Giant *Drosophila* Neurons Differentiated from Cytokinesis-Arrested Embryonic Neuroblasts

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SUMMARY

The relative contributions of the intrinsic and extrinsic factors in determining neuronal differentiation are not fully understood yet. We found that isolated neuroblasts from *Drosophila* gastrulae were able to differentiate neuron-specific properties in culture even when cell divisions were inhibited. The resultant giant multinucleated neurons displayed thickened neurites with a variety of distinct branching patterns. Neuronal antigens were expressed as in normal cultured neurons, and action potentials could be evoked by current injection within two days after plating. These results indicate that the factors for initiating specific differentiation programs for basic neuronal form and function are present in a neuroblast already. The cells of increased sizes in this culture system are more accessible to physiological and cell biological analyses and could facilitate future studies of the *Drosophila* nervous system.

INTRODUCTION

Cells in cleavage-arrested early embryos of ascidians and nematodes can develop in correct lineage pattern and temporal order several tissue-specific enzymes, antigens, ion channels, and ultrastructural features (Whittaker, 1973, 1987; Hirano et al., 1984; Cowan and McIntosh, 1985). For instance, in cell division-arrested early ascidian blastulae, muscle acetylcholine esterase and brain tyrosinase develop only in the subsets of blastomeres that belong to the appropriate lineages (Whittaker, 1973). These studies clearly demonstrate differential segregation of cytoplasmic determinants for developmental potentials of daughter cells (Conklin, 1905). Cleavage-inhibited blastomeres have been dissociated to determine whether the various differentiated features require cell–cell interaction or other position-dependent external factors in earlier embryonic stages (Laufer et al., 1980; Okado and Takahashi, 1988). However, effects of cleavage arrest on cellular differentiation of more complex features in late embryos have not been studied extensively, especially in species other than those with typical mosaic development. For example, some neuronal differentiation programs might be initiated solely by intrinsic factors already present in the neuroblast, even though morphological and functional differentiation normally occurs in daughter cells after a sequence of neuroblast divisions. One approach to this problem is to ask whether complex neuronal form and function can be expressed when cytokinesis of neuroblasts is arrested. Here, we report that isolated neuroblasts from *Drosophila* gastrulae are able to differentiate neuronal morphology and other neuron-specific features, even when cell divisions are inhibited throughout their development in culture.

METHODS

The wild-type strain, Canton-S, of *Drosophila melanogaster* was used throughout this study. Single-embryo cultures were prepared according to previously described procedures (Seecof et al., 1971; Koana and

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Miyake, 1982; Sakai et al., 1989). Embryos were dissociated and plated on poly-L-lysine coated or uncoated glass cover slips. Before plating, cytochalasin B (2 μg/ml; Aldrich Chemical Co.) was added to M3(BF) medium, which contained 10% heat-inactivated fetal bovine serum, penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and insulin (2.6 mU/ml). Time-lapse studies of the developmental changes in neuronal morphology were performed during the first week following plating. Some cultures were observed up to 2 weeks. Records of phase-contrast photographs were analyzed.

For immunofluorescent staining, cultures were fixed for 10 min in a phosphate buffer solution containing 4% formaldehyde. Fluorescein-conjugated goat anti-horseradish peroxidase (anti-HRP) was from Organon Teknika Corp. Mouse anti-α-tubulin (Amersham International) and MAb 8C5 (provided by Dr. S. C. Fujita) were visualized by fluorescein- and rhodamine-conjugated goat anti-mouse IgG (Organon Teknika Corp.), respectively. The staining procedure was identical to that described previously (Fujita et al., 1982). The cultures plated on the coverslips were mounted on glass slides and viewed by epifluorescence.

