Neuron/Glia Relationships Observed Over Intervals of Several Months in Living Mice

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Abstract. Identified neurons and glial cells in a parasympathetic ganglion were observed in situ with video-enhanced microscopy at intervals of up to 130 d in adult mice. Whereas the number and position of glial cells associated with particular neurons did not change over several hours, progressive differences were evident over intervals of weeks to months. These changes involved differences in the location of glial nuclei on the neuronal surface, differences in the apparent number of glial nuclei associated with each neuron, and often both. When we examined the arrangement of neurons and glial cells in the electron microscope, we also found that presynaptic nerve terminals are more prevalent in the vicinity of glial nuclei than elsewhere on the neuronal surface. The fact that glial nuclei are associated with preganglionic endings, together with the finding that the position and number of glial nuclei associated with identified neurons gradually changes, is in accord with the recent observation that synapses on these neurons are normally subject to ongoing rearrangement (Purves, D., J. T. Voyvodic, L. Magrassi, and H. Yawo. 1987. Science (Wash. DC). 238:1122–1126). By the same token, the present results suggest that glial cells are involved in synaptic remodeling.

Materials and Methods

Young adult male mice (CF1 strain; 25–30 g) were anesthetized with chloral hydrate (0.6 g/kg, i.p.) and placed on the stage of a microscope modified to allow observation of neurons in living animals (Purves et al., 1986; Purves and Lichtman, 1987; Purves et al., 1987). The right sublingual and submandibular salivary ducts were exposed surgically under a dissecting microscope; in mice, the salivary duct ganglia are relatively small collections of parasympathetic neurons (up to several hundred) covered by a thin, transparent capsule. As in some other parasympathetic ganglia in small mammals, these neurons do not have dendrites (Snider, 1987). Once the ducts and their associated ganglia had been freed from overlying connective tissue, these structures were gently lifted on a reflective, chrome-plated support to stabilize them for microscopic observation (Purves and Lichtman, 1987; Purves et al., 1987). The ducts and ganglia were examined under epillumination at low power with a Newvicon video camera (model 67MI; DAGE-MTI, Inc., Wabash, MI) mounted on a compound microscope; the light source was a 50-W Hg bulb, attenuated by a variable neutral density filter. Illumination of the ganglia in this manner allowed us to see the superficial neurons and glial cells by virtue of asymmetric illumination-contrast (Fig. 1) (Purves et al., 1986; Purves and Lichtman, 1987).

A small portion of the surface of one of the salivary duct ganglia was then observed under higher power with a 100× water immersion objective (NA 1.2; E. Leitz, Inc., Rockleigh, NJ) and one to five neurons were selected for detailed study. The criterion for selection of a given neuron was location near glial nuclei, taken together with the observation that glial nuclear position gradually changes, implies that glial cells are active participants in the process of synaptic rearrangement.

Figure 1. Video image of a salivary duct ganglion cell and an associated glial cell in a living mouse. The ganglionic neurons are relatively large mononuclear cells, the longest dimension of which usually ranges between 20 and 40 μm. The glial cells associated with these neurons are evident in situ primarily by virtue of the glial nucleus and the greater amount of glial cytoplasm in this region (arrow). In this and subsequent figures, the original image has been improved by averaging, a local background-subtraction convolution, and contrast enhancement.

of the glial nuclei associated with each neuron could be determined. Video images of up to six focal planes/neuron were digitized, enhanced, and stored as a permanent record. For this purpose we used a Trapix 5500 image processor (Recognition Concepts, Inc., Incline Village, NV) and IMAGR, a general purpose computer language for image processing (Purves and Voyvodic, 1987). A series of images at lower powers was also obtained to facilitate the location of the neurons of interest at a later time. Once satisfactory images had been stored, the wound was closed and the animal allowed to recover.

To view the same cell once again, this entire procedure was repeated after an interval of up to 130 d. The previously identified neurons were found using the images that had been saved in computer disk files, and a second set of images was acquired. The two sets of images were then compared to assess the stability of the association between neurons and glia. Different animals were used for each time interval. A total of 181 neurons from 90 animals were examined in this way; the large number of mice used reflects the fact that, on average, only two neurons were followed in each animal (since mice do not survive prolonged anesthesia).

For electron microscopic examination of ganglia, the salivary ducts were removed from anesthetized mice after superfusion of the ganglia with 2% glutaraldehyde and 2% paraformaldehyde in phosphate buffer. The ducts and their associated ganglia were left in the fixative overnight. After postfixation for 1 h in 1% osmium tetroxide and 0.1% cacodylate buffer, the tissue was dehydrated and embedded in araldite. Thin sections were stained with lead citrate and uranyl acetate and viewed with an electron microscope (model EM10; Carl Zeiss, Inc., Thornwood, NY).

