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SUBSTANCE P-LIKE IMMUNOREACTIVITY IN NEURONS IN DISSOCIATED CELL CULTURES OF MAMMALIAN SPINAL CORD AND DORSAL ROOT GANGLIA[1]

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Abstract

Dissociated cell cultures prepared from fetal mouse spinal cords and dorsal root ganglia were stained for endogenous substance P using the peroxidase-antiperoxidase technique. Substance P-like immunoreactivity was localized within a small percentage of rounded or multipolar neuronal somata and in varicose processes. The substance P-positive multipolar neurons were derived from spinal cord, while the small rounded neurons were possibly of spinal cord and/or sensory ganglion origin. Large dorsal root ganglion neurons were unreactive.

These results are consistent with *in vivo* findings and indicate the feasibility of electrophysiologic studies in culture to analyze the synaptic connections between substance P neurons and their target cells.

The undecapeptide, substance P, has been localized by immunocytochemistry in neuronal somata in spinal sensory ganglia (Hökfelt et al., 1975a, b, 1976) and in occasional cell bodies in the dorsal spinal cord (Hökfelt et al., 1977; Ljungdahl et al., 1978). Immunoreactive fibers are found in spinal cord laminae containing sensory fibers and their terminations (Hökfelt et al., 1975a, b). This peptide is believed to function in the spinal cord in the processing of sensory information related to nociception (Henry, 1976; Henry et al., 1980; Randić and Miletić, 1977).

In the present study, neurons of the fetal mouse spinal cord (SC) and dorsal root ganglia (DRG), maintained in dissociated cell culture, were examined for immunoreactivity to substance P. This study was undertaken to determine whether substance P-containing neurons were present in SC-DRG cultures and, if so, to define which morphologic features might distinguish them. The SC-DRG cell cultures, showing essentially two-dimensional anatomy, have been used effectively for electrophysiologic studies of synaptically connected cells (Macdonald and Nelson, 1978; Nelson et al., 1977; Ransom et al., 1977a, b) and for detailed analyses of the morphology and synaptic relationships of physiologically studied and selectively labeled neurons (E. A. Neale et al., 1978; Nelson et al., 1978). The ability to identify substance P neurons in cultures would allow further analyses of (*a*) electrophysiologic characteristics of the substance P neuron, (*b*) synaptic relationships with other peptide-containing neurons, and (*c*) possible coexistence of substance P with a particular neurotransmitter and/or an additional peptide.

Materials and Methods

Tissue cultures. Dissociated cell cultures were prepared from 13- to 15-day fetal mouse spinal cords and dorsal root ganglia as previously described (Ransom et al., 1977c). For immunocytochemistry, cultures grown in 35-mm plastic culture dishes were used routinely, although, occasionally, cultures grown on glass coverslips were stained. Fifteen cultures, from four dissections, ranging in age from 5 to 12 weeks, were treated with nerve growth factor (2.5 S NGF at a final concentration of 40 ng/ml) for at least 3 days prior to study. After two rinses in 0.1 M sodium phosphate buffer, pH 7.3, cultures were fixed for 90 min at room temperature with freshly

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prepared 4% paraformaldehyde in phosphate buffer, pH 7.3. Following fixation, the cultures were rinsed with and stored refrigerated in phosphate-buffered saline (PBS).

Immunocytochemistry. Fixed cultures were treated with two changes (5 min each) of PBS containing 0.125% Triton X-100. The cultures then were reacted with rabbit antiserum to substance P (RD_2L–Leeman) diluted 1:1000 for 13 to 19 hr at 4°C in a moist chamber, after which, they were reacted sequentially with sheep antiserum to rabbit γ-globulin (1:100) and rabbit peroxidase-antiperoxidase complexes (Sternberger, 1979) at a 1:400 dilution as previously described (Zimmerman and Antunes, 1976) for periods of 90 min each. All antisera were diluted with PBS containing 0.25% Triton X-100. The cultures were washed between each step with PBS containing 0.125% Triton X-100. Reaction products were formed with a solution of the tetrahydrochloride of 3,3′-diaminobenzidine (0.5 mg/ml) in Tris-HCl buffer, pH 7.6, containing 0.01% H_2O_2.

As controls for staining specificity, cultures from each experiment were reacted for 22 hr at 4°C with specific antiserum (1:1000) containing 1 to 10 μg of synthetic substance P (Immuno Nuclear Corp., Stillwater, MN) per ml. After the immunoperoxidase reaction was complete, the cultures were osmicated, dehydrated, and embedded in Epon for convenient storage.

Results

Dissociated cell cultures, prepared from fetal mouse spinal cords and dorsal root ganglia and maintained for more than 4 weeks, were stained for substance P-like immunoreactivity. Substance P was detected in cultures fixed by several methods, although the initial fixation in freshly prepared 4% paraformaldehyde yielded optimal cell preservation, strong staining, low background, and minimum contamination with nonspecific precipitates. Antiserum was tested at several concentrations; a 1:1000 dilution yielded distinct staining with low background. There was no immunoreactivity in cultures treated with antiserum preabsorbed with synthetic substance P at 1 μg/ml or in cultures treated with normal rabbit serum (1:1000).