The culture neurons were studied by the whole-cell recording technique. The patch-clamp circuit (Axopatch 1-B, Axon Instruments) and recording methods were the same as described by Hamill et al. (1981). The cultured cells plated on coverslips were immersed in normal saline (128 m*M*, NaCl; 2 m*M*, KCl; 4 m*M*, $MgCl_2$; 1.8 m*M*, $CaCl_2$; 35.5 m*M*, sucrose; 5 m*M*, HEPES, pH 7.1 titrated with NaOH). The pipets were filled with an internal solution (144 m*M*, KCl; 1 m*M*, $MgCl_2$; 0.5 m*M*, $CaCl_2$; 5 m*M*, EGTA; 10 m*M* HEPES, pH 7.1 titrated by KOH) and their resistance ranged from 3 to 5 MΩ. Test-pulse generation and data acquisition and analysis were performed with the pCLAMP software system (Axon Instruments) on an IBM-XT computer. Signals were digitized at a sampling rate of 0.5–2.0 kHz and were also recorded on an FM tape recorder (Store-4D, Lockheed Electronics).

RESULTS

In the fruit fly, *Drosophila*, the earliest identifiable precursors of the central nervous system are neuroblasts, which begin to segregate from the ectoderm during germ-band elongation (Poulson, 1950; Thomas et al., 1984; Campos-Ortega and Hartenstein, 1985). After segregation, neuroblasts divide several times to produce ganglion mother cells that divide once more to differentiate as neurons. Thus, neuroblasts in dissociated cultures generally produce neuronal clusters with neurites radiating from them (Seecof et al., 1973; Wu et al., 1983; Furst and Mahowald, 1985). Single-embryo cultures were prepared from individual early gastrulae, which were dispersed just before germ-band elongation (about 4.5-h-old) and were grown in a medium containing 2 μg/ml cytochalasin B. Among drugs that inhibit mitosis and cytokinesis, cytochalasin B has been used most commonly to study the effects of cell division arrest (Whittaker, 1973, 1987; Hirano et al., 1984; Cowan and McIntosh, 1985). Only one day after plating, the cytochalasin B-treated cultures became strikingly different from normal ones. In the treated cultures, most neuroblasts differentiated into single neurons with somata 2–3 times the normal diameter and with well-extended thick neurites exhibiting a wide variety of branching patterns (Fig. 1). The soma diameter of some treated cells reached 21 μm (12.0 ± 2.9 μm, mean ± SD in 61 cells). Distinct mono-, bi-, and multipolar cell types have been observed repeatedly and could be categorized in the treated cultures. Their morphological development reached a steady state in 4 days and the cultures could be maintained for at least 2 weeks.

Next we investigated whether division-arrested neuroblasts can express other neuronal properties. Antibodies against horseradish peroxidase (anti-HRP) recognize neuron-specific surface molecules in different insect species and have been used to identify insect neurons in their early development (Jan and Jan, 1982; Bastiani et al., 1986; Bentley and Toroian-Raymond, 1986; Palka et al., 1986). In 1–13-day-old cultures, cytokinesis-arrested giant cells with neuronal arborizations were stained as intensely by anti-HRP as neurons in normal cultures, while other cell types, such as muscle cells, remained unstained (Fig. 2). Therefore, the morphological differentiation of these giant cells was accompanied by the accumulation of neuron-specific antigens. Differentiated neurites are known to contain abundant axially-arranged microtubules, which consist of tubulin subunits. In addition to the somata, the unusually thick processes of these giant neurons also exhibited anti-α-tubulin staining, suggesting well-developed longitudinal bundles of microtubules typical of normal neurites [Fig. 3(A,B)].

The use of a nuclear-specific antibody (Fujita et al., 1982), MAb 8C5, clearly demonstrated the multinucleated nature of these neurons [Fig. 3(D)]. The isolated soma of a cytochalasin B-treated neuron contained a few (mostly 2 or 3, up to 5) large nuclei. The increase in nuclear size suggests the possibility of each nucleus being polyploid. In contrast, neuronal clusters derived from single neuroblasts in normal cultures showed many (up to 18) smaller nuclei, each contained in

