced by NCM. Colchicine at 10^{-6} M completely blocked the aggregation of AChR by NCM, while cytochalasin B at concentrations as high as 10^{-4} M had little effect on AChR aggregation. Methylamine, which may disrupt receptor cytoskeletal interactions (Davies *et al.* 1980), also blocked NCM-induced AChR aggregation (Table 1). Sodium azide itself, over a 4 h time course, prevented the induction of AChR aggregation by NCM, but did not lead to a decrease in the number of AChR aggregates found in myotubes not treated with NCM. When free calcium in the culture medium was complexed with EGTA, however, the number of AChR aggregates was significantly decreased below that of cultures with free calcium, both in cultures treated with NCM and control cultures (Table 2).

The colchicine block of NCM-induced AChR aggregation, together with the lack of blocking activity by cytochalasin B, raises a number of questions. Although it is not surprising that microtubules are likely involved in muscle cell modulation of its receptor topography, the failure to implicate actin-containing microfilaments leaves open the question of the motile-force-generating mechanism in the polarized movement of AChR. Although apparently analogous to AChR aggregation, the capping

Table 2. *Inhibition of acetylcholine receptor aggregation*

(Rat myotube cultures were treated as described in Table 1. When EGTA was added to the incubation medium, the concentration of Mg^{2+} was increased to 3 mM.)

Inhibitor	Concentration	Aggregates/myotube	
		Control	NG108-15 CM
–	—	0·60	1·94
Colchicine	1×10^{-4}	0·59	0·76
Cytochalasin B	1×10^{-5}	—	2·50
—	—	0·98	1·36
Cytochalasin B	1×10^{-4}	0·68	1·58
Sodium azide	1×10^{-3}	0·83	0·87
EGTA ($+Mg^{2+}$)	3×10^{-3}	0·32	0·46

of lymphocyte integral membrane proteins by antibodies is blocked by cytochalasins, but agents which effect the state of microtubule formation, when used alone, are without effect (dePetris, 1977). The difference in the action of these cytoskeletal modulators on the muscle cell AChR aggregation and lymphocyte capping have led to the proposal that these two processes occur by different mechanisms (Bloch, 1979). A further complication is the fact that neither class of cytoskeletal modulators has an effect on the lateral mobility of the diffuse AChR of the rat myotube (Axelrod *et al.* 1978). However, the studies on AChR aggregation were done at 37 °C, whereas the lateral mobility measurements were made at 25 °C, a temperature at which it is reported azide has no effect on the state of AChR aggregation (Bloch, 1979).

In addition to the polarized movement of AChR in NCM-induced aggregation and the block of this process by drugs which affect microtubule polymerization, there is additional evidence that the myotube control of the topographic distribution of AChR is by means of a trans-membrane control mechanism involving myotube cytoskeletal elements. By use of a detergent extraction procedure, we have obtained evidence that AChR is attached to the myotube cytoskeleton, and that this attachment is modulated by NCM (Prives *et al.* 1980). Myotube cultures were extracted with 0·5% Triton X-100 in a buffer of moderate ionic strength, which was shown to extract the lipids and soluble cytoplasmic proteins from cells but which leaves the myotube cytoskeleton relatively intact and attached to the cytoskeletons of extracted fibroblasts and to the collagen coated tissue culture plate (Ben-Zeev *et al.* 1979).

Myotube cultures were labelled with rhodaminated α-btx, the surface distribution of AChR on selected myotubes was determined by fluorescence microscopy, and the same myotubes were examined after 5 min extraction with Triton X-100. On both chick and rat myotubes, the detergent extraction appeared to remove the diffuse AChR, but left a large portion of the aggregated AChR attached to the extracted myotube cytoskeleton. To quantitate the difference in the rate of extraction of AChR from diffuse and aggregated areas, the experiment was repeated while monitoring the fluorescence of small areas of the myotube with a photomultiplier tube (Fig. 1). Due to the continuous illumination of the myotube, there was photobleaching of the rhodamine α-btx probe, which followed first-order kinetics in both diffuse and aggregated areas. When detergent was added to the myotube culture, the rate of this photobleaching decay was not altered in areas of AChR aggregation. In areas of diffuse AChR