The general morphology of the neurons in "mature" SC-DRG cultures has been described (Nelson et al., 1981; Ransom et al., 1977c). Briefly, one large (~40-μm-diameter) bulbous cell type was identified unequivocally by electrophysiologic and morphologic criteria as a DRG neuron. Large (>30-μm-diameter) multipolar cells in which spontaneous excitatory and inhibitory synaptic activity was recorded were assumed to be SC neurons. The remainder of the neurons were small and multipolar, presumably derived from SC, or were small and rounded and could have originated from either SC or DRG. Cultures prepared from DRG alone and grown in the continuous presence of NGF contained large neurons with round somata of rather uniform size; no multipolar neurons were found in these "pure" DRG cultures. Cultures prepared from SC tissue from which the DRGs were removed and regardless of whether NGF were present contained all of the cell types mentioned except the large bulbous neuron.

These SC-DRG cell cultures were strongly immunoreactive for substance P and staining was observed in both cell bodies and in neuronal processes (Fig. 1). Nonneuronal "background" cells were clearly nonreactive. Staining was detected most easily when preparations were viewed with dark-field optics (Fig. 1*B*) although morphologic details were best studied using bright-field and phase contrast microscopy.

A frequent staining pattern was that of an isolated network of beaded processes with no stained cell body in evidence. A small percentage of neuronal somata were clearly stained, however, and these cells fell into one of three morphologic types. The most commonly occurring neuronal soma that displayed substance P-like immunoreactivity was densely stained, small (~20 μm diameter), and rounded and associated with a complex of delicate beaded processes which could not, however, be traced unambiguously to a point of emergence from the soma (Figs. 2*A* and 3, *A* to *E*). A second stained cell type closely resembled the small round cell in size, shape, and dense uniform stain, although several processes were seen clearly to emanate from the soma (Fig. 3, *F* to *I*). A third cell type, the least frequently seen, was the distinctly multipolar neuron (Figs. 4*B* and 5) ranging in diameter from ~15 to 30 μm and with up to seven emerging cell processes. The brown reaction product within these somata appeared perinuclear and punctate and paler than that in the small round cells. Because of their multipolar morphology, the second and third stained cell types are most probably SC neurons. On morphologic criteria alone, it would be difficult to classify the first cell type as either SC or DRG. However, large DRG neurons clearly were not immunoreactive for substance P (Fig. 3*C*, *arrow*) and typically were devoid of contacts by stained processes.

Generally, all processes emerging from a given cell were equivalently stained; staining was not distinctive to one process that might be identified as the axon. Immunoreactive processes were unfailingly varicose (Fig. 6*A*) and easily discriminated from the unstained processes with which they were associated (Fig. 6*C*). Relationships between stained and unstained cells ranged from contacts comprised of only a few stained swellings (Fig. 4*C*) to rather heavy investments in which unstained neuronal somata were enclosed by "baskets" of stained processes (Figs. 2*B* and 6*B*). Apparent autaptic contacts between a neuron and a branch of its own axon (van der Loos and Glaser, 1972) were seen occasionally (Figs. 4*B* and 5*D*).

Discussion

There are a number of issues involved in the identification of the neurons showing substance P-like immunoreactivity in dissociated SC-DRG cell cultures.

Reactive somata comprised a small percentage (less than 5%) of the total neuronal population. Of those neurons with reaction product in the cell body, a minority showed the characteristic morphology of multipolar SC neurons. That substance P-like immunoreactivity was demonstrated in SC neurons is consistent with reports that cell bodies in intact SC (laminae I to V) could be stained after local injection of colchicine (Hökfelt et al., 1977; Ljungdahl et al., 1978). Colchicine treatment of SC-DRG cultures did not appear to increase the frequency of stained neurons. Similarly, colchicine was not greatly effective in increasing either the intensity or frequency