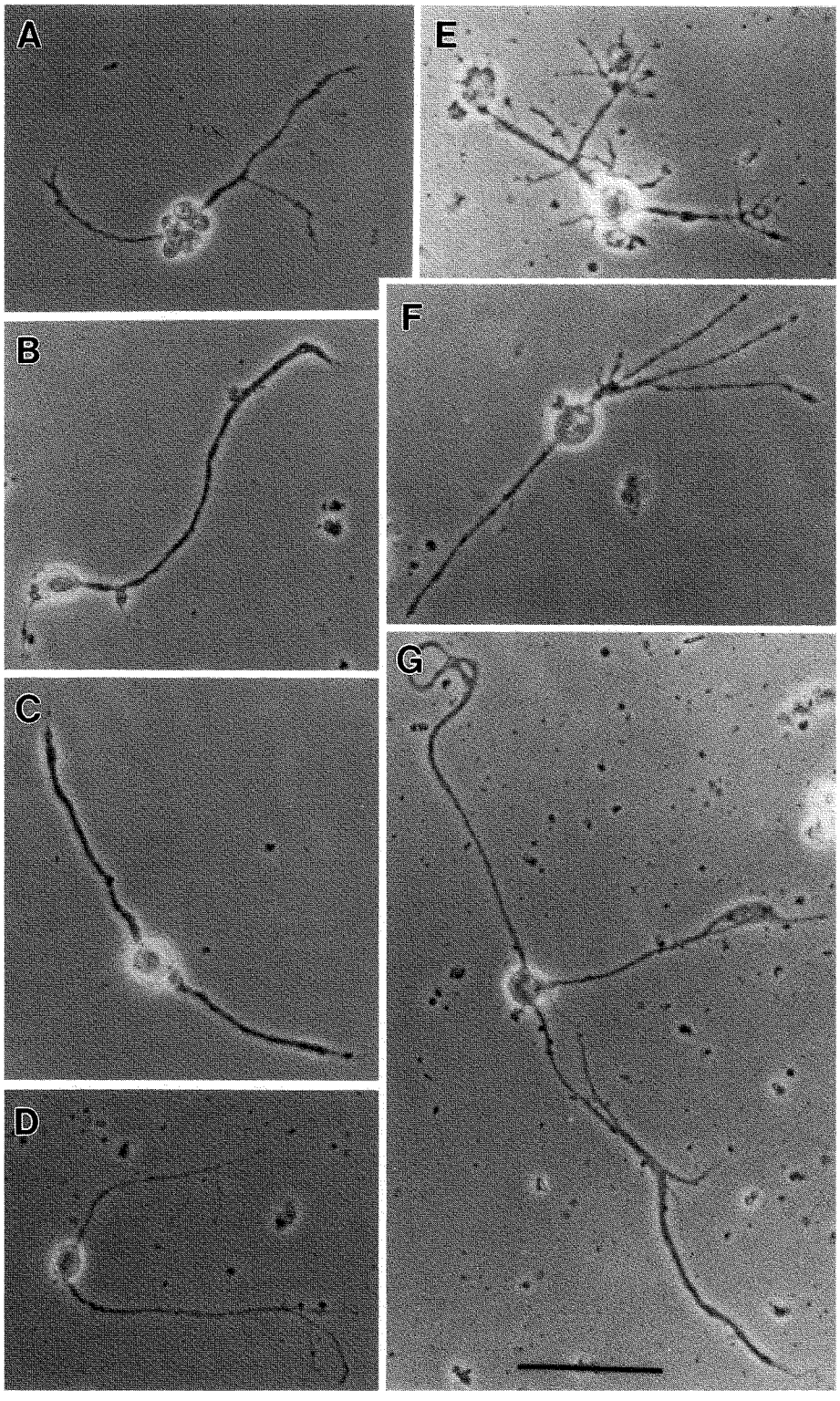

Figure 1 Giant *Drosophila* neurons differentiated from cell division-arrested gastrula neuroblasts. (A) A neuronal cluster differentiated from a neuroblast in the control culture (without cytochalasin B). (B–G) Single neurons derived from cytochalasin B-treated neuroblasts. Mono-, bi-, and multipolar cells with different branching patterns were observed repeatedly, presumably corresponding to different forms of normal cell types. Bar, 50 μm.