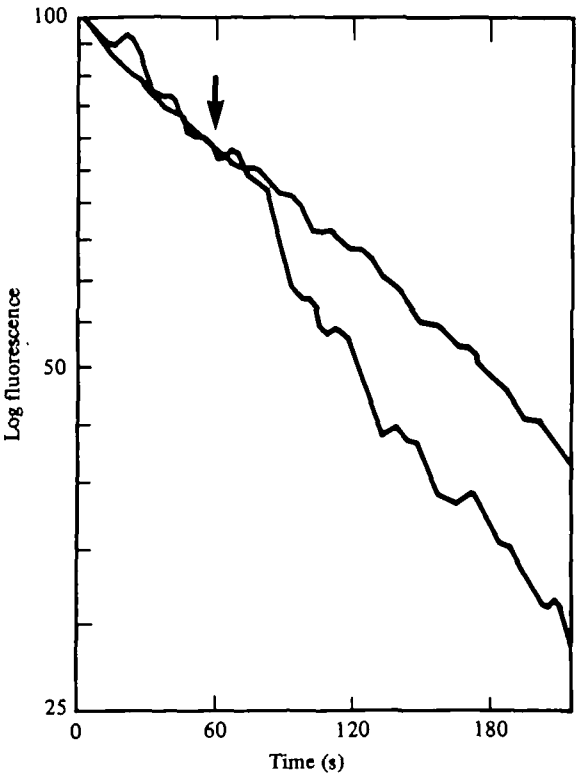


Fig. 1. Rat myotubes grown on glass coverslips were labelled with rhodamine α-btx, and the fluorescence of a spot 37 μm in diameter on the myotube membrane was led off to a photomultiplier tube during the continuous perfusion of the culture with a solution of 0·3 M sucrose, 50 mM-NaCl, 1 mM-$MgCl_2$, 10 mM Hepes, 1 mM EGTA, and 0·5 μg/ml tetrodotoxin. The arrow represents the time at which 0·5 % Triton X-100 was introduced into the perfusion chamber. The upper trace is a representative example of the photobleaching and extraction observed at a large endogenous AChR aggregate. The lower trace was taken at an area of diffusely distributed AChR. Both traces have been normalized to give 100% fluorescence at time 0.

however, there was a rapid decrease in fluorescence intensity, followed after approximately 1 min by a slower decay of fluorescence with the same time course as the photobleaching. The initial rate of fluorescence photobleaching before the addition of detergent was extrapolated and used to correct the rates of detergent extraction of aggregated or diffuse AChR. When the rates during the first minute of detergent extraction were compared, the rate of extraction of aggregated AChR was 25-fold slower than diffuse AChR (Table 3). Diffuse AChR is thus composed of both a quickly and slowly extracting fraction of receptors, whereas the entire population of aggregated AChR is slowly extracted in detergent solutions. It is tempting to attribute the relative stability of aggregated AChR in detergent solutions to the attachment of the receptor to a submembrane cytoskeletal element. If this is the case, then varying degrees of cytoskeletal attachment may also explain why there are two populations of diffuse AChR, both with respect to detergent extraction and lateral mobility.

The myotube cultures were further studied by labelling myotube AChR with

Table 3. *Rate of detergent extraction of aggregated and diffuse AChR*

(A summary of the detergent extraction kinetics of diffusely distributed and aggregated AChR. The rate of photobleaching was determined from the last minute before the addition of detergent and used to correct the following curve. The first-order rate constants for detergent extraction were determined for the 1 min period following the addition of detergent. All experiments were conducted at 25 °C.)

	Aggregated receptors	Diffuse receptors
Half-time (s)	5415·2	185·8
TAU $\times 10^{-4}$ (s^{-1})	1·28	37·3
S.E. $\times 10^{-4}$	1·16	9·28
n	5	6