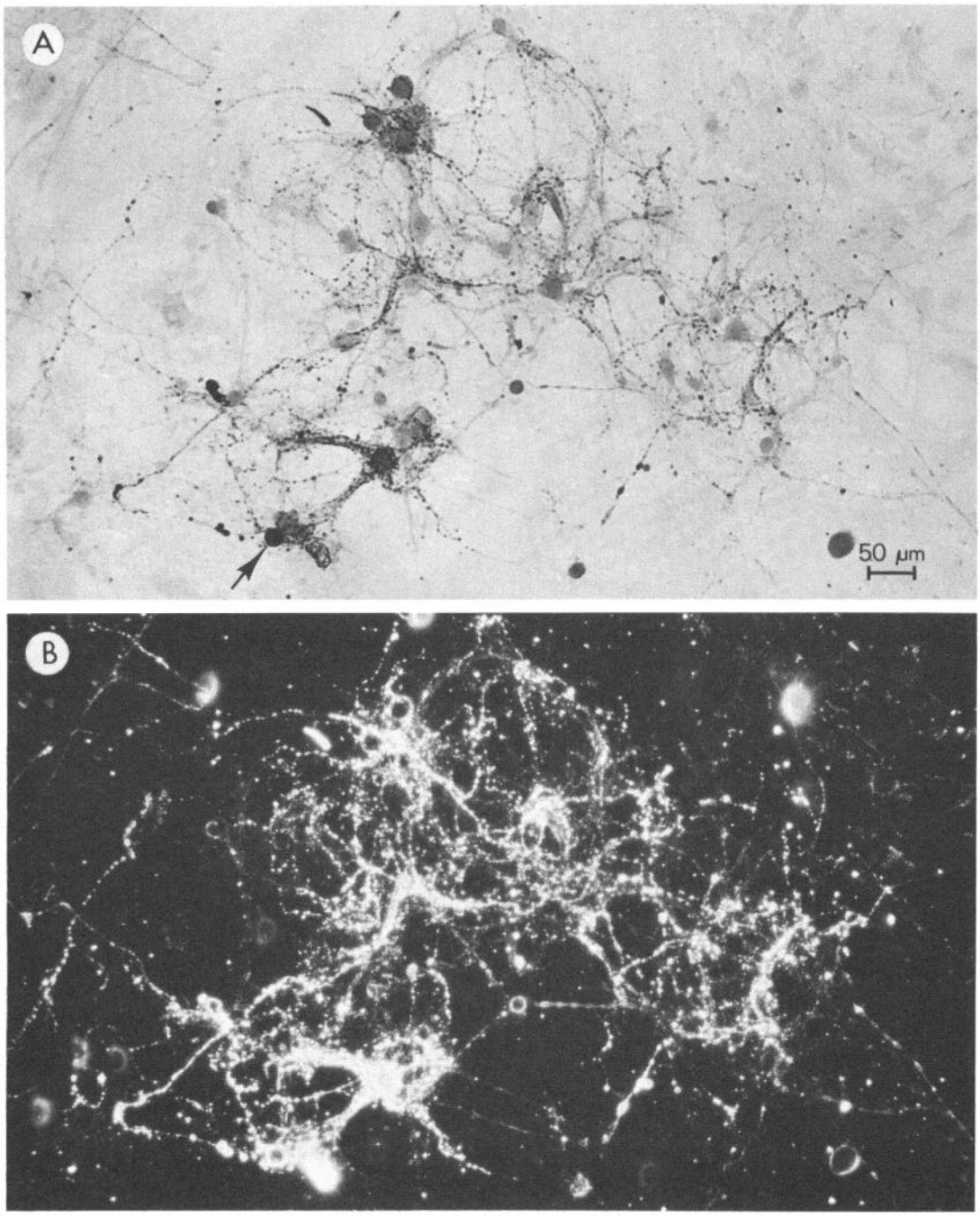

Figure 1.[3] A neuronal network photographed using bright-field (*A*) and dark-field (*B*) optics. Positively stained neuronal processes course among nonreactive neurons. An immunoreactive neuronal soma is indicated by the *arrow* in *A*. Selected areas of this field are shown at higher magnification in Figure 2. Magnification × 120.

of immunofluorescent cell bodies in cultured chick spinal ganglia stained for substance P (Schultzberg et al., 1978).

The majority of stained neuronal somata were small and rounded. Such cells might correspond to the small diameter substance P-containing neurons of the intact DRG (Hökfelt et al., 1975a, b, 1976) or, alternatively, they might represent intrinsic SC neurons. Pure DRG cultures, continuously treated with NGF and reacted immunocytochemically for substance P, showed staining of neuronal processes which did not occur with substance P-absorbed antiserum and an apparent nonspecific staining of neuronal somata which persisted after reaction

[3] Figures 1 to 6 are photomicrographs of dissociated cell cultures prepared from fetal mouse spinal cords and dorsal root ganglia and maintained for at least 4 weeks prior to immunocytochemical reaction for substance P. Cultures were fixed with 4% paraformaldehyde (except where noted) and reacted with a 1:1000 dilution of antiserum directed against substance P and made visible with the peroxidase-antiperoxidase technique. The cultures were osmicated and embedded in Epon before photomicrography.