a single soma, distributed within individual clusters [Fig. 3(C)]. To demonstrate that these giant neurons were single cells, each confined by a continuous surface of plasma membrane, they were subject to lowered osmotic pressure. An eightfold reduction in osmotic pressure was achieved by the addition of distilled water to the culture medium. Under this condition, the multinucleated soma appeared as a single enlarged sphere [Fig. 4(C,D)]. Although tightly associated neurons in untreated cultures sometimes looked like single larger neurons, their individual somata nevertheless turned into separate bubbles after osmotic shock [Fig. 4(A,B)]. In addition, in both control and treated cultures, spherical membrane blebs appeared along the neurites, especially at their tips. The thickened processes of the treated neurons showed larger blebs than untreated ones [Fig. 4(B,D)], representing neurites with increased diameters rather than fasciculated bundles. In a previous study of chemically-induced cell fusion of cultured *Drosophila* neurons, the effectiveness of this method has been shown by staining the membrane with the fluorescent lipid probe, di-I. The continuity of plasma membrane in the swollen cells was confirmed by direct visualization with fluorescent microscopy (Suzuki and Wu, 1984).

The growth cone, a specialized structure at the

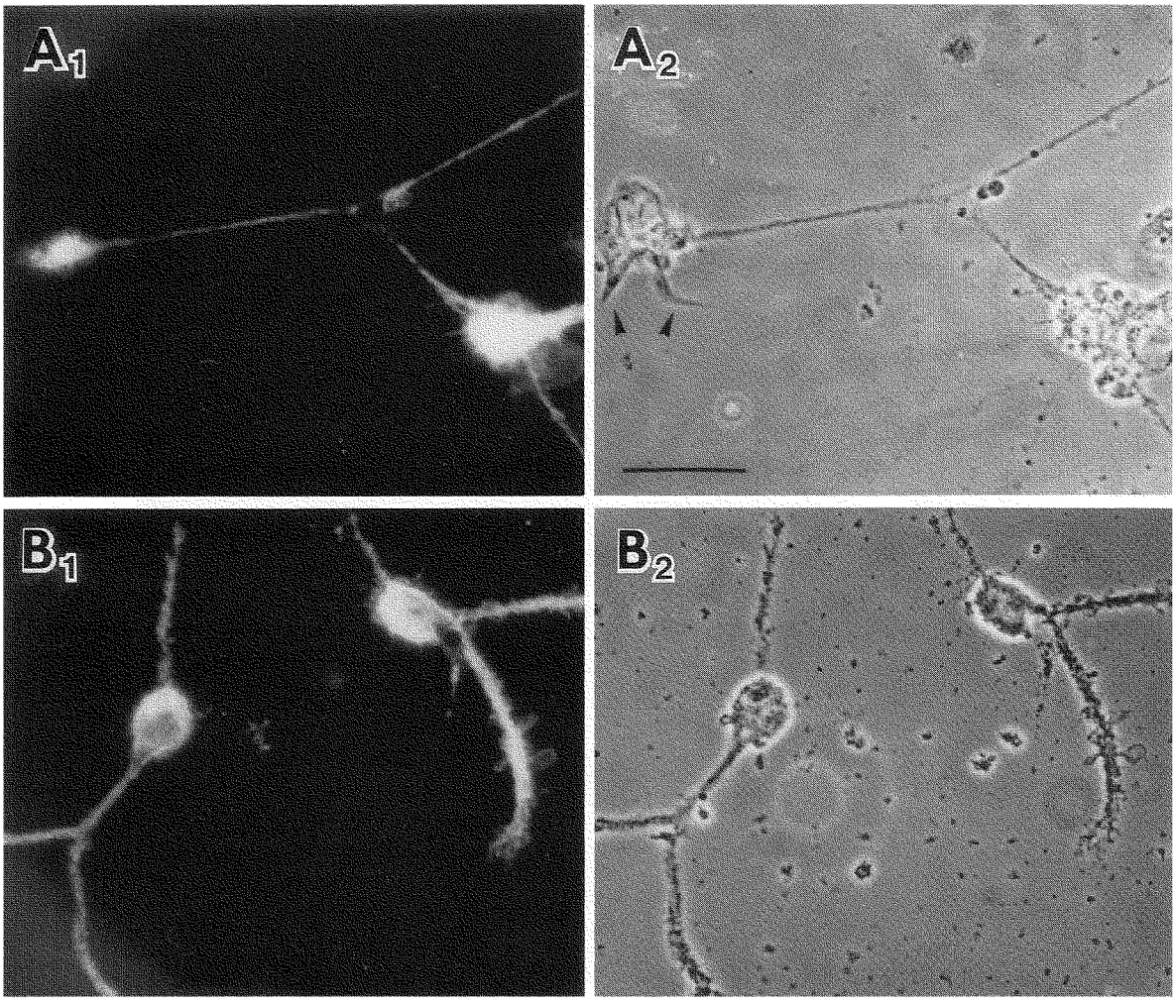

Figure 2 Expression of neuronal antigens in neurons derived from normal and cytokinesis-arrested neuroblasts. (A_1, A_2) Antibodies against anti-horseradish peroxidase (anti-HRP) selectively stained neurons (in clusters) in the control culture, but not muscle and other cell types (arrowheads). (B_1, B_2) Neurons derived from cell division-arrested neuroblasts also were stained intensely by anti-HRP, indicating the expression of neuron-specific antigens. Fluorescent and corresponding phase-contrast micrographs are shown. Bar, 50 μm.

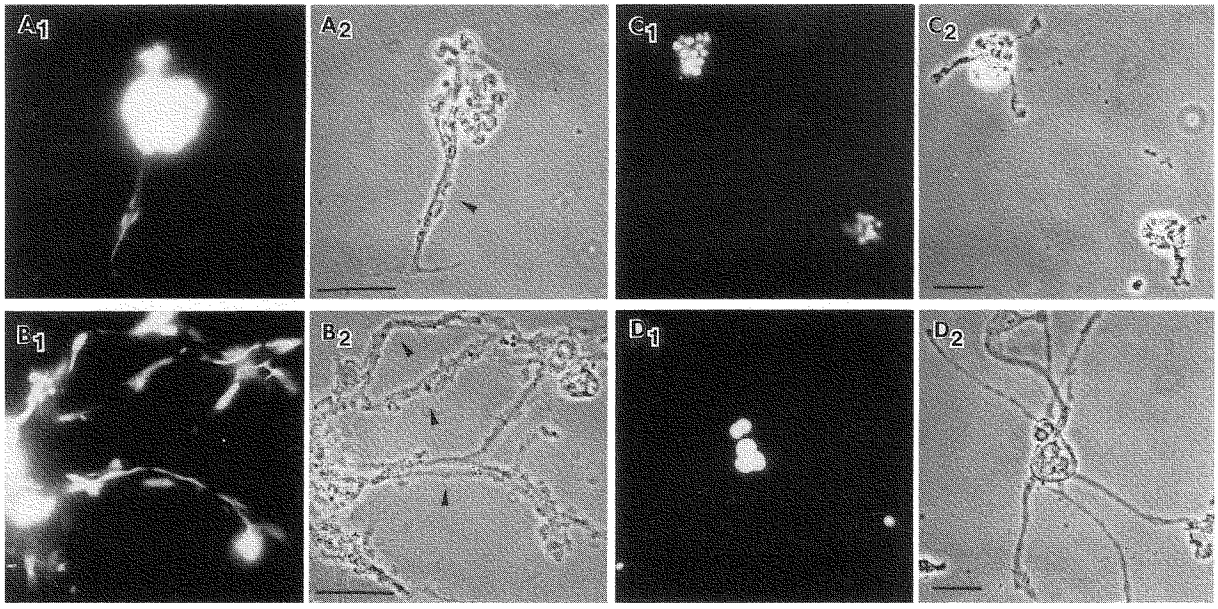

Figure 3 Indirect immunofluorescent characterization of normal and multinucleated neurons. Fluorescent and corresponding phase-contrast micrographs are shown. (A_1, A_2) Monoclonal anti-α-tubulin stained neuritic bundles (arrowheads) as well as somata of neuronal clusters in the control culture. (B_1, B_2) Cytochalasin B-treated neurons showed similar staining by anti-α-tubulin. (C_1, C_2) In the control culture, many smaller nuclei, which distributed among the individual cells in individual clusters, were stained by a nucleus-specific monoclonal antibody, MAb 8C5. (D_1, D_2) This antibody revealed that single neurons in the treated culture contained a few large nuclei. Two single neurons in contact with each other at their somata are shown. Bars, 30 μm.