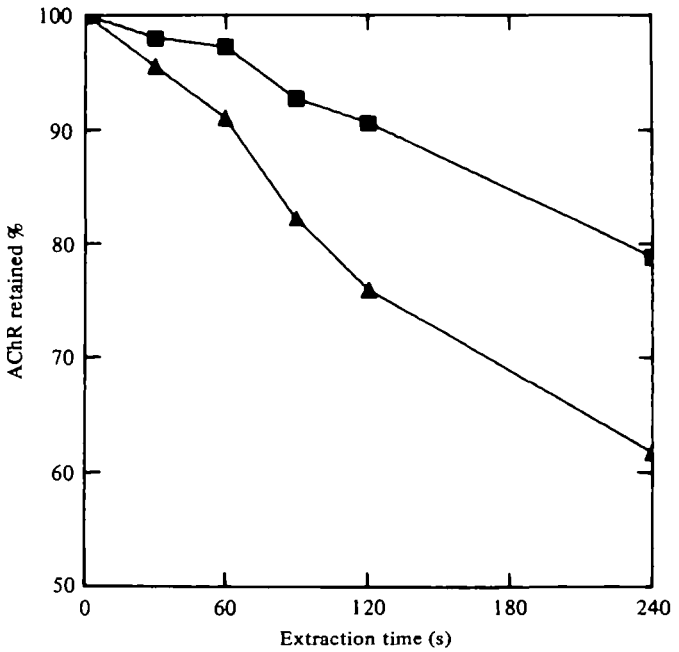
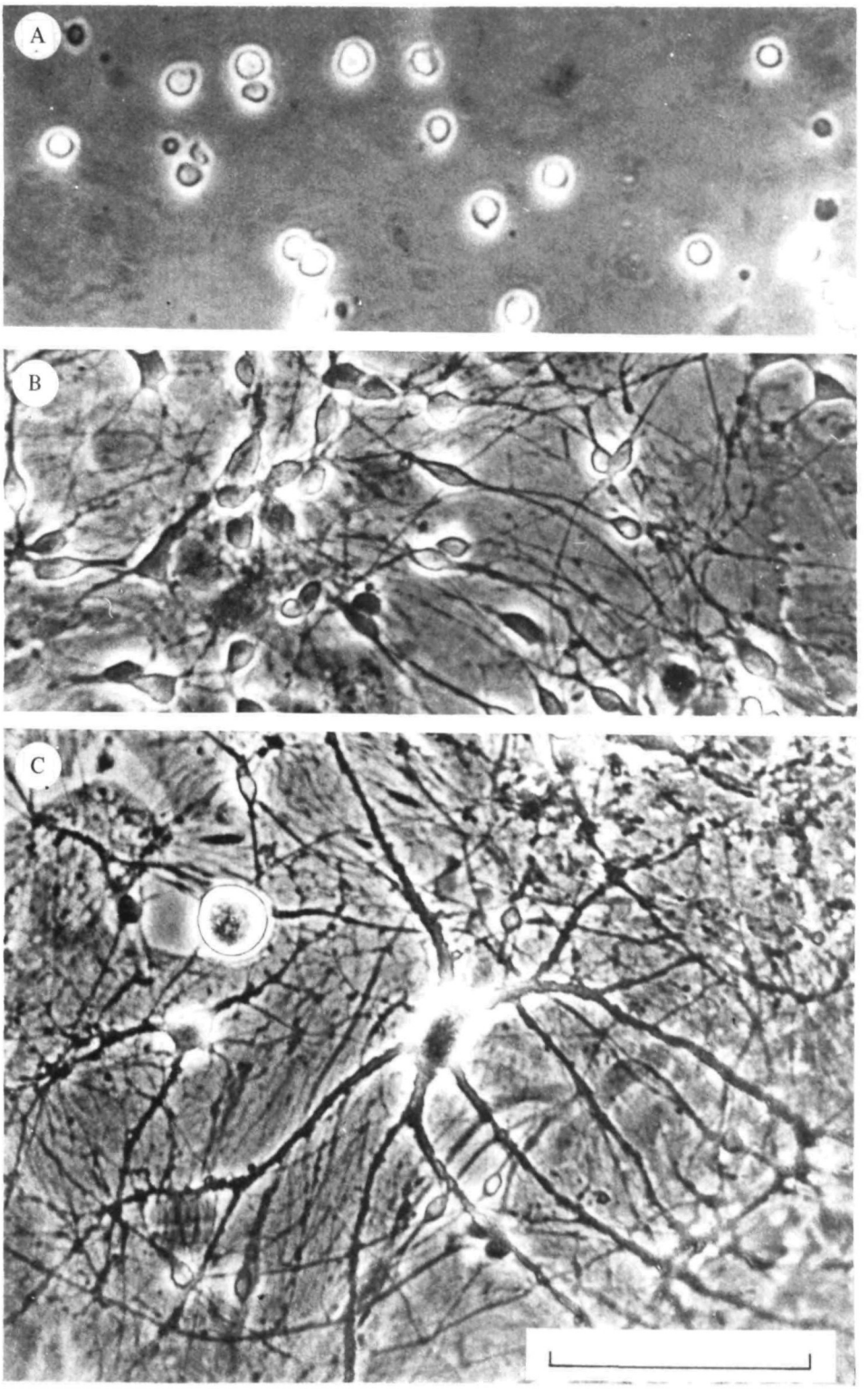


Fig. 2. The effect of NCM on the detergent extraction kinetics of rat muscle cultures. The cultures were treated for 24 h with control medium (triangles), or 25-fold concentrated NCM (squares). After labelling the cultures with [^{125}I]α-btx, the detergent extraction medium described in the legend to Fig. 1 was added, and at the times indicated, the radioactivity of the supernatant and that remaining with the myotube culture was counted. Each time point is the average of two tissue-culture plates.