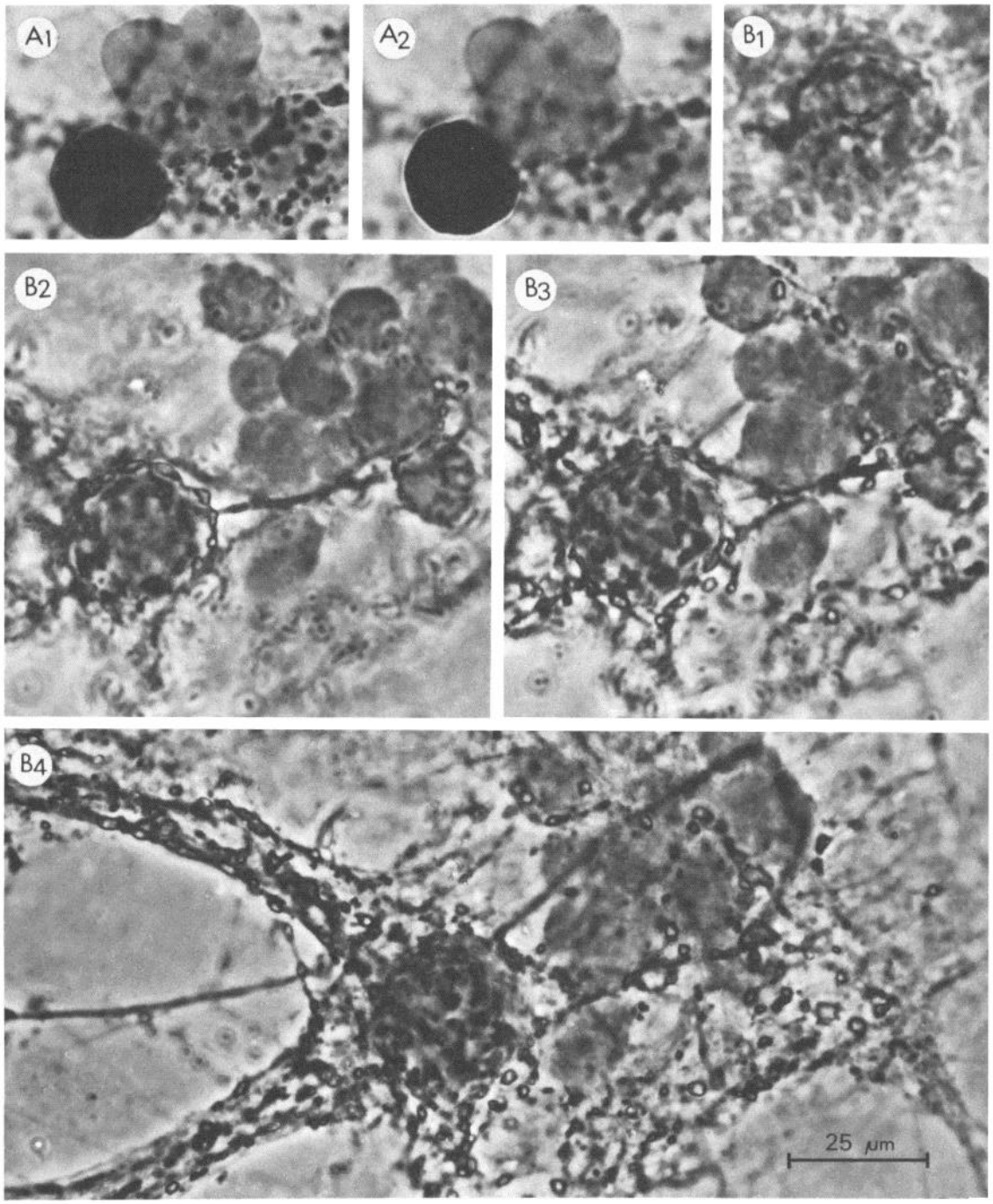

Figure 2. Neuronal soma and processes showing substance P-like immunoreactivity. A_1 and A_2, The densely stained cell body is one among a cluster of similar, though nonreactive, rounded somata and is shown in two focal planes using bright-field optics. B_1 to B_4, Immunoreactive neurites lie in close association with unstained neurons. With phase contrast optics, the larger stained varicosities appear "bright." Varicose processes are wrapped around a large neuronal soma, and labeled swellings stud the surfaces of other smaller neurons visible in the field. Both are shown in several focal planes using phase contrast optics. Magnification × 800.

with the absorbed antiserum (E. A. Neale, E. Matthew, E. A. Zimmerman, and P. G. Nelson, unpublished data). The specific reactivity of processes and not somata might be characteristic of DRG neurons in mixed SC-DRG cultures. It is difficult to correlate the observations obtained from the mixed SC-DRG cultures with those obtained in cultures of DRG alone. The small immunoreactive cells in the mixed cultures may reflect DRG neurons whose differentiation had been influenced by coculture with SC elements. In contrast, the morphology of large DRG cells in pure cultures of sensory neurons may reflect the absence of trophic influences from SC cells as well as continuous treatment with NGF.

The neurons in culture generally showed staining of all

neurites emerging from a reactive soma. This neurite staining might be explained, in the case of DRG neurons, by the synthesis of substance P in the cell body and transport along both central and peripheral processes. Although detailed morphologic analyses of intrinsic substance P-containing SC neurons are not available, a dendritic distribution of substance P in central neurons has been noted (Chan-Palay, 1979) and is similar to that seen in the small multipolar neurons in SC-DRG cultures.