tips of growing neurites, generally, is considered to be important for neurite extension and branching. Cytochalasin B is known to inhibit cytokinesis by preventing actin filament polymerization. Since actin filaments comprise one of the major cytoskeleton components in the growth cone, it is interesting to ask how the continuous presence of cytochalasin B affects the growth cone structure and neurite outgrowth in this system. We found that some cytochalasin B-treated neurons showed club-shape growth cones (Fig. 1) at the tip of neurites without extended lamellipodia and filopodia seen in normal neurons (Wu, 1988). Neurite extension and lower order branching, however, did not appear to be inhibited. When returned to normal medium following a 1- or 2-day cytochalasin B treatment, the growth cones of these neurons resumed normal morphology and motility (Wu, 1988), leading to ramification of the neurite arbor without altering the existing lower order branches (data not shown).

One important specialization of neurons is the ability to generate action potentials. It is known that larval neuroblasts are not excitable under normal culture conditions (Wu et al., 1983). There has been no report of intracellularly recorded action potentials in cultured *Drosophila* neurons because of the technical difficulties imposed by their small sizes. However, by using patch-clamp electrodes, spike activities can be detected extracellularly (Byerly and Leung, 1988), and inward Na^+ and Ca^{2+} currents can be recorded (Byerly and Leung, 1988; O'Dowd and Aldrich, 1988) from embryonic neurons differentiated in culture. To determine whether membrane excitability development is inhibited by the arrest of cell divisions, we performed whole-cell recordings of membrane potentials using extracellular patch pipets (Hamill et al., 1981). Gigaohm seals formed readily on these cells and the input resistance ranged from 0.5–1.8 GΩ in the whole-cell configuration. Most of the cells studied were capable of generating action potentials upon application of depolarizing currents (Fig. 5). Among the 81 cells recorded (age 2–15 days), prolonged current injection (500 ms) produced repetitive action potentials in 42 cells. In 20 cells, this elicited only a single regenerative spike at the onset, and the rest of the 19 cells