[^{125}I]α-btx, and determining the rate of release of radioactivity into the detergent extraction solution. In chick myotube cultures at all ages studied, the rate of AChR extracted was fit by two exponentials, and the proportion of rapidly extracting AChR declined as the myotube cultures matured. In rat myotube cultures the rate of extraction did not clearly have two kinetic components and was, in general, more closely approximated by single first-order kinetics. The general rate of detergent extraction in rat myotubes also declined with the age in culture. These developmental changes in the rates of AChR extraction in both chick and rat myotube cultures parallel


A
B
C

developmental organization in the density and complexity of the myotube cytoskeleton in the region near the cell surface.

When rat myotube cultures were treated for 24 h with NCM, the rate of detergent extraction was significantly reduced (Fig. 2). In experiments in which the concentration of NCM was varied, and its effect on both the number of AChR aggregates and the rate of AChR detergent extraction was assessed, the two phenomena exhibited similar dose–response curves. The time courses of the effect of NCM on the two phenomena were also similar: within 2 h after the addition of NCM to rat myotube cultures there was an increase in the number of AChR aggregates and a decrease in the rate of detergent extraction. The maximal increase in the number of AChR aggregates was observed after 2 h of NCM treatment and the maximal depression of detergent extraction was observed after 12 h of NCM treatment. Although this may indicate that AChR are first induced to aggregate by NCM, and then later are attached to the myotube cytoskeleton, another interpretation is possible. There may be a continuing transition of AChR from a diffuse to an aggregated form during NCM treatment. During this process small AChR aggregates may fuse to form larger clusters, and thus the total number of induced AChR aggregates may appear to decrease after an initial maximum, even though the total number of aggregated AChR and their state of aggregation is increasing. The increased attachment of AChR to the cytoskeleton and hence the depression in the rate of detergent extraction may be simultaneous with this process, with perhaps an increase in the complexity of AChR cytoskeletal attachment as small aggregates or AChR speckles are linked to myotube motile elements which produce polarized motion and the formation of larger aggregates.

II. BLOCKADE OF ELECTRICAL ACTIVITY PREVENTS NORMAL NEURONAL MATURATION

Dissociated cell culture systems undergo a high degree of morpho-differentiation *in vitro*, since the single-cell innoculum is derived from immature tissue (Vaughn, Sims & Nakashima, 1977). The cultured preparations are initially quite simple morphologically and the 1- to 2-month-old preparations are relatively complex both morphologically and functionally (Fig. 3) (Ransom *et al.* 1977*b*). During this period of development *in vitro*, various manipulations of the culture medium can be made. A simple but drastic test of a possible linkage between electrical activity and neuronal maturation can be made by adding tetrodotoxin (the specific blocker of voltage-dependent sodium channels) to the culture medium (Kao, 1966). We have found that this completely eliminates the action potentials and associated synaptic activity that

Fig. 3. Photomicrographs of mixed mouse spinal cord (SC) dorsal root ganglion (DRG) cell cultures at different stages of maturation *in vitro*. Cell suspensions from 13·5-day mouse fetuses were prepared as described elsewhere (Ransom *et al.* 1977*b*). (A) At about 2 h after plating neurones show only rudimentary processes and few cell–cell contacts. (B) Eight days after plating, neurones have developed prominent processes and many cell–cell contacts. The neurones sit on a background of non-neuronal flat cells. (C) Five weeks after plating large SC neurones with processes are present (represented by the multipolar cell at right centre of field). Phase bright spherical DRG cell (left) can be readily distinguished. Calibration bar in C represents 100 μM in all panels.

