Summary

Two different monoclonal antibodies to the Thy-1 antigen, T11D7 and 2G12, were used to purify and characterize retinal ganglion cells from postnatal rat retina. Although Thy-1 has been reported to be a specific marker for ganglion cells in retina, retinal cell suspensions contained several other types of Thy-1-positive cells as well. Nevertheless, a simple two-step "panning" procedure allowed isolation of ganglion cells to nearly 100% purity. We found that postnatal ganglion cells differed in antigenic, morphological, and intrinsic electrophysiological characteristics, and that these properties were correlated with one another. Minor variations of this panning protocol should allow rapid, high yield purification to homogeneity of many other neuronal and glial cell types.

Introduction

Ideally, in vitro analysis of cellular interactions requires the isolation and culture of utterly purified cell types. Until recently, cellular neurobiology has tended to lag in this approach, largely because of the difficulty in defining and identifying cell types. Such barriers are increasingly being surmounted and, in putatively less complex regions of the CNS such as the retina and optic nerve, many cell types and specific markers have now been identified. Partial purification of specific types of central neurons has previously been possible only for some regions of the brain. The large difference in size of cerebellar granule cells and Purkinje cells has allowed purification by density gradients (Barkley et al., 1973; Hatten, 1985). In other cases labeling by retrograde transport of a fluorescent marker injected into a fiber tract or target nucleus has permitted the use of fluorescent-activated sorters, for instance in the case of cortical motor neurons (Armson and Bennett, 1983; Calof and Reichardt, 1984; O'Brien and Fischbach, 1986). Such methods sacrifice yield for purity, may be time-consuming and require expensive instrumentation, and in general are not well suited to the purification of cells that constitute only a small percent of a cell suspension.

Another method, antibody-mediated plate adhesion ("panning"), allows rapid purification of cells in high yield and to high purity when a specific antibody to a surface component exists (Barker et al., 1973; Mage et al., 1977; Wysocki and Sato, 1978). This method has mainly been used to purify cells from the immune system, but has also been used to purify Schwann cells (Assouline et al., 1983). Although there are now many monoclonal antibodies that recognize specific neural cell types, the panning method has not yet been applied to purification of cells from the central nervous system.

We now report its use to purify retinal ganglion cells from postnatal rats. Since rat retinal ganglion cells have been reported to express specifically the Thy-1 antigen (Beale and Osborne, 1982; Barnstable and Drager, 1984), we initially tried a one-step panning with the anti-Thy-1 antibody, 2G12 (Barnstable and Drager, 1984), to effect their purification. However, we found that postnatal day 8 (P8) retina contains Thy-1-positive cells that are not ganglion cells. Thus it was necessary to use a two-step panning protocol: the first to deplete macrophages and Thy-1-positive contaminants that are not ganglion cells and the second to select Thy-1-positive ganglion cells. This procedure allowed rapid isolation of a population of pure retinal ganglion cells with excellent viability. Minor variations of this protocol should allow purification to homogeneity of many other neuronal and glial cell types.

In mammals, ganglion cells have been shown to vary in their electrophysiological response to illumination in intact retinas (e.g., Kuffler, 1953). Ganglion cells in culture have been reported to be homogeneous in electrical behavior (Lipton and Tauck, 1987), suggesting the possibility that the variable responses in vivo arise extrinsically by their dynamic interaction with other retinal cell types. However, we observed a wide variation in the intrinsic electrophysiological properties of acutely isolated ganglion cells prior to culture. In addition, we found that in developing retina, the intensity of surface Thy-1 labeling was highly variable among retinal ganglion cells, and that their intrinsic electrophysiological properties correlated with both soma size and intensity of Thy-1 labeling.

Results

Preparation of Retrogradely Labeled Rat Retinal Suspensions

Rat retinal ganglion cells were retrogradely labeled by injections of the fluorescent dye, fast blue (Bentivoglio et al., 1980; Kuypers et al., 1980) into both superior collicular brachia. Forty-eight hours later, at P8, the retinas were dissected, and dissociated into a single cell suspension using the protocol of Huettner and Baughman (1986). This protocol is derived from those of Lam (1972) and especially Baden et al. (1978), and produced high retinal cell yields (in both postnatal and adult animals).
Table 1. Summary of Cell Types That Adhere to Anti-Thy-1 Panning Plates

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell ID</td>
<td>macrophage</td>
<td>endothelial</td>
<td>unknown</td>
<td>RGC</td>
</tr>
<tr>
<td>2G12+</td>
<td>no</td>
<td>yes</td>
<td>yes*</td>
<td>yes</td>
</tr>
<tr>
<td>T11D7+</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Diameter (µm)</td>
<td>3-20</td>
<td>9-30</td>
<td>7-9</td>
<td>10-18</td>
</tr>
<tr>
<td>Process-bearing</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Fast blue</td>
<td>occasional</td>
<td>no</td>
<td>no</td>
<td>100%</td>
</tr>
<tr>
<td>Neurofilament*</td>
<td>NT</td>
<td>no</td>
<td>no</td>
<td>100%</td>
</tr>
<tr>
<td>Sodium current</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Macrophage antiserum*</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Percent of 2G12 adherent cells</td>
<td>12</td>
<td>9</td>
<td>43</td>
<td>36</td>
</tr>
<tr>
<td>Percent of T11D7 adherent cells</td>
<td>32</td>
<td>12</td>
<td>0</td>
<td>56</td>
</tr>
</tbody>
</table>

* These cells were labeled in retinal suspensions using indirect immunofluorescence; however, in contrast to type B and D cell that were strongly labeled with 2G12, type C cells were lightly labeled.

As previously reported (Linden and Perry, 1983), 100% of ganglion cells could be labeled reliably by this procedure: All cells that were judged to be retinal ganglion cells, on the basis of Thy-1 expression, morphology, and sodium current expression, contained fast blue. Using the same criteria, we found no evidence of fast blue in cells other than ganglion cells, except for a small percent of macrophages (see Table 1).