For studies of glia by fluorescent labeling, glass microelectrodes were filled with 5% (6)-carboxyfluorescein (wt/vol) in 0.44 M KOH (pH 7.6; electrode resistance 130–180 MΩ) (Purves et al., 1986). Using a 40× water immersion objective (NA 0.75; Carl Zeiss, Inc.), the electrode tip was centered over the perinuclear region, and the glial cell impaled by oscillating the amplifier briefly. After a cell had been impaled, hyperpolarizing current pulses were passed through the electrode for 2 min (2–3 nA, 50 ms, 5 Hz). The labeled glial cells were then viewed with epifluorescence illumination (H2 cube; E. Leitz, Inc.) and digitized video images recorded.

Results

The Relation of Neurons and Glia Determined by Intracellular Marking

To assess the general relation of glial cells and ganglionic neurons, we injected a total of 75 glial cells (21 animals) with fluorescent dye (Fig. 2). In all cases, the neuron associated with the injected glial cell was completely surrounded by a thin fluorescent sheath at the end of the 2-min injection period. The glial nuclei associated with a neuron also stained intensely with fluorescent dye (see Fig. 2); it was thus evident that individual neurons are associated with up to three glial nuclei, presumably representing different glial cells. These nuclei were usually distributed on the neuronal surface and did not bear a consistent relationship to the axon hillock. In 19 of the 75 neurons examined, glial cells associated with one or more neighboring neurons also became visible after several minutes (not shown). Thus glial cells are sometimes dye coupled to glia associated with nearby neurons.
associated with a neuron, the glia cells are strongly dye coupled. Furthermore, it is evident that only a portion of the glial cell—the nucleus and the perinuclear cytoplasm—is seen in situ in the absence of dye injection (cf. Fig. 1). Accordingly, when we refer to glial position in what follows, we necessarily mean the position of the nucleus and the perinuclear portion of the cell that can be observed in the living animal.

Imaging Neurons and Their Associated Glia over Short Intervals

When identified neurons were located one or a few hours after the first imaging procedure, the same number of associated glial cell nuclei was always found in the positions originally occupied (Fig. 3 and Table I). Not infrequently, a glial nucleus was better seen at one viewing or the other, due, for example, to a slight rotation of the ganglion. Nevertheless, the fact that we could in all cases see the same arrangement of neurons and glia at short intervals indicated that any changes observed over longer intervals were unlikely to arise from any limitations of our imaging methods.

Changes in Neuron/Glial Relationships over Longer Intervals

When identified neurons were observed after longer intervals, substantial differences were found in the number and position of the associated glial cells. The changes included the following: (a) the appearance of an additional glial cell nucleus; (b) the disappearance of a glial cell nucleus initially seen; or (c) a glial cell nucleus in a new position. With respect to the last of these categories, a glial nucleus was judged to be in a new location when it occupied a position at the second viewing that was $\geq 10 \mu m$ distant in the horizontal plane from the position of any glial nucleus observed at the first viewing. Positional differences in the vertical dimension were more difficult to judge, and were therefore not considered.

The incidence of altered neuron/glia relationships increased with the interval between viewings. After 1 wk, the relation between identified neurons and their associated glia had, by the criteria listed above, changed for $\sim 16\%$ of the neurons studied (Fig. 4 and Table I). By 3 wk, the portion of neurons that showed a change in the number and/or posi-

<table>
<thead>
<tr>
<th>Interval</th>
<th>Neurons showing an increase in associated glial nuclei</th>
<th>Neurons showing a decrease in associated glial nuclei</th>
<th>Neurons showing a change in position of associated glial nuclei</th>
<th>Neurons showing a change in number and/or position of associated glial nuclei</th>
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<tr>
<td>1–6 h</td>
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<tr>
<td>110–130 d</td>
<td>n=39, n=16</td>
<td>38.5%</td>
<td>10.3%</td>
<td>51.3%</td>
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Table I. Proportion of Identified Neurons That Show a Change in the Number and/or Position of Their Associated Glial Cell Nuclei at Various Intervals
Figure 5. Video images of an identified ganglion cell and its associated glial cells observed over a period of several weeks. In this example, a glial nucleus (arrow, right-hand panel) is apparent at a new location after the 21-d interval between observations; the glial nucleus at the original position (arrow, left-hand panel) was no longer evident. Approximately the same focal plane from the focus-through series (see Fig. 4) is shown in the two images.