Frequently, extensive and intricate fiber networks of strongly immunoreactive beaded processes without associated stained somata were seen under the stated reaction conditions. The lack of somatic staining might be explained by the presence of a precursor molecule whose

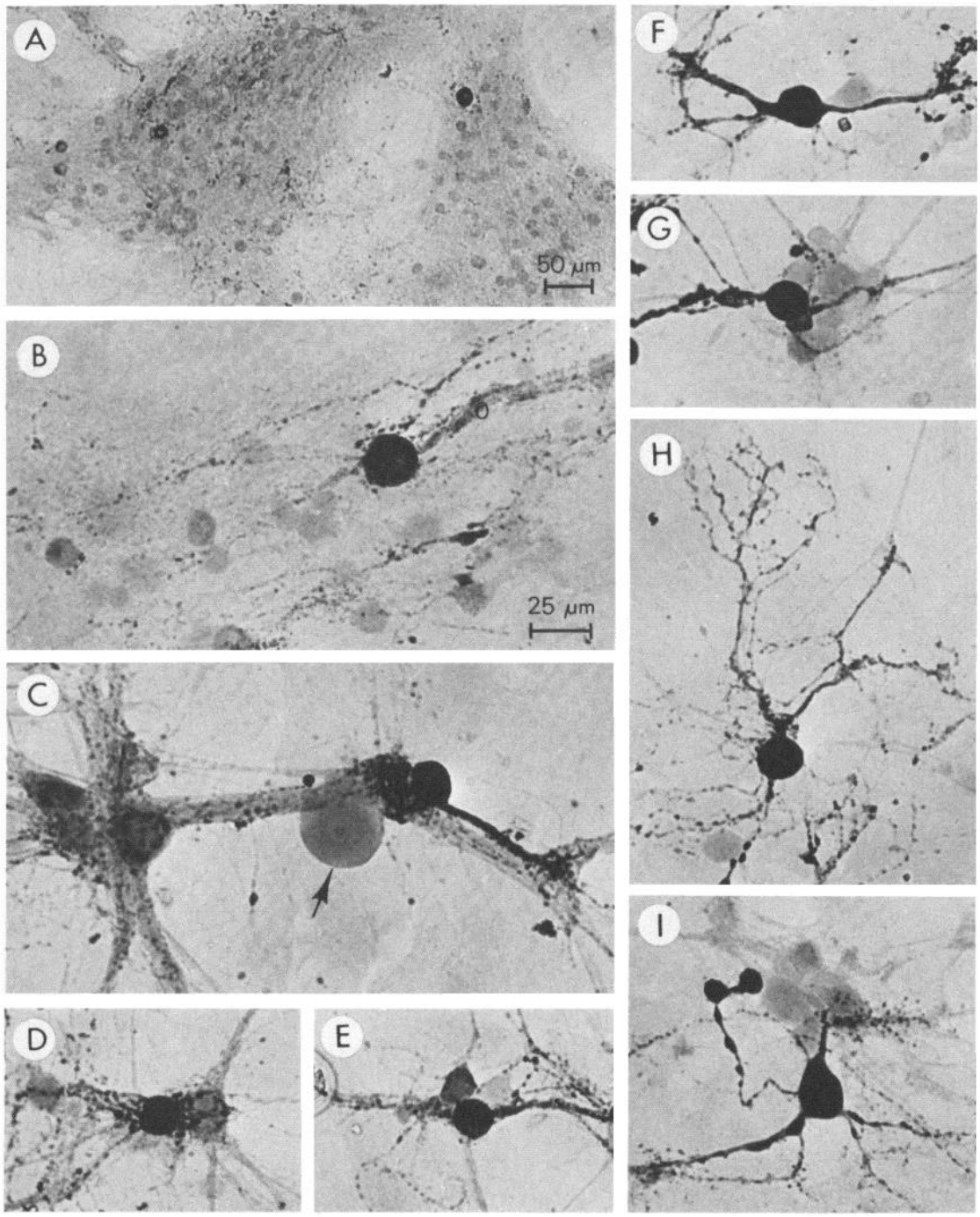

Figure 3. Morphologic variation among neurons displaying immunoreactivity for substance P. *A*, Low magnification view of closely packed neurons, two of which show positive reactivity. *B* to *E*, Positively stained rounded neuronal somata, ~20 μm in diameter, associated with delicate beaded processes. Large dorsal root ganglion neurons, such as that shown in *C* (*arrow*), are consistently unstained. *F* to *I*, Positively stained somata, generally rounded in form, but from which several stained processes clearly emerge. Bright-field optics were used. Magnification: *A*, × 120; *B* to *I*, × 320.