Control panning plates, prepared by coating with either anti-IgM or anti-IgG followed by an irrelevant primary antibody of the appropriate isotype, did not cause adherence of any retinal cells, except for macrophages (see below), which are able to bind to the plate either by Fc receptors or nonspecifically.

Retinal cells could also be panned with Thy-1 antibodies of the IgG isotype, if the cells rather than the panning dish were incubated with the Thy-1 antibody. In contrast, with T11D7, an IgM, adherence was only achieved if the primary antibody was placed on the plate.

Retinal Cells Adhere to Plates Coated with Thy-1 Antibodies

As previously reported, many retinal cells adhered tightly to plates coated with Thy-1 antibodies (Leifer et al., 1984; also see MacLeish et al., 1983). As was found for immune cells (Mage et al., 1977; Wysocki and Sato, 1978), the largest number of cells adhered if an indirect antibody coating procedure was used. Panning plates, made from non-wetting polystyrene, were first coated with affinity-purified antibodies to mouse IgG, followed by incubation in 2G12 ascites. When retinal suspensions were placed on these plates, cells adhered almost instantly, although we usually allowed the cells to settle on the dish for 30-60 min at room temperature.

The yield was typically 20 to 25 million cells per P8 retina.

As previously reported (Linden and Perry, 1983), 100% of ganglion cells could be labeled reliably by this procedure: All cells that were judged to be retinal ganglion cells, on the basis of Thy-1 expression, morphology, and sodium current expression, contained fast blue. Using the same criteria, we found no evidence of fast blue in cells other than ganglion cells, except for a small percent of macrophages (see Table 1).

Control panning plates, prepared by coating with either anti-IgM or anti-IgG followed by an irrelevant primary antibody of the appropriate isotype, did not cause adherence of any retinal cells, except for macrophages (see below), which are able to bind to the plate either by Fc receptors or nonspecifically.

Retinal cells could also be panned with Thy-1 antibodies of the IgG isotype, if the cells rather than the panning dish were incubated with the Thy-1 antibody. In contrast, with T11D7, an IgM, adherence was only achieved if the primary antibody was placed on the plate.

Retinal Cells Adhere to Plates Coated with Thy-1 Antibodies

As previously reported, many retinal cells adhered tightly to plates coated with Thy-1 antibodies (Leifer et al., 1984; also see MacLeish et al., 1983). As was found for immune cells (Mage et al., 1977; Wysocki and Sato, 1978), the largest number of cells adhered if an indirect antibody coating procedure was used. Panning plates, made from non-wetting polystyrene, were first coated with affinity-purified antibodies to mouse IgG, followed by incubation in 2G12 ascites. When retinal suspensions were placed on these plates, cells adhered almost instantly, although we usually allowed the cells to settle on the dish for 30-60 min at room temperature.

MRC-OX-7 (Mason and Williams, 1980), an anti-rat Thy-1.1-specific mouse monoclonal IgG antibody, also bound retinal ganglion cells to the plate. In addition, retinal cells adhered to plates coated with the anti-mouse Thy-1.1-specific T11D7 mouse monoclonal, an IgM antibody (Lake et al., 1979).

Control panning plates, prepared by coating with either anti-IgM or anti-IgG followed by an irrelevant primary antibody of the appropriate isotype, did not cause adherence of any retinal cells, except for macrophages (see below), which are able to bind to the plate either by Fc receptors or nonspecifically.

Retinal cells could also be panned with Thy-1 antibodies of the IgG isotype, if the cells rather than the panning dish were incubated with the Thy-1 antibody. In contrast, with T11D7, an IgM, adherence was only achieved if the primary antibody was placed on the plate.

The Adherent Cells Are Not Pure Ganglion Cells

After panning a retinal suspension on a 2G12-coated plate, the cells were divided into two fractions: those that adhered to the plate, and those that did not. The nonadherent cells were examined for the presence of retinal ganglion cells. An occasional fast blue-containing cell could be found, but these were extremely rare, even when 200 million retinal cells were panned on a single dish. Both adherent and nonadherent cell groups appeared the same (see Table 1) regardless of whether 2G12 or MRC-OX-7 was used in the panning procedure.

Four morphologically distinct cell types adhered to a 2G12 panning plate; they will be referred to as A, B, C, and D (Figure 1A). Table 1 summarizes the phenotype of each cell type. Cell type D constituted about 35% of the cells. One hundred percent of type D cells contained the retrograde label and were therefore identified as retinal ganglion cells. Also, they were the largest adherent cells and most bore processes.
Figure 1. Retinal Cells Adherent to Anti-Thy-1 Panning Plates Are Heterogeneous

(A) Cells adherent to a 2G12 panning plate are heterogeneous. A P8 retinal suspension was panned on a 2G12 plate as described in Experimental Procedures. The adherent cells consist of four morphological types labeled A, B, C, and D. Type D cells are retinal ganglion cells, while types A, B, and C are contaminating cell types (see text).

(B) Cells adherent to a T11D7 panning plate are also heterogeneous, but have one less contaminating cell type: type C cells did not adhere. Calibration: 25 μm.

Type A Cells Are Macrophages

Cell type A appeared flat and vacuolated and constituted about 12% of adherent cells. These cells were initially identified on morphological grounds as macrophages or microglial cells. Consistent with this idea, these cells adhered to control plates containing either no antibody, secondary antibody alone, or secondary antibody plus an irrelevant primary antibody (anti-galactocerebroside). They were also labeled by a rabbit antiserum to rat macrophages (see below), but not by any of the anti-Thy-1 antibodies. Most did not contain fast blue, but some were moderately labeled, perhaps by phagocytotic incorporation.

Type B Cells Are Endothelial Cells

Cell type B constituted a variable proportion of the adherent cells, typically about 9% (Table 1). In contrast to the ganglion cells, which had smooth membranes and were process-bearing, the type B cells had a rough appearing membrane and lacked processes (Figures 1A, 1B, and 3B). Some glial cells and lymphocytes may express Thy-1, however no adherent cells were labeled by an antiserum to GFAP or by a monoclonal antibody to rat lymphocytes. Type B cells did label with an antiserum to rat macrophages (Figure 2). They were also labeled specifically with rhodamine-conjugated Bandeiraea simplicifolia lectin I, and with the brain endothelial cell-specific antibody, MRC-OX-45 (Arvieux et al., 1986; see below).