Figure 4. Focus-through series of video images of an identified ganglion cell and its associated glial cells observed after an interval of ~1 wk. Comparison of focal planes obtained during the initial imaging procedure (A) with those obtained 9 d later (B) demonstrates the appearance of a glial nucleus that was not previously seen (large arrows). A second glial nucleus associated with the neuron was still present in the same position at the end of this period of observation (small arrows).

Association of Glial Nuclei and Preganglionic Nerve Terminals

Examination of neurons and their associated glial cells by EM confirmed the arrangement of neurons and glia deduced from dye injection. Each neuron was fully ensheathed by a thin lamella of glial cytoplasm (Fig. 7); the sheath was sometimes interrupted by complex interdigitations that presumably represented regions where the lamellae of two glial cells came together. In confirmation of an impression gained from studies in which preganglionic terminals were vitally stained (Purves et al., 1987), we also noted that vesicle-filled preganglionic nerve terminals appeared to be more prevalent in the vicinity of the glial nuclei than in regions removed from this site. The preganglionic nerve terminals tended to establish synaptic contacts in a complex of short finger-like extensions of the neuronal soma interdigitated with processes arising from the glial cell (Fig. 8).

The apparent relationship of glial nuclei and preganglionic terminals was evaluated more systematically by detailed examination of 192 neuronal profiles in electron micrographs taken from 10 ganglia. Each of these profiles was selected for the following characteristics: (a) the presence of a neuronal nucleus (thus indicating a plane of section through the mid-portion of the neuron); (b) the presence of a single associated glial nucleus; and (c) the presence of at least one vesicle-filled profile around the perimeter of the neuronal profile. The circumference of each neuron was traced from the photomicrograph using a digitizing tablet, and the positions of the glial nucleus and each vesicle-filled profile mapped onto the tracing (Fig. 7b). The neuronal circumferences were normalized so that we could plot the location of preganglionic terminals on the cell body surface as a function of relative distance from the glial nucleus (Fig. 9). This analysis showed that the incidence of vesicle-filled profiles was about fivefold greater in the region immediately adjacent to a glial nucleus than in the region of the cell surface furthest removed from this site.
Figure 6. Video images of an identified ganglion cell and two associated glial cells visualized after an interval of several months. Approximately the same focal plane is shown in the two images. The glial nuclei present at the first observation (arrows) are no longer apparent at the second. Another glial nucleus at a different location in a deeper focal plane (not shown) was also evident at the final observation 124 d later. The great majority of neurons studied after 110–130 d showed changes in the number and/or position of their associated glial nuclei (Table I).

Discussion

Our interpretation of these findings can be summarized as follows. Observations of identified nerve cells in situ have shown that neuronal processes in mammalian autonomic ganglia normally undergo continual remodeling in adult animals. Thus, the dendrites of mouse superior cervical ganglion cells extend and retract over intervals of weeks to months (Purves et al., 1986), and preganglionic nerve terminals on salivary duct ganglion neurons gradually change their configuration over intervals of several weeks (Purves et al., 1987). Glial cells, or at least the portions of glial cells that can be readily seen in the living animal, also change their relationship to neurons over time. Because preganglionic terminals usually occur on neuronal fingers that are intertwined with glial cell processes in the vicinity of the glial nucleus, some coordination of synaptic and glial change is implied. Accordingly, it seems likely that glial cells participate, perhaps actively, in synaptic remodeling.

Uncertainties That Affect the Interpretation of These Results

One important issue that bears on this interpretation is whether the changes we have observed represent the normal behavior of these neurons and their associated glia, or are the result of our intervention.

There are several reasons why these altered neuron/glial relationships are unlikely to be artifactual. First, the ganglia are minimally disturbed during the imaging procedure and the transparent capsule covering the principal neurons is left intact. Second, the neurons and glia appeared healthy at the second viewing; neither inflammatory cells within the ganglion nor neuronal loss was noted. Perhaps most important, the changes we observed were gradually progressive over longer intervals. If these changes in neuron/glial associations had been a response to injury one would not expect such rearrangements to continue weeks or months later.