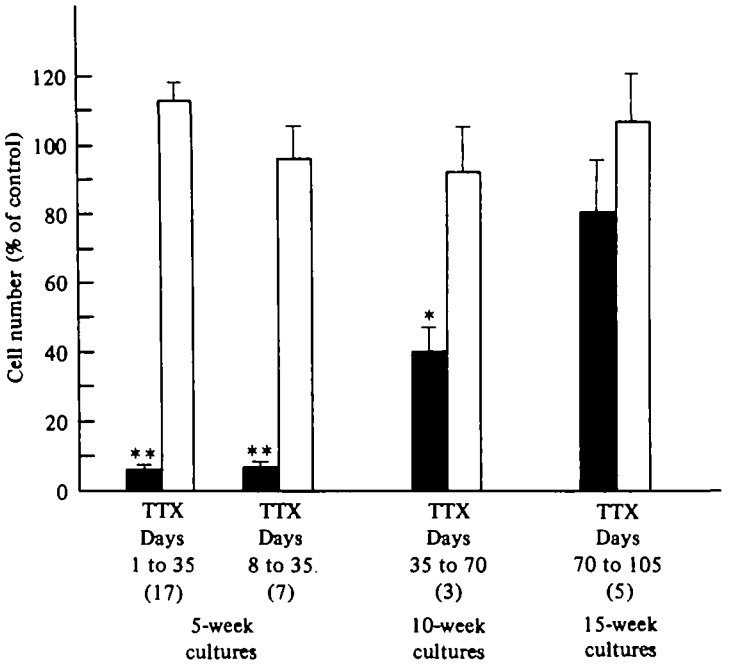


Fig. 4. Composite histograms of counts of SC and DRG neurones from a number of experiments calculated as percent of control. Error bars represent S.E.M. All SC neurones with a greatest diameter greater than 30 μM and all DRG neurones greater than 22·5 μM were counted on 35 mm diameter culture dishes. Numbers in parentheses are total numbers of plates in TTX treated groups. Tetrodotoxin treatment from day 1 or day 8 until day 35 markedly reduced SC neurone counts. Treatment of cultures with TTX from day 35–70 resulted in less marked (but statistically significant) reduction of SC neurone counts. Treatment initiated at day 70 *in vitro* produced no significant reduction in SC neurone counts. No reduction in DRG neurone counts was seen with any of the treatment schedules. ■, SC cells; □, DRG cells. * $P < 0{\cdot}01$; **, $P < 0{\cdot}001$. (From Bergey *et al.* 1980.)

are a prominent feature of untreated cultures (Ransom *et al.* 1977*a*). The consequences of the deletion of electrical activity are dramatic. The number of large spinal cord (SC) neurones surviving in the cultures is reduced to 5–10% of control plates. Dorsal root ganglion (DRG) neurones are unaffected (Bergey, MacDonald & Nelson, 1978; Bergey *et al.* 1980).

Is this vulnerability to impulse blockade a function of the developmental stage of the neurones? To address this question we instituted the TTX treatment at various periods of time following the initial cell plating. We found that the TTX effect was not detectable in fully mature cultures and that early stages of development were the most susceptible to impulse blockade. The results of these experiments are summarized in Fig. 4. A 4-week TTX treatment period was begun at day 1, 8, 35 and 70 in culture and counts of large SC and DRG neurones made in control and experimental plates at the end of the treatment period. Treatment begun at either day 1 or 8 produced a pronounced decrease in SC neurone counts, and treatment begun at day 35 produced a lesser but still significant decrease. When treatment was delayed until day 70, no significant decrement in SC neurone counts was observed. DRG neurone counts were not significantly affected by any of the treatment schedules

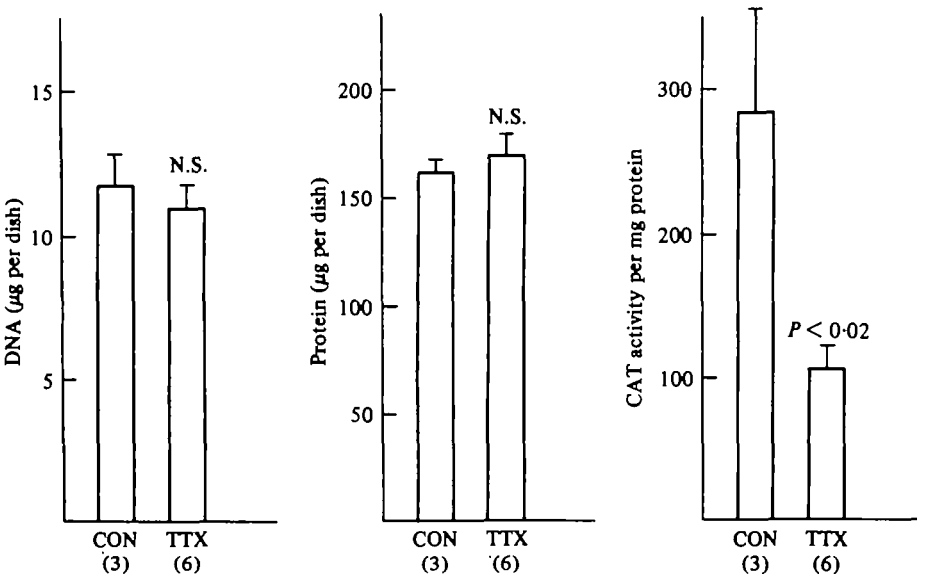


Fig. 5. Biochemical effects of TTX treatment on SC-DRG cultures from day 0–35 *in vitro*. CAT activity is expressed as picomol ACh hydrolyzed per minute. (From Bergey *et al.* 1980.)