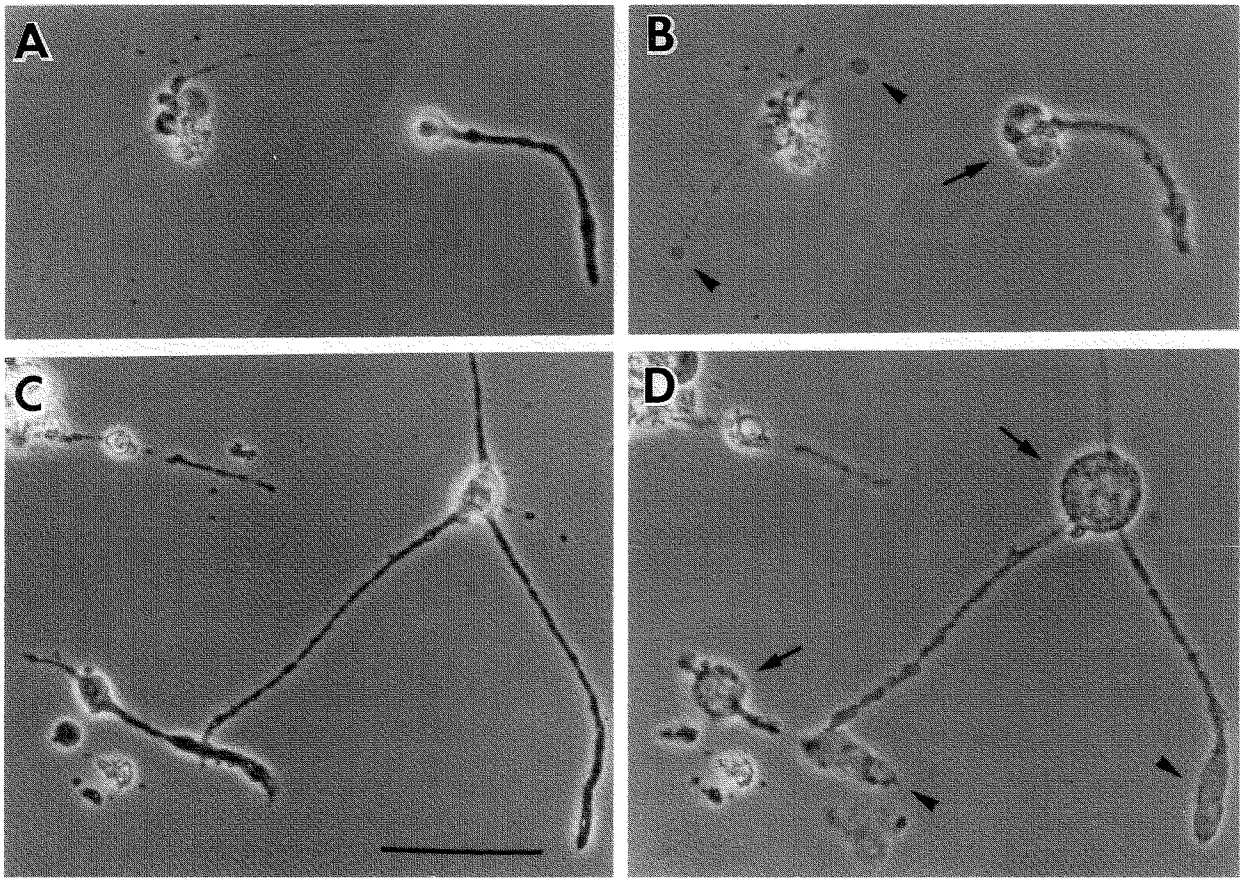

Figure 4 Confirmation of continuity of surface membrane by lowered osmotic pressure. (A, B) Somata of tightly associated neurons in the control culture turned into individual spheres (arrow) by an eightfold reduction in osmotic pressure. Smaller spherical blebs can be observed along neurites and at their tips (arrowheads). (C, D) Following the same osmotic shock, the somata of the cytochalasin B-treated neurons changed into single enlarged spheres (arrows) containing multiple nuclei (which are not well resolved in the single focal plane of this micrograph). Note the large blebs at the tip of neurites (arrowheads). Bar, 50 μm.

showed outward rectification without indications of regenerative potentials. The action potentials appeared to be generated, at least in part, by an inward Na^+ current because they were reversibly blocked by 25–200 n*M* tetrodotoxin in saline.

DISCUSSION

The present study demonstrates that individual cytokinesis-arrested neuroblasts from *Drosophila* gastrulae are able to differentiate in isolation complex neuronal morphology, membrane excitability, and neuron-specific antigens. It remains to be explored further whether all essential features of normal neurons are expressed in these giant multinucleated cells. Studies of *Drosophila* neurons have been hampered often by their small sizes, even though the rapidly accumulated genetic and molecular biological data call for their cellular correlates. The giant *Drosophila* neurons described here could provide a more accessible model system for studying a variety of basic physiological and cell biological processes, including morphological experiments and patch-clamp recordings in subcellular regions.

An interesting observation is that altered growth cone morphology in the presence of cytochalasin B does not prevent neurite development in *Drosophila* neuroblasts. Similar observations in growth cone and neurite morphology have been reported in cytochalasin B-treated cultures dissociated from dorsal root ganglia of chick embryos (Marsh and Letourneau, 1984). Thus, in both ver-

tebrate and invertebrate cultures, neurite extension and branching do not depend on the intact structure of growth cones. This is also consistent with the previous in situ study in grasshopper embryos, where cytochalasin B altered growth cones without blocking neurite elongation (Bentley and Toroian-Raymond, 1986).

It is worth noting that cytokinesis-arrested neuroblasts did not generate neurons of uniform morphology in the homogeneous culture environment. The various distinct types of neuronal branching patterns observed in cytochalasin B-treated cultures parallel those in dissociated *Drosophila* central nervous system cultures from third instar larvae (Wu et al., 1983; Kim and Wu, 1987; Wu, 1988). The significance of this variety of morphological categories is not known. However, it is tempting to suggest that the different patterns displayed by cytochalasin B-treated neurons may reflect different forms among normal cell types, i.e., the arrested neuroblasts are determined already in their fate to become specific types of neurons. If this is true, the above observation also raises the possibility that the expression of specific neuronal morphology does not depend on cell sizes. Verification of these notions requires direct comparisons of neuronal types present in normal and cytochalasin B-treated cultures. Such comparisons are not yet possible. To date, all reported embryonic cultures in *Drosophila* produce neuronal clusters because gastrula neuroblasts are used to initiate cultures (Seecof et al., 1971, 1973; Cross and Sang, 1978; Koana and Miyake, 1982; Furst and Mahowald, 1985; Salvaterra et al., 1987; and Sakai et al., 1989). It is difficult to delineate individual neuronal arbors in the cluster, and information concerning the morphological development of isolated neurons in normal embryonic cultures is still lacking.