Another concern is that we could observe only the portion of the glial cell that included the nucleus and surrounding cytoplasm; the lamellar glial sheath that surrounds each neuron is invisible at the light microscopic level in the absence of intracellular dye injection. Thus, the extent to which the extranuclear portion of the glial sheath might change is not known. In consequence, we cannot say whether relocation of a nucleus indicates nuclear movement, or relocation of the entire glial cell. Moreover, since we had no method of marking individual glial cells for long periods, we do not know whether the changes observed represent movements of glial cells which remain associated with the neuron under study, or the migration of glial cells from one neuron to another. Finally, we have no information about glial proliferation or death. It is known that a few glial cells in autonomic ganglia of adult cats and rats incorporate tritiated thymidine (Schwyn, 1967; Dropp and Sodetz, 1971), indicating that some glial proliferation occurs normally in these animals. Finding an additional glial nucleus associated with an identified neuron could thus result from either cell division or migration. Similarly, loss of a glial nucleus could result from either death or migration. In spite of these uncertainties, it is evident that the relationship of neurons and glial cells in this ganglion is a highly dynamic one.
Figure 7. Electron microscopical appearance of a ganglion cell and an associated glial cell, showing the method for evaluating the location of axon terminals with respect to the location of a glial nucleus. (a) The glial sheath of salivary duct ganglion neurons is attenuated around the majority of the neuronal perimeter (see also Fig. 2). Near the nucleus, however, the glial cytoplasm is more abundant (see tracing in b). The boxed areas are enlarged to show the thickness of the glial sheath away from the perinuclear region (arrowheads; lefthand box), and a preganglionic terminal in the thickened perinuclear region (arrowhead; righthand box). (b) Vesicle-filled profiles (asterisks) are frequently found on the region of the neuronal surface near the glial nucleus. The location of synapses with respect to the position of glial nuclei was analyzed by tracing the perimeter of each neuronal profile and glial nucleus on a digitizing tablet; the positions of vesicle-filled profiles were then mapped onto the neuronal perimeter (arrows). The center of the glial nucleus (black dot) was also mapped onto the neuronal perimeter (open arrow; see Fig. 9).

A Possible Role for Glial Cells in Synaptic Maintenance and Remodeling

Our suggestion that these glial cells play a part in synaptic maintenance and remodeling is indirectly supported by several other observations. For instance, it is now known that glial cells, at least in some circumstances, are a rich source of growth factors. Thus Schwann cells, a cell type similar or identical to the glial cells in autonomic ganglia (Gabella, 1976; Pannese, 1981), synthesize nerve growth factor (NGF)\(^1\) and other trophic molecules to which some growing axons...

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\(^1\) Abbreviation used in this paper: NGF, nerve growth factor.
Figure 8. A higher power electron micrograph of synaptic contacts made in the vicinity of a glial nucleus (g). Preganglionic synaptic contacts (arrow) were usually found on fingers (f) projecting from the neuronal soma (n); such fingers were typically intertwined with similar projections arising from the glial cell.

Figure 9. The tendency of preganglionic axon terminals to be located in the vicinity of glial nuclei. The incidence of preganglionic synapses on neuronal profiles was plotted as a function of distance from the glial nucleus (0 on the abscissa); the histogram was then made by normalizing the neuronal circumferences for all 192 profiles studied and measuring the distance of each preganglionic terminal from the centerpoint of the glial nucleus (see Fig. 7 b). The incidence of nerve terminal profiles is about fivefold higher in the vicinity of a glial nucleus than in the region furthest removed from the nucleus. Note that relatively fewer nerve terminals lie directly beneath the nucleus, perhaps for mechanical reasons (see also Fig. 7 a).

respond (Riopelle et al., 1981; Heumann et al., 1987; Villegas-Perez et al., 1988).

Another relevant line of evidence concerns the response of autonomic neurons to postganglionic axotomy. When the connection between ganglion cells and their peripheral targets is interrupted by surgical or pharmacological means, the majority of synapses on ganglion cells are lost over a period of ~1 wk and are recovered coincident with regeneration of the postganglionic axons to peripheral targets (Matthews and Nelson, 1975; Purves, 1975, 1976; Brenner and Johnson, 1976). Moreover, trophic agents such as NGF appear to be involved in synaptic maintenance (Njå and Purves, 1978). Thus, exogenous NGF can largely prevent the synaptic detachment that occurs after postganglionic axotomy, whereas treatment of animals with NGF antiserum mimics the effects of axotomy, causing a loss of ganglionic synapses. The recent
demonstration that axotomy stimulates the synthesis of NGF and NGF receptors by Schwann cells in mammalian peripheral nerves (Heumann et al., 1987; Taniuchi et al., 1988) adds further interest to the possibility that the glia in autonomic ganglia are involved in normally occurring synaptic rearrangement.

If we are correct in concluding that glial cells in autonomic ganglia participate in the formation and maintenance of ganglionic synapses, an important function for these cells will have been identified that may be relevant to the function of glial cells elsewhere in the nervous system.

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