Biochemical studies indicated a neurone-specific effect of the TTX treatment. Total protein and DNA were not affected but the total and specific activity of choline acetyltransferase was decreased to 30% of control levels by TTX treatment from day 1–35 *in vitro* (Fig. 5). Most of the DNA and protein in the cultures is contributed by background fibroblast and glial cells; these cells are apparently unaffected by the TTX treatment.

Some neurones survived in all the experiments. Some indication of their lesser degree of maturation is given in Fig. 6. The TTX-treated cells were fragile and it was difficult to obtain stable intracellular recordings from them but it was possible to demonstrate in some cases that in the absence of TTX, the neurones were electrically excitable and exhibited ongoing synaptic activity. Thus formation and maintenance of synaptic connexions between neurones are not absolutely dependent on electrical activation of the neurones (Obata, 1977).

Neurones in cell culture from the mouse cerebral cortex are also strongly dependent on electrical activity for maintenance of their normal developmental pattern. Morphologic changes produced in such cortical cell cultures by TTX treatment for 1 week (day 10–17 *in vitro*) are shown in Fig. 7. Particularly impressive was the reduction in the network of neuronal processes associated with TTX treatment.

A more quantitative measure of neuronal development is provided by an assay involving binding of ^{125}I-labelled tetanus toxin to the cultured cells. This material binds relatively non-selectively to all neuronal membranes, with less affinity for other cellular membranes (Dimpfel, Neale & Haberman, 1976; Mirsky *et al.* 1978). Data from one experiment are shown in Table 4, documenting a substantial decrease (to 20% of control values) in the neurone-specific toxin binding produced by TTX treatment.

Table 4. *Mouse brain cell cultures*

(^{125}I-labelled tetanus toxin binding to mouse brain cell cultures. About 110000 counts were added to each dish and incubated for ½ h. Plates were rinsed 3 times; the cells were then solubilized in NaOH and counted in a gamma counter. We are grateful to Dr William Habig for participation in this experiment.)

Condition	[^{125}I]tetanus toxin counts bound per dish	Protein μg/dish	^{125}I (counts per μg protein)	'Specific' counts per μg protein
Control A	10452	612	17·1	11·1
Control B	9665	636	15·5	9·5
Mixed labelled and unlabelled toxin (1:600)	4193	678	6·2	—
Tetrodotoxin 10^{-6}; 1 week	3630	461	8·3	2·3
Neurone-free cultures	1561	248	5·7	—

Another treatment that produced a substantial (although incomplete) decrease in neuronal activity did not drastically affect neuronal maturation and synapse formation in SC-DRG cultures. Adding 0·4 mM Gamma-amino butyric acid (GABA) to the culture medium produced an enduring marked decrease in activity. The incidence of EPSPs was decreased nearly fourfold and burst generation was completely eliminated. No striking deficit in cell number was noted with a 4-week treatment period and following replacement of GABA-containing media with normal media, normal ongoing spike and synaptic activity was observed (Nelson *et al.* 1977).

Conversely, treatment with glycine-containing medium produced a significant increase in paroxysmal, bursting electrical activity. Again, no dramatic shift in the developmental pattern was noted in these experiments and a few hours after returning chronically glycine-treated cultures to control media, a normal pattern of spontaneous activity was seen (Nelson *et al.* 1977).

What aspects of neuronal spike and synaptic activity are important in regulating neuronal development? The TTX treatment results in a global deficit in activity and gives little information regarding this question. More selective blockade of different aspects of neuronal activity will be required to get such information. Blockade of different types of synapses should be possible. Tetanus toxin produces a general blockade of synaptic activity while leaving chemosensitivity and Ca^{2+} and Na^{+} dependent action potentials intact (Bergey & Nelson, unpublished observations). Various neurotransmitters and neuromodulators, as well as conditioned medium from active cultures, can be added to the medium bathing TTX treated neurones. Such intervention may reveal the relative importance, developmentally, of the different components that go into the functioning of active synaptic networks.