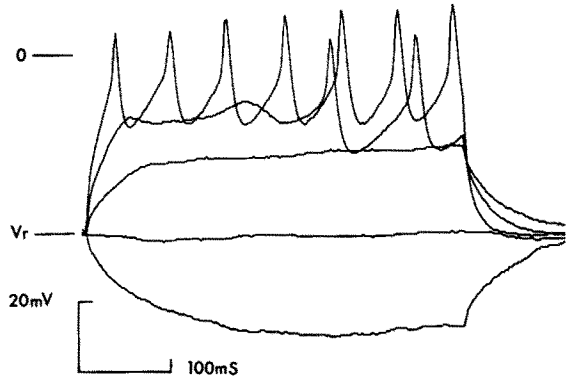

Figure 5 Action potentials elicited by current injection. The superimposed traces show membrane potential responses recorded in the whole-cell configuration during application of −40, +40, +80, +120 pA current pulses. The resting potential (V_r = −65 mV) and zero potential are indicated.

In addition to morphological differentiation, the cell division-arrested neuroblasts are capable of continuing differentiation of molecular markers. Dissociated gastrular cultures (5-h-old) have been reported to show a very low level of anti-HRP staining in a small number of "neuroblast-like" cells (Salvaterra et al., 1987), which is too weak to be discerned in situ (Jan and Jan, 1982). It is not clear whether this weak staining reflects the presence of all 17 membrane glycoproteins recognized by anti-HRP antibodies so far identified (Snow et al., 1987). The intense anti-HRP staining observed in the giant cells described here may represent either the accumulation of the antigen species already present before neuroblast division or the appearance of a new species after cytochalasin treatment. After completion of the present study (Wu et al., 1989), a report has appeared describing the developmental effect of cytochalasin B applied at later stages (7 and 11 h) of neuroblast development (Huff et al., 1989). Under these conditions, cultures of isolated neuroblasts give rise to cell clusters of about 4 or 8 neurons, instead of up to 20 in untreated cultures. Nevertheless, the proportion of clusters expressing serotonin remains unaltered in these experiments, suggesting that *Drosophila* neuroblasts are determined already for subsequent expression of this transmitter (Huff et al., 1989).

The experiments in cleavage-arrested ascidian and nematode blastomeres indicated that differentiation of some tissue-specific markers is determined by internal factors that segregate in early cleavages (Whittaker, 1973, 1987; Hirano et al., 1984; Cowan and McIntosh, 1985). Manipulation of dissociated blastomeres demonstrated that certain differentiated features, such as neural-specific ion channels in tunicates (Okado and Takahashi, 1988) and topogenesis in nematodes (Laufer et al., 1980), require additional cell–cell interaction through contact or other external cues present at defined embryonic stages. Recent studies of cell lineage in the vertebrate central nervous system suggest that the dividing precursor cells that give rise to neurons are multipotential (Price, 1989). In the retina, the precursor cells are capable of generating neurons, photoreceptors, and glial cells (Holt et al., 1988). Furthermore, postmitotic cells, which would become neurons in situ, can turn into photoreceptors in dissociated cultures, indicating that

cell-cell interactions may be important in determining the fate of individual postmitotic cells (Adler and Hatlee, 1989). In contrast, results from this study and others (Huff et al., 1989) suggest that *Drosophila* neuroblasts may be committed early in development to a rather narrow range of cell fate. The present study on cytokinesis-arrested neuroblasts shows that further segregation into daughter cells and cell–cell interaction are not required for the expression of primary neuronal forms and some other neuron-specific properties, and that the necessary cellular induction events, if any, most likely take place before neuroblast divisions.

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