There is some disagreement as to whether rat retinal capillaries express Thy-1 (see Beale and Osborne, 1982; Perry et al., 1984). Because capillaries in the retina form a flat tangential network (Michaelson, 1954), they are difficult to visualize in routine cryostat cross sections. We therefore isolated retinal layers on Millipore filter paper according to a technique modified from the procedure of Shiosaka et al. (1984) by Yeh and Strabowski (1986). In this procedure, the fifth layer to be isolated (see Experimental Procedures) contains the entire optic nerve fiber layer, including the superficial capillary plexus and the ganglion-cell layer (Yeh, unpublished data). Continuous fast blue-labeling in ganglion cells was observed in the fifth layer (Figure 3D), but not in other layers. The anti-rat macrophage antiserum, 2G12, MRC-OX-45, and rhodamine-conjugated Bandeiraea simplicifolia lectin I (BSLI) each produced specific labeling of the entire capillary plexus of the optic nerve fiber layer (Figure 3). In the case of both BSLI and the macrophage antiserum, other scattered isolated cells were also brightly labeled. These cells are most likely macrophages and microglia (Streit and Kreutzberg, 1987; Hume et al., 1983). Only the capillary plexus was labeled with MRC-OX-45. In control layers labeled with secondary antibodies only, or with an irrelevant antibody such as anti-galactocerebroside substituted for the primary, no labeling was observed.

The Thy-1 labeling pattern was observed with the antibodies 2G12 and MRC-OX-7. The capillary plexus and
Figure 3. Type B Cells Are Endothelial

Retinal layers were prepared according to the technique of Yeh and then immunohistochemically labeled. The fifth isolated layer contains the optic nerve fiber layer and the retinal ganglion cell layer and is shown here. A: MRC OX-45 labeled only the capillary plexus. B: Anti rat macrophage antiserum labeled the capillary plexus as well as scattered isolated cells. C: Rhodamine-conjugated Bandeiraea simplicifolia lectin labeled the capillary plexus and a few scattered cells. D: 2G12 labeled the ganglion cell neuropil, optic nerve fiber bundles, and the capillary plexus. In this layer, out-of-plane fast blue contained in the ganglion cells is also shown. In A, B, and D the primary antibody was detected with a peroxidase-conjugated secondary antibody. Calibrations: A, 640 μm. B, C, D, 30 μm.

the optic nerve fiber bundles were labeled (Figure 3D), as was the deep capillary plexus in the inner nuclear layer (data not shown). A diffuse label of the neuropil in the ganglion cell layer and inner plexiform layer was also observed.

Type C Cells Have Not Been Identified

Type C cells were of medium size, were smooth, and did not bear processes. At P8 they constituted about 43% of the adherent cells, and were never labeled with fast blue (Table 1). However, we were unable to find a marker that surface labeled type C cells differently than type D cells. It is possible that type C cells may be another kind of retinal neuron (amacrine or bipolar, see Perry et al., 1984; Hinds and Hinds, 1983).

Could these cells represent "degenerating" ganglion cells that have withdrawn their optic axons but have not yet died? Several experiments suggest not: first, type C cells were not labeled by a neurofilament antibody, SMI-33, which labeled the retinal ganglion cells (Table 1). In addition, since a large percent of ganglion cells die between days P2 and P5, we tested whether fast blue injected at P2 could be detected in type C cells on P5. Retrograde transport was operative at P2, as evidenced by the presence of the fast blue label in 100% of type D cells, yet no type C cells contained fast blue. Finally cells of identical morphology were observed when adult retinal suspensions were panned on 2G12 panning plates.

A summary of the morphological, antigenic and electrophysiological phenotype of the four adherent cell types is presented in Table 1. Together these observations indicate that only type D cells are retinal ganglion cells. Virtually all of the retinal ganglion cells adhered to the 2G12 panning plate, but at least three contaminating cell types were also adherent. Two of them, types B and C, adhered to the panning dish because they expressed Thy-1 on their surfaces: immunohistochemical labeling of retinal suspensions with 2G12 produced labeling of cell types B, C, and D (not shown). Therefore, using a one-step panning protocol, a population of retinal ganglion cells was obtained with a yield of 100% but a purity of at best 35%.
Punificahon of Retinal Ganglion Cells by Panning

PREPARE RETINAL CELL SUSPENSION WITH PAPAIN

INCUBATE CELLS IN RABBIT ANTI-RAT-MACROPHAGE ANTISERUM

INCUBATE CELLS ON FIRST PANNING PLATE

INCUBATE NONADHERENT CELLS ON SECOND PANNING PLATE

WASH NONADHERENT CELLS OFF PLATE

INCUBATE NONADHERENT CELLS WITH TRYP SIN (OR DISPLACE FROM BINDING SITES WITH 2G12)

COLLECT PURIFIED RETINAL GANGLION CELLS

Only a Subset of 2G12-Adherent Cells Adhere to T11D7 Plates
When the anti-Thy-1 monoclonal antibody T11D7 was used instead of 2G12 on the panning plate, there were several differences (Figure 1B). First, ganglion cell yields, as judged by labeling with fast blue, were only 25%-50% of that obtained with 2G12. Second, only contaminating cell types A and B adhered; cell type C was never observed on T11D7 plates.