DISCUSSION

Considerable information is becoming available about the regulation of key steps in the formation of stable synaptic networks. Specification of neurotransmitter biosynthesis is effected by soluble fractions elaborated by target and supporting cells in

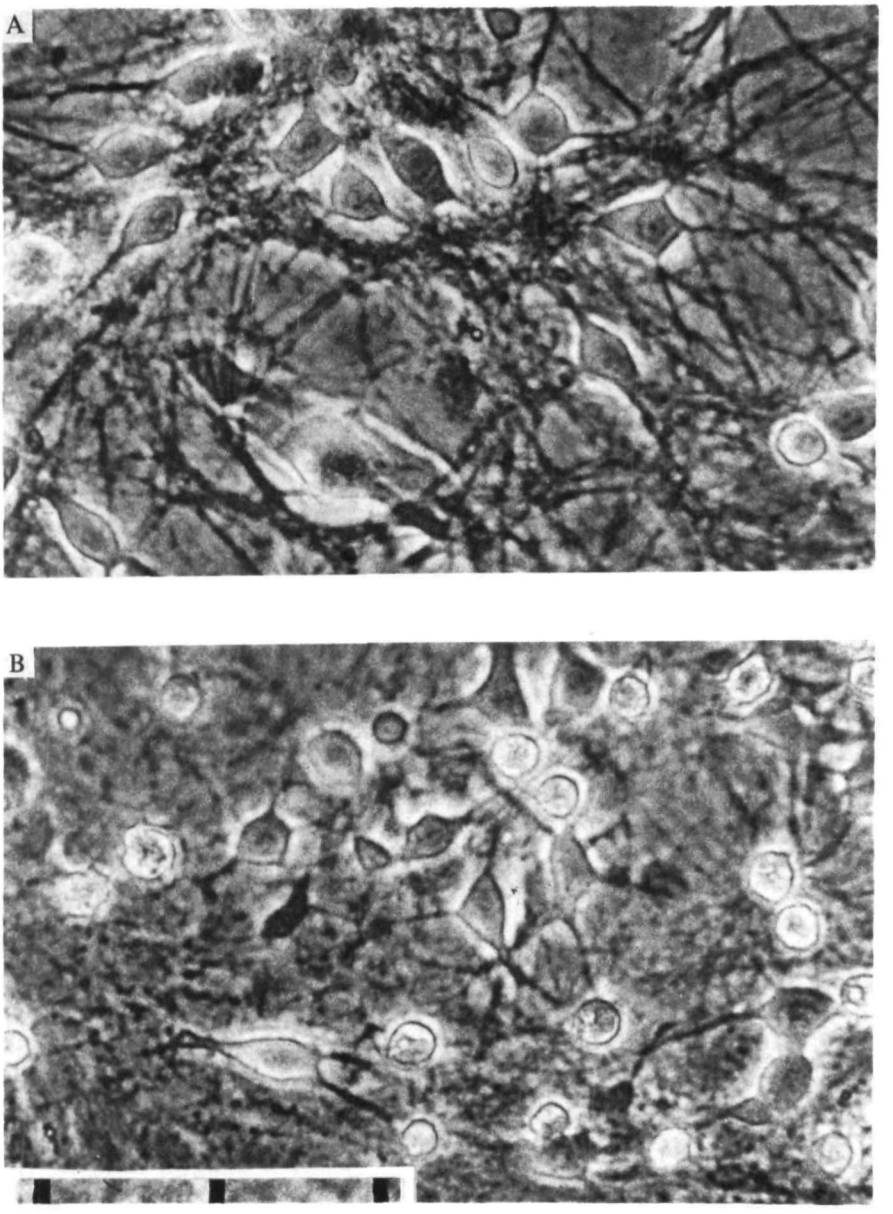


Fig. 7. Photomicrographs of sister cultures prepared by the methods described earlier (Godfrey *et al.* 1975). (A) control plate at 17-day *in vitro*. (B) A culture treated with 10^{-6} M TTX from day 10–17 *in vitro*. Calibration marks represent 50 μM.