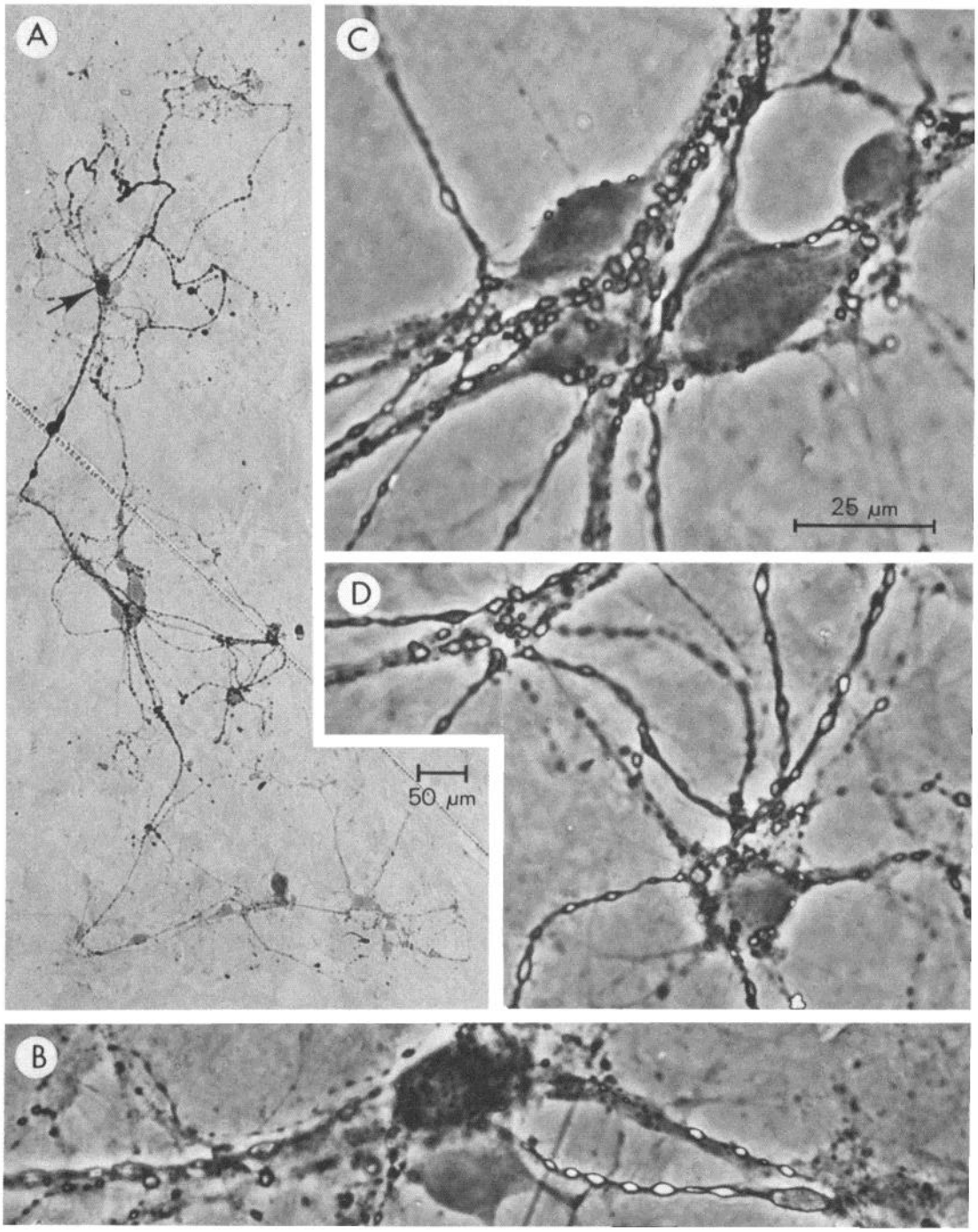

Figure 4. A field of neurons in a culture stained for substance P. *A*, Low magnification view of an immunoreactive multipolar neuron (*arrow*) and its processes. Portions of this field are shown at higher magnification in *B* to *D*. *B*, The stained soma, lying adjacent to another unstained neuron, exhibits a pale, diffuse reaction. The beaded processes are stained more intensely than the soma and frequently appear to impinge upon the soma from which they originated, suggestive of autaptic contacts. *C* and *D*, Several nonreactive neurons in close association with positively stained varicose fibers. Phase contrast optics were used for *B* to *D*. Magnification: *A*, × 120; *B* to *D*, × 800.

reactive sites are not exposed until later steps in molecular "processing" (Hökfelt et al., 1975b) which occurs along neurites (Gainer et al., 1977) or, alternatively, by a relatively rapid synthesis and transport away from the cell body (Hökfelt et al., 1975b).

While some of the varicosities observed in these preparations may correspond to conventional chemical synaptic boutons or perhaps to sites of accumulation and/or release of neuropeptides (see Barber et al., 1979), it is also likely that many of the varicosities are "induced" by some aspect of the immunocytochemical reaction. The disposition of labeled swellings in Figure 4*C* is suggestive

of specific intercellular contacts. Labeled swellings along processes showing similar associations with nonreactive neural elements were seen when selected neurons were injected with horseradish peroxidase (HRP) (E. A. Neale et al., 1978; Nelson et al., 1978). Such swellings exhibited the ultrastructural criteria for chemical synapses and corroborated the electrophysiologic findings of synaptic connectivity between pairs of studied neurons. The HRP-injected neurons were large cells with long, uniform diameter axonal processes and nonbeaded dendrites. In contrast, neurons stained for substance P immunoreactivity were small and showed beading of all labeled processes. Some swellings were quite large (Fig. 3*I*) and occasionally appeared unconnected. In Figure 6, *A* and *C*, beading appears exaggerated in the immunoreactive neurites, whereas nonreactive processes show relatively smooth contours. Neither paraformaldehyde fixation nor HRP histochemistry alone induced beading. Although