Retinal Ganglion Cells Can Be Purified to Homogeneity with T11D7
While the one-step panning with 2G12 or with T11D7 helped to identify Thy-1-positive retinal cells, it did not provide a method to obtain pure preparations of ganglion cells. To get pure ganglion cells, we added a second panning step to the protocol (Figure 4). The contaminating cell types A and B were first depleted from a retinal cell suspension containing fast blue-labeled ganglion cells (Figure 5A) by panning with the anti-macrophage antiserum. The first pannig dish was coated with affinity-purified goat anti-rabbit IgG, the cells incubated in rabbit anti-rat macrophage antiserum, and the labeled cell suspension was then incubated on...
Figure 6. T11D7 Labels a Subset of Retinal Ganglion Cells
A P8 retinal suspension that had been retrogradely labeled with fast blue was labeled with T11D7. The cell suspension was incubated in T11D7 supernatant followed by incubation in Texas Red-conjugated anti-mouse IgM, mu chain-specific. (A) Fast blue label. Two ganglion cells containing fast blue are shown. (B) T11D7 label. Only the larger of the two ganglion cells is brightly labeled with T11D7. The other is not detectably labeled. Calibration: 15 μm.

Intensity of Thy-1 Labeling Varies among Ganglion Cells
Analysis of purified ganglion cells obtained from the T11D7 panning dishes, repeatedly showed that only about 25%–50% of ganglion cells could be isolated whereas all ganglion cells adhered to 2G12, and that type C cells never adhered to T11D7 plates. As these antibodies are both Thy-1-specific, we wondered how these differences could be explained. Since T11D7 is an antibody raised against mouse Thy-1 while 2G12 was raised against rat Thy-1, could the two antibodies be recognizing different forms or conformations of the Thy-1 molecule (Williams and Gagnon, 1982)? Alternatively, could the difference in yield be accounted for by a difference in affinity of the two antibodies for Thy-1? This latter possibility seemed particularly plausible since 2G12 and MRC-OX-7 are IgGs and T11D7 is an IgM antibody.

We labeled P8 retinal suspensions with the various Thy-1 antibodies. We found, as did Beale and Osborne (1982), that MRC OX 7 (raised against rat Thy-1) strongly and equally labeled all fast blue–containing cells. The identical result was obtained with 2G12 or with NEI-014, an IgG raised against mouse Thy-1 (NEN). In contrast, when suspensions were labeled with T11D7 using indirect immunofluorescence, only 22% of ganglion cells were labeled (Figure 6). When we increased the sensitivity of our detection by using a more concentrated supernatant and a Texas Red-conjugated secondary antibody to detect T11D7 binding to cells, we increased the percentage of labeled cells to 75%. However we observed that 22% of cells were labeled brightly, 53% were labeled lightly, and 25% were not labeled at all (Figure 7A).

Size histograms of fast blue–containing cells revealed that the largest cells were consistently the most brightly labeled cells (Figure 7). Brightly labeled cells had the largest average diameter, 13.3 ± 1.4 μm, lighter cells were intermediate in size, 11.5 ± 0.7 μm, and negative cells were the smallest, 10.9 ± 0.8 μm.

Figure 7. Intensity of Thy-1 Labeling Is Correlated with Cell Size
A retinal suspension from P8 rats that had been retrogradely labeled with fast blue was labeled with T11D7 supernatant. Fast blue–containing cells were graded as brightly, lightly, or negatively labeled. Size histograms were constructed by measuring cell diameters with an eyepiece micrometer. Size units are given in micrometers.
These experiments suggest that large ganglion cells may express a greater density of Thy-1 on their surfaces than small ganglion cells, and that the difference between T11D7 and 2G12, NEI-014, and MRC-OX-7 labeling (and adherence to panning dishes) occurred because of a difference in affinity for the Thy-1 molecule.

Intrinsic Electrophysiological Differences Were Observed among Ganglion Cells

We used whole-cell, tight-seal recording, and specific ion isolation solutions to compare electrophysiological properties of T11D7-labeled and unlabeled rat retinal ganglion cells. Cells of both types at P8 had sodium currents that ranged from 1 to 16 nA peak inward current and had sustained "L-type" calcium currents that ranged from 100 to 500 pA. We also observed potassium currents that consisted of both sustained and rapidly inactivating components. There was no conspicuous difference in the types of voltage-sensitive currents expressed by T11D7-positive and negative cells.

It has previously been reported that ganglion cells can exhibit sustained or transient visual responses (Kuffler, 1953; Boycott and Wässle, 1974). Studied in the current-clamp configuration, all of the P6-P8 cells we recorded from generated at least a single regenerative action potential, and most fired repetitively with sustained currents that consisted of both sustained and rapidly inactivating components. There was no conspicuous difference in the types of voltage-sensitive currents expressed by T11D7-positive and negative cells.

We observed an apparent continuum of spike adaptation: some cells fired at a high rate before adapting, others fired more slowly, while still others adapted quickly at all currents injected (Figure 8A). In addition, in many cells a delay was observed prior to the onset of spiking when shallow current steps were used (Figure 8A, second trace); this appears to be an additional type of adaptation. This delay decreased with larger depolarizing currents, but was not studied further.

Many lightly and some negatively labeled cells showed intermediate behavior (middle traces), often with long silences of hundreds of milliseconds before the onset of firing. Many lightly labeled cells also spike adapted, as illustrated in the lower trace. The second trace was obtained from a lightly labeled cell that exhibited a prominent delay prior to onset of spiking. Some action potentials appear clipped because of digitizing error.

We observed an apparent continuum of spike adaptation: some cells fired at a high rate before adapting, others fired more slowly, while still others adapted quickly at all currents injected (Figure 8A). In addition, in many cells a delay was observed prior to the onset of spiking when shallow current steps were used (Figure 8A, second trace); this appears to be an additional type of adaptation. This delay decreased with larger depolarizing currents, but was not studied further.

Figure 8. Electrophysiological Properties of Ganglion Cells

(A) Current-clamp recordings obtained by whole-cell recording from cells in suspensions labeled with fast blue and T11D7. Changes in membrane potential (top 4 traces) elicited by current test steps (bottom trace) are shown. Bath (external) solution contained (in mM): NaCl 140, CaCl2 2, MgCl2 1, HEPES 5, Dextrose 3 (pH 7.4). The pipette (internal) solution contained: KCl 140, Ca 10^{-4} M buffered with EGTA 2, MgCl2 1, HEPES 5 (pH 7.4). Four examples of the spectrum of behavior are shown, from no adaptation to strong adaptation. All brightly labeled cells fired repetitively (top trace) over most of the range of current injected, while most negatively labeled cells spike adapted at all currents (bottom trace). Many lightly and some negatively labeled cells showed intermediate behavior (middle traces), often with long silences of hundreds of milliseconds before the onset of firing. Many lightly labeled cells also spike adapted, as illustrated in the lower trace. The second trace was obtained from a lightly labeled cell that exhibited a prominent delay prior to onset of spiking. Some action potentials appear clipped because of digitizing error.