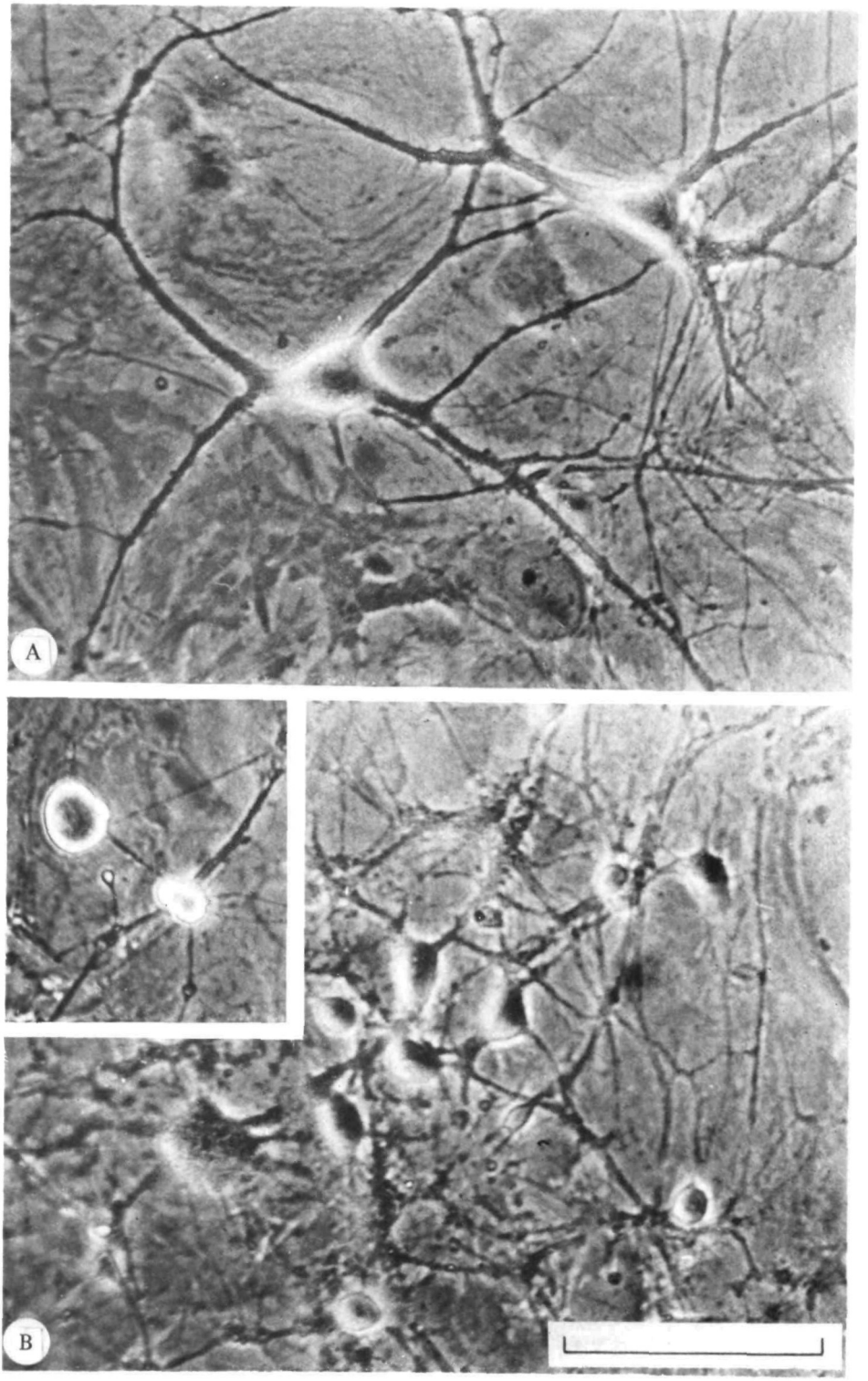


Fig. 6. Photomicrographs of sister SC-DRG cultures at 5 weeks in culture under control (A) or TTX (B) conditions. Control plates show large SC neurones with thick processes. In TTX-treated plates, even at the highest cell density area (shown), few large SC neurones were present and these few were often granular, not phase bright and with short processes. Many areas of TTX-treated plates were devoid of SC neurones. A morphologically normal DRG neurone in a TTX-treated culture is shown in the insert. Calibration bar is 100 μM. (From Bergey *et al.* 1980.)

conjunction with the electrical activity of the neurones. Neuronal factors markedly affect post-synaptic metabolism, in particular synthesis and turnover of receptor molecules and degradative enzymes. A complex cell biologic machinery for control of receptor topographic distribution in the cell membrane is becoming amenable to detailed study. Interactions between surface membrane molecules and underlying cytoplasmic structural systems may be of particular importance.

In the cell culture system it would appear that the trophic function of neurones is affected markedly by their electrical activity, particularly during early developmental stages of the nerve cells. This conclusion is based on the marked deficit in development produced by the blocker of spike activity, tetrodotoxin. The cell cultures may exhibit a sensitivity to perturbation not evidenced by explant or intact tissue. Indeed, explants of CNS tissue did not exhibit detectable deficits associated with pharmacologic blockade of action potentials (Crain *et al.* 1968; Model *et al.* 1971). The role that phenomena revealed in cell culture play in normal development clearly must be evaluated in more intact systems (Meyer, Burkart & Jockush, 1979). Nevertheless, as illustrated in other papers in this symposium, the cell cultures represent valuable, sensitive model systems for analysing important developmental interactions.

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