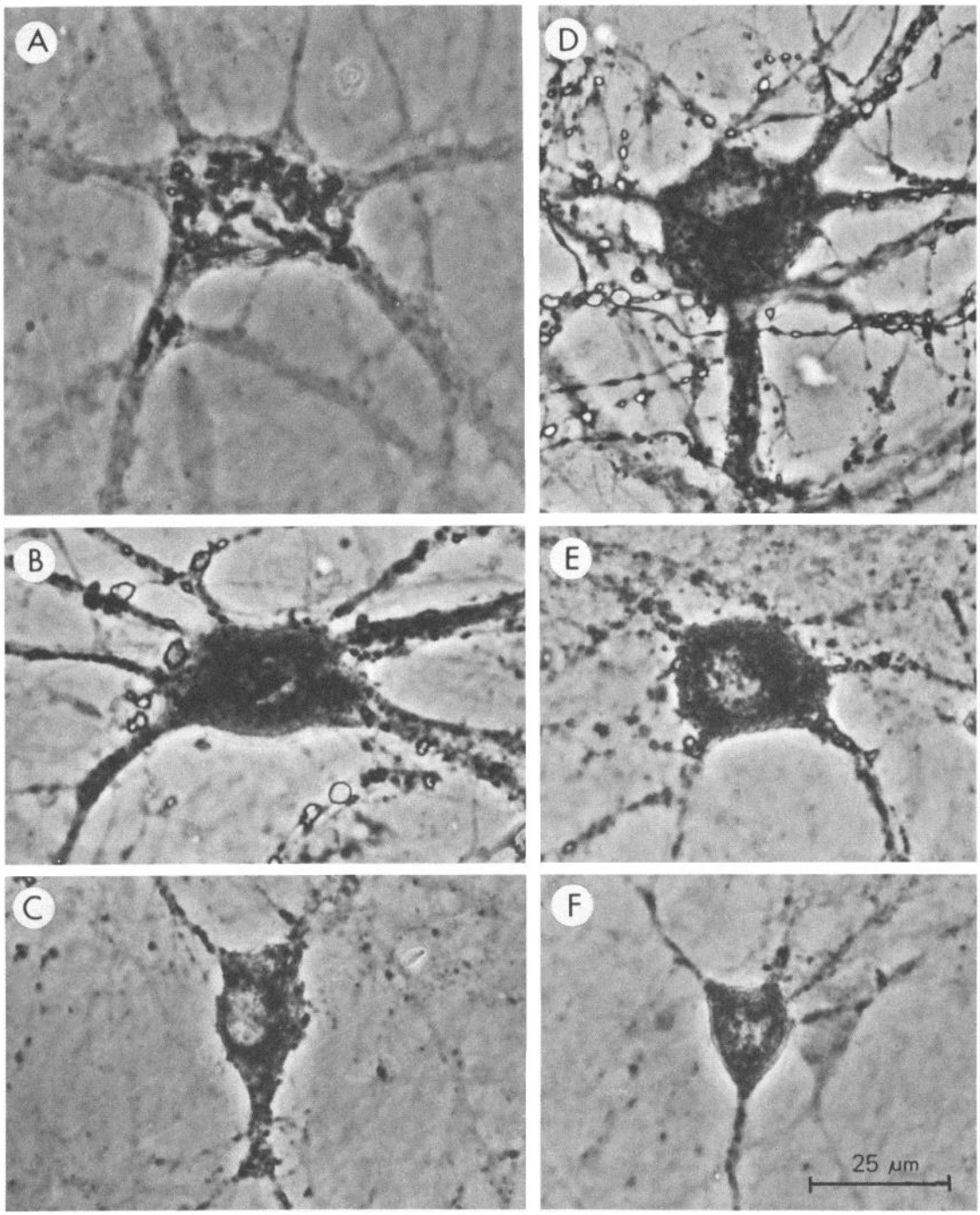

Figure 5. Substance P-like immunoreactivity expressed in neurons that are distinctly multipolar and range in soma diameter from ~15 to 30 μm. Relative to that shown in Figure 3, the staining is pale, the reaction product appears somewhat granular, and nuclei are seen as unstained. *A*, *C*, *E*, and *F* were fixed initially in Bouin's solution. Phase contrast optics were used. Magnification × 800.