(B) Maximal firing rate is correlated with intensity of labeling. The maximal firing rate is plotted for negatively, lightly, and brightly labeled cells. The cells that spike adapted at all currents injected are illustrated as points plotted below the zero line.

(C) Maximal firing rate is correlated with the diameter of the ganglion cell. Cells that adapted at all currents are not plotted. Brightly labeled cells are plotted as squares, lightly labeled cells as triangles, and negative cells as crosses.
Spike Adaptation WasCorrelated with Soma Size and Tl ID7 Labeling Intensity

Ganglion cells (age P7) were labeled both by retrograde transport of fast blue and by Tl ID7 labeling using indirect immunofluorescence. Tl ID7 labeling was graded as brightly, lightly, or negatively labeled prior to electrophysiological recording, and soma size measured using an eyepiece micrometer. Intensity of Tl ID7 labeling strongly correlated with the degree of spike adaptation: the brightly labeled Tl ID7-positive cells fired at the highest sustained frequencies (Figure 8B and Table 2). Lightly labeled cells fired at a slower rate, and most negatively labeled cells adapted at all currents injected. If the firing rate of cells that adapted at all currents injected was considered to be zero, then average peak firing rate increased from negatively to brightly labeled cells (Table 2). Sodium current density alone cannot explain the difference in firing behavior (Table 2). The parameter that best correlated with the maximal firing rate of the cell was the soma diameter (Figure 8C).

Two artifacts that could result in apparent electrophysiological differences were ruled out. It was possible that relabeling of the cells with the antibody could have induced the difference. However, control recordings in the absence of Tl ID7 labeling (performed on cells identified either with fast blue but no Tl ID7 labeling, or without fast blue and Tl ID7 labeling but identified solely on ganglion cell morphology) revealed identical results. Alternatively, differential localization of channel types between soma and dendrites could result in an apparent difference in behavior, if large cells were able to retain larger amounts of dendritic processes than smaller cells during dissociation. To examine this, the ratio of dendrite to soma area was estimated by dividing the total membrane area calculated from the cell capacitance by the soma area estimated from the soma diameter. This ratio was not statistically different among brightly, lightly, and negatively labeled cells (Table 2).

In some CNS neurons, specific channel types have been shown to underlie spike adaptation. A progressive increase in activation of a calcium-activated potassium current is implicated in some cells (Madison and Nicoll, 1984), but seems not to be responsible here, because the baseline failed to hyperpolarize progressively during the course of the adaptation. In other cells an "M" potassium current is responsible (Adams et al., 1982), but we did not observe this current in retinal ganglion cells with voltage-clamp protocols consisting of long steps of shallow hyperpolarizations (20 out of 20). Thus, the mechanism of spike adaptation in retinal ganglion cells is not yet understood.

Discussion

Panning Is a Generally Useful Procedure for Purification of Neural Cell Types

Although our starting retinal suspensions contained only 0.57% retinal ganglion cells, by using a panning procedure we have been able to isolate these cells to greater than 99.5% purity, with a yield ranging from 25% to 50%. Retinal ganglion cells have previously been purified from rat retina using density gradients (Kornguth et al., 1981; Beale et al., 1983; Sarthy et al., 1983). The best degree of purification was 50%-70%, achieved by Sarthy et al. (1993). Using fluorescence-activated sorting of retrogradely labeled cells, Armson and Bennett (1983) achieved a 75% purification. Neither of these papers reported the yield of purified ganglion cells.

The protocol we have developed is a variation of the antibody-mediated plate adhesion protocol developed by Barker et al. (1975), Mage et al. (1977), and Wysocki and Sato (1978). All of these authors exploited the technique for isolation of cells in the immune system. The panning technique has many advantages for purification of central neurons as well: the yield is high, the procedure is inexpensive and rapid (requiring about 5 hr), and the purified cells exhibit excellent viability. Minor variations of this procedure should be generally applicable to purification of other neural cell types. In other parts of the nervous system macrophages and microglial cells may need to be eliminated with an additional panning step.

Thy-1 Is Not a Specific Marker of Ganglion Cells

Other investigators have concluded that Thy-1 is a specific marker of retinal ganglion cells in the rat. Beale and Osborne (1982) reported after indirect immunohistochemical examination of P8 retinal sections that "Thy-1 is found chiefly or exclusively on ganglion cells." Barnstable and Drager (1984) examined postnatal rat retinal suspensions that had been double-labeled by retrograde transport of granular blue and 2G12 immunohistochemistry and found "almost complete coincidence of the two labels." Similarly, Leifer et al. (1984) reported that examination of suspensions of rat retinal cells "revealed virtually complete overlap of the two markers." Our results are not consistent with these reports: 60% of the 2G12 or MRC-OX-7 surface-labeled cells in our experiments with P8 rats were not retinal ganglion cells.
Perry et al. (1984) suggested that Thy-1 may be present on other rat retinal cell types in addition to ganglion cells. They found that if ganglion cells were eliminated by transection of the optic nerve, some Thy-1 staining of the inner plexiform layer remained. They concluded that 30% of Thy-1 in the retina was found "on cells which contribute processes to the inner plexiform layer, presumably amacrine, bipolar, or Muller cells." Interpretation of their results has been confounded by the observation that changing patterns of Thy-1 expression are found at some sites in nervous tissue after axonal injury (Morris, 1985). However, in our study, we have confirmed the presence of Thy-1-positive cells other than ganglion cells in retinas during the normal course of development.

At least two types of retinal cells (types B and C) express Thy-1 but are not ganglion cells. These cells were judged not to be ganglion cells: they were antigenically distinct (type B cells labeled with anti-macrophage antiserum, MRC-OX-45, and Bandeiraea lectin; type C cells do not express neurofilaments) and both were electrophysiologically distinct (type B and C cells were not excitable and did not express either voltage-dependent calcium or sodium channels). Most important, type B and C cells were never labeled retrogradely following injections of fast blue into the lateral geniculate or superior colliculus. The type B cells are clearly endothelial cells: they have the surface antigenic phenotype anti-rat macrophage+, RSI++, MRC-OX-45+, Thy-1+, and they form capillary nets in the retina. While the identity of the type C cells has eluded us so far, it is possible that they are some type of retinal neuron.

It is unclear why some investigators have observed a coincidence of Thy-1 and retrogradely labeled ganglion cells in rat retinal suspensions. Perhaps the cell types we observed were not released in high numbers by previous procedures.

Intensity of Thy-1 Labeling Varies among Ganglion Cells

The two different Thy-1 antibodies, 2G12 and T11D7, produced different patterns of ganglion cell labeling and adherence to panning dishes. 2G12, MRC-OX-7, and NEI-014 labeled and produced adherence of all ganglion cells while T11D7 allowed labeling and adherence of only a subset of ganglion cells.

We observed a correlation between intensity of T11D7 labeling and the size of the ganglion cells: the largest diameter ganglion cells tended to be more brightly labeled, while smaller ganglion cells were lightly or entirely unlabeled. Several artifacts could produce such a difference. Size alone does not explain it: although difficult to quantitate, the variability of brightness among cells (at least 10-fold) appeared far greater than the differences in size (only 2-fold). However, as suggested above, it is possible that Thy-1 surface labeling was most intense in the neuropil on ganglion cell dendrites and that larger cells retained a disproportionately high share of resorbed neuropil.

The simplest explanation is that this difference is due to a varying density of Thy-1 on the surfaces of ganglion cells and by the different affinities for Thy-1 of the two antibodies. Our data would also be consistent with two possibilities: density of surface Thy-1 may differ in specific subsets of ganglion cells, or instead may vary continuously as a gradient among ganglion cells.

Only one of the two Thy-1-positive nonretinal ganglion cells (type B cells) was found on T11D7 panning plates, whereas both types (B and C) were found on 2G12 or MRC-OX-7 plates, suggesting type C cells express a smaller density of Thy-1. This possibility is also suggested by the much lighter immunohistochemical labeling of type C cells (see Table I).

Intrinsic Electrophysiological Differences among Retinal Ganglion Cells

The characteristics we observed with acutely dissociated retinal ganglion cells differ from those reported for cultured ganglion cells (Lipton and Tauck, 1987). Although freshly dissociated cells had the same basic ion channel types, the densities of current we observed were many times greater than those observed in cultured cells. In addition, Lipton and Tauck (1987) reported that excitability behavior was identical among all cells in culture, whereas we found that excitability behavior varied significantly among cells. Acutely dissociated ganglion cells appeared to exhibit a continuum of electrical behavior, large diameter cells were capable of firing at the most rapid sustained rates, had the lowest thresholds, and were often brightly T11D7-labeled. While smaller cells fired at slower rates or fired only single action potentials, had higher thresholds, and often had less intense T11D7 labeling.

The absence of these electrophysiological differences in culture may reflect alterations of channel expression in vitro, as we have found for certain types of glial cells (Barres et al., 1987). Alternatively, perhaps these differences do not exist in vivo and are an artifact of the dissociation procedure, since the cells are released with a variable amount of dendritic processes intact. However, this is unlikely since many of the currents generated at the extremes of long dendritic branches are electrically remote from the patch electrode, and thus are probably negligible. Second, we were able to estimate how much surface area of the dendrite was present relative to that of the soma. This ratio did not vary among small, intermediate, and large ganglion cells.

The mechanism of spike adaptation in those cells remains unclear but intriguing. While our studies are preliminary, two known mechanisms appear not to be operative (see Results). Since the same basic types of voltage-dependent channels are present in all ganglion cells, the different firing behavior may be accounted for either by varying localization or relative densities of channel types, or by a cell to cell variation in the kinetics of one or more potassium current types as has been previously demonstrated in cochlea (Art and Fettiplace, 1987).

While an extensive body of literature has documented differences in electrical discharge patterns of ganglion
cells in intact mammalian retinas (Kuffler, 1953; Boycott and Wasse, 1974; Levick, 1975; Fukuda, 1977), the issue has not been addressed adequately as to whether these differences arise extrinsically from the pattern of connections among retinal cell types and ganglion cells or from intrinsic electrophysiological differences among ganglion cells. Our data indicate that, at least in developing retina, ganglion cell differ in intrinsic electrophysiological properties. Fawcett et al. (1984) suggested that different patterns of electrical activity among ganglion cells might be involved in elimination of incorrect retinotectal connections during development; thus the electrophysiological differences we have observed could potentially have functional significance during development. Our experiments raise two additional questions: how are intrinsic electrophysiological differences among postnatal retinal ganglion cells generated, and do these differences also occur in adults?

Experimental Procedures

Retrograde Labeling

Animals were anesthetized with ether. Four microliters of a fast blue solution (1% in water, Sigma) were injected into the superior collicular brachium bilaterally. In early experiments granular blue was used. However, we found that animals suffered acute and chronic neurologic symptoms after these injections, regardless of the commercial source of granular blue. Injections were performed over a period of 1 min with a 10 μl Hamilton syringe through the skull, which had been exposed on the dorsal surface by making a small longitudinal incision in the skin midline. Injections were performed without the aid of a stereotactic device, but in mice animals the needle could be accurately positioned 2 mm lateral to the sagittal sinus, just anterior to its intersection with the transverse sinus, at a depth of 4 mm. With these coordinates, the injection site was at the intersection of the dorsal midbrain and diencephalon (see Potts et al. 1982).

Dissection of Rat Retinas

All important aspects of the panning purification procedures are summarized below. A more detailed, step by step, purification protocol is available on request.