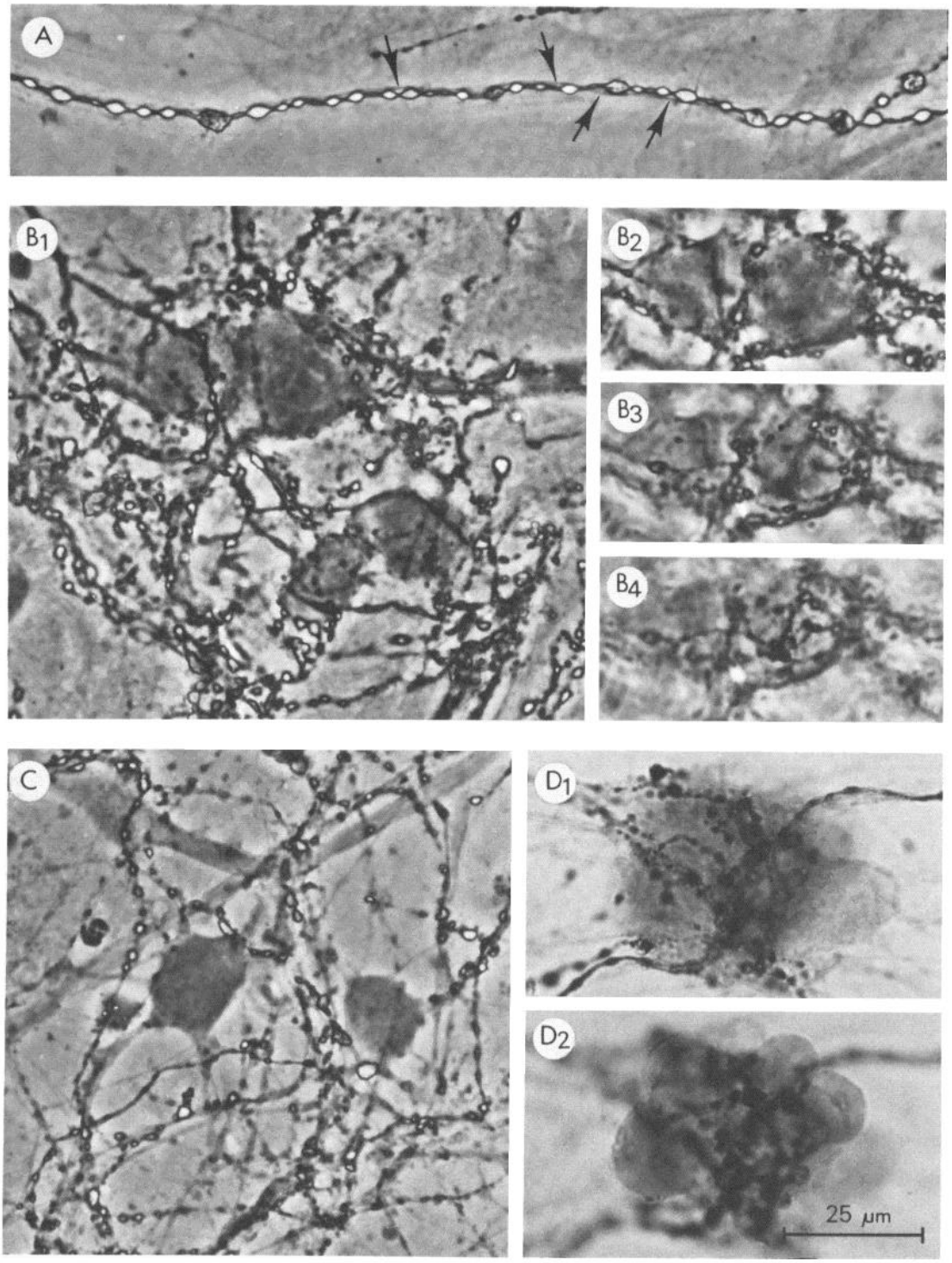

Figure 6. Positively stained neuronal processes. *A*, Stained neurites are clearly varicose and frequently are seen closely applied to nonreactive processes (*arrows*). The swellings vary in size and usually are seen to be connected by fine strands. B_1 to B_4, Stained processes encrust nonreactive neural elements. *C*, Although stained and unstained processes intermingle, the two are easily discriminated. D_1 and D_2, Stained neurites course through a cluster of neuronal somata. For *A* to *C*, phase contrast optics were used; for *D*, bright-field optics were used. Magnification × 800.

this degree of beading might be characteristic of substance P neurons, it is also possible that some aspect of the immunocytochemistry has exaggerated or even induced the varicosities.

Dissociated neuronal cell cultures provide an opportune system for the study of morphologic, pharmacologic, and electrophysiologic aspects of neuronal function, and it was hoped that immunocytochemical analysis might

provide a useful morphologic marker for pre-selection of specific peptide-containing cells. However, with the exception of their generally small size, it was not possible to characterize the substance P neurons unequivocally. Nonetheless, these *in situ* immunocytochemical techniques allow initial electrophysiologic analysis of selected small neurons to be followed by subsequent identification of substance P cells. The enrichment of cultures for small neurons (Schnaar and Schaffner, 1981) ultimately could increase the possibilities for study of substance P neuronal physiology.

Neurons in dissociated SC-DRG cell cultures contain a variety of neuropeptides (Matthew et al., 1979; J. H. Neale et al., 1978; Nelson et al., 1980). The anatomical resolution inherent to the culture system is most advantageous for sequential or double label staining procedures to study the relationship of substance P with other peptides or transmitters (Hökfelt et al., 1980a, b).

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