One to ten rats from a Long/Evans natural litter (Charles River) were used. Although we typically used rats on postnatal day 8 to 10, this procedure worked well for the dissection and dissociation of neonatal to adult retinas. Retinas were removed from the eye in situ. The animal was decapitated, and skin overlaying the eyes was removed with a #11 scalpel blade while the cornea was held with a pair of rat-tooth, micro-dissecting forceps. The lens and vitreous humor were removed with forceps. The retina was then gently lifted away with a small spatula. Retinas were stored at room temperature in Earle's Balanced Salts Solution (EBSS) containing calcium and magnesium (pH 7.4), until retinas were dissected from all animals.

Preparation of Retinal Cell Suspensions

This tissue was dissociated enzymatically to make a suspension of single cells, essentially as described by光学学数 (1986). Briefly, the tissue was incubated at 37°C for 75 min in a papain solution (30 U/ml; Worthington) equilibrated with 95% O₂ and 5% CO₂. This solution also contained Earle's Balanced Salts and calcium, magnesium, EDTA, sodium bicarbonate, glucose, and L-cysteine. The tissue was then triturated sequentially with a 1 ml pipette in a solution containing ovomucoid (0.2%, Calbiochem-Behring) and bovine serum albumin (BSA; 0.1%, Sigma) to yield a suspension of single cells. After centrifugation at 800 × g, cells were resuspended in Eagle's Minimum Essential Medium (MEM). During this procedure the cells were never exposed to glutamate, asparate, or glutamine, and never allowed to be cooled lower below room temperature to avoid toxic effects on ganglion cells.

Preparation of Panning Plate

Secondary antibodies included affinity-purified goat anti-mouse IgM, murine-specific (Hyclone), affinity-purified goat anti-mouse IgG Fc (Accurate), and affinity-purified goat anti-rabbit IgG H+L (Cappel). Primary antibodies included monoclonal supernatant IgM antibody against mouse Thy1.1 (T1D7b, American Type Culture Collection, TIB 103), monoclonal ascites IgG antibody against rat Thy1.1, 2G12 (Barnstable and Drager, 1984), which was generously provided by Dr. Colin Barnstable, and monoclonal ascites IgG antibody against rat Thy1.1, MRC-OX-7 (Sveste). Each of two petri dishes (100 x 15 mm; Fisher, 775712) was incubated with 5 ml of Tris buffer solution (pH 9.5), with 10 μg of secondary antibody (either anti-mouse IgG Fc or IgM) for 12 hr at 4°C. Each dish was then washed three times with 8 ml of phosphate-buffered saline (PBS) and incubated with 10 ml of Thy 1.1 IgM monoclonal supernatant or 5 ml of 2G12 (ascites fluid diluted 1:2000) for at least 1 hr at room temperature. The supernatant was removed and the plate again washed three times with PBS.

In order to prevent nonspecific binding of cells to the panning dish, 5 ml of MEM with BSA (0.2%) was placed on each plate for 20 min.

Panning Procedure

The retinal suspension was incubated in anti-rat-macrophage antiserum (Axell, 1:100) for 20 min, centrifuged, resuspended in MEM and incubated on a 150 mm anti-rabbit IgG panning plate at room temperature for 45 min. The plate was swirled after 20 min to ensure access of all cells to the surface of the plate. If cells from greater than eight retinas were being panned, the nonadherent cells were transferred to another 150 mm anti-rabbit IgG panning plate for another 30 min. The nonadherent cells were removed with the suspension, filtered through 15 μm Nitril mesh (Fetko), and placed on the T1D7 panning plate. The cells were incubated on the plate as described above. After 1 hr, plates were washed eight times with 6 ml of PBS and swirled moderately vigorously to dislodge nonadherent cells. Progress of nonadherent cell removal was monitored under the microscope. The washing was terminated when only adherent cells remained.

Removing Adherent Cells from the Plate

A standard 100 mm petri plate binds about 35 μg total antibody (Mage et al., 1977). In most cases we expect this to far outnumber the actual number of binding sites on the cell, and we found in practice that a saturating antibody concentration produced such strong adherence of the cells to the plate that they could not be dislodged without damage. In earlier panning reports, this problem was solved by reducing the amount of antibody bound to the plate, so that cells would adhere less strongly and could be rinsed off with a vigorous jet of buffer (Mage et al., 1977; Wysocki and Sato, 1978).

In our hands, dilutions large enough to allow easy removal of the cells from the plate bound a low density of cells, probably because many were lost during the extensive washing required to eliminate nonadherent cells completely. We found, as did previous investigations, that high concentrations of irrelevant secondary or primary antibodies would not compete the adherent cells from their binding sites. We have found three acceptable ways to elute adherent cells from their sites while preserving high viability.

Papain

Adherent cells could be removed by incubation in a papain solution (45 U/ml; 5 ml per 100 mm plate) prepared in a bicarbonate-buffered saline at 37°C in a 5% CO₂ atmosphere, as described above. Cell detachment was followed under the microscope. The cell suspension was then collected and mixed gently with an equal volume of ovomucoid solution prepared as above, which was slightly acidic so that most of the papain solution in the absence of CO₂ resulted in a final pH near 7.4. The cells were then spun at 800 × g for 10 min and resuspended in MEM containing 10 mM HEPES.

Trypsin

A trypsin solution (0.125%) was prepared by diluting trypsin 10x stock (GIBCO) to 1:20 in PBS containing calcium and magnesium, phenol red, and 10 mM HEPES adjusted to pH 7.4. Cells on the panning dish were incubated with this solution for 15 min at room temperature. The trypsin was inactivated by adding an equal volume of a trypsin inhibitor solution (Soysbean trypsin inhibitor. Sigma, Neuron 800
0.2%) and cells were dislodged by gently pipetting trypsin solution around the plate. The cells were then spun and collected as above.

**Antibody Competition**

Cells adherent to a T11D7 panning dish were competed from their binding sites by incubation in a 2G12 solution (1:100 or 1:1000) in Eagle's MEM containing BSA (0.2%) at room temperature. Decreasing the amount of antibody increased the length of time of elution of cells, which varied from 5 to 30 min. Elution by displacement with 2G12 had the virtue of being the most gentle, but it caused clumping of the cells because of its divalent structure.

Centrifuged, resuspended in a small volume of PBS, and air-dried of cells, which varied from 5 to 30 min. Elution by displacement antibody used to pan the cells. The procedure was identical to that described above except that all antibodies were dissolved in a solution containing BSA (0.2%) and HEPEs (10 mM) to decrease nonspecific binding. Controls, with the primary antibody omitted or substituted with fresh hybridoma growth medium, a control monoclonal antibody, or preimmune rabbit serum, revealed negligible background.

The essential requirement for labeling cells recovered from a panning dish was to use a primary antibody of a different species or isotype than the primary used to pan for the cells. If not, residual antibody on the cells was detected by the secondary antibody used in the indirect immunohistochemistry. The secondary antibodies (Cappel) used for indirect immunofluorescence were fluorescein- or rhodamine-coupled Fab or F(ab')2 fragment goat anti-mouse IgG or IgM-specific, or similarly coupled goat anti-rabbit IgG. For labeling utilizing the ABC technique, biotinylated horse anti-mouse IgG or IgM-specific (Vector, 1:200) or biotinylated goat anti-rabbit IgG (Jackson, 1:20000) was used.

Primary antibodies included MRC-OX-45, generously provided by Alan Williams, 2G12 ascites, generously provided by Colin Barnstable, SM-31 anti-neurofilament antibody (Sterneberger-Meyer), rabbit anti-rat macrophage antiserum (Axell), rabbit anti-GAP (Accurate), mouse anti-rat lymphocyte monoclonal (Sera-tec), and mouse anti-mouse Thy-1.1 (NEI-014, New England Nuclear).

**Immunohistochemical Labeling of Cells Adherent to the Panning Plate**

Labeling of cells still adherent was successful only when the antibody used to label the cells was from a different species than the antibody used to pan the cells. The procedure was identical to that described above except that all antibodies were dissolved in a solution containing BSA (1%).

**Preparation of Retinal Layers on Filter Paper**

P8 retinas were dissected and separated into five layers according to the technique of Yeh and Strabowski (1986; see also Shiosaka et al., 1984), except that papain was used instead of trypsin. In brief, the retinas were incubated in papain (150 U/ml) in EBSS containing EDTA for 12 min at room temperature and then rinsed in an ovomucoid solution (above). They were transferred to PBS, and allowed to flatten onto the bottom of a 35 mm petri dish, ganglion cell layer side up. A piece of Millipore HVLP paper was gently placed on top of the retina, and then the entire retina was inverted to place the photoreceptor layer on the paper. The first four retinal layers were peeled off by sequentially placing pieces of filter paper over the retina, then gently lifting them off. The remaining fifth layer was the ganglion cell layer that was remaining. The entire process was accomplished within 60 sec.

**Immunohistochemical Labeling of Retinal Layers**

The retinal layers on filter paper were immediately fixed. For surface labeling they were placed in 2% paraformaldehyde for 30 min, and for internal labeling they were fixed and permeabilized in 5% acetic acid and 95% ethanol at 4°C for 20 min. The layers were then rinsed in a Tris-saline buffer (50 mM Tris, 0.85% NaCl [pH 7.4]), and blocked with 100% goat serum or 3% BSA for 30 min. Immunohistochemistry with indirect immunofluorescence or peroxidase labeling was then performed as described above. Layers were mounted in an excess of polyvinyl alcohol containing glycerol (1:15) prior to viewing. The polyvinyl alcohol caused the filter paper to become transparent, allowing microscopic transillumination.

**Electrophysiological Recording**

**Tight-Seal Recording**

A small aliquot of cells (10 µl) was placed in the recording chamber, which contained an appropriate bath solution (volume 500 to 750 µl). Standard procedures for pipette preparation, seal formation, and whole-cell recording were used (Hamill et al., 1981; Corey et al., 1984). Micropipettes were drawn from hard borosilicate capillary glass (Drummond), coated with Silgard to reduce their capacitance, and fire-polished to a bubble number of 4.0 to 4.5 (corresponding to an internal tip diameter of about 0.6–0.8 µm; Corey and Stevens, 1983). Pipette capacitance and series resistance were electronically compensated by the patch clamp, a Yale Mark V. All experiments were done at room temperature, approximately 24°C.

**Data Acquisition and Analysis**

Voltage stimuli were generated and responses recorded with a PDP 11/73 computer (INDEC). Analog signals were filtered with an 8-pole, low-pass Bessel filter before being digitized and recorded by the computer. In each experiment, linear capacitive and leakage currents were measured and subtracted before storage of data. The BCLAMP program set was used for acquisition and analysis of whole-cell data.

**Solutions**

Solutions for current clamp experiments, the bath (external) solution contained (in mM): NaCl 140, CaCl2 2, CaCl2 2, HEPES 5, MgCl2 0, MgCl2 1, Dextrose 3 (pH 7.4). The pipette (internal) solution contained KCl 140, HEPES 5, CaCl2 0, MgCl2 0, 8 buffered with EGTA to 2 to a final calcium concentration of 10-7 M, and MgCl2 1. For voltage clamp experiments, these solutions were used to see both inward and outward current components. Solutions designed to isolate specific inward currents were also used. For sodium current isolation, the bath solution contained: NaCl 140, CaCl2 10, MgCl2 0, MgCl2 0, 8 buffered with EGTA to 2 to a final calcium concentration of 10-7 M, and MgCl2 1. For calcium current isolation, the bath solution contained: CaCl2 10, NaCl 140, HEPES 5, Dextrose 3, TTX 10 µM (pH 7.4). For both sodium and calcium current isolation, the pipette solution contained: CsCl 140, MgCl2 1, CaCl2 0 added, Ca buffered to 10-9 M with EGTA 10, and HEPES 5 (pH 7.4).

**Acknowledgments**

We are indebted to Ursala Drager and Richard Masland for frequent and instructive conversations, and to Colin Barnstable and Alan Williams for their generous gifts of monoclonal antibodies. We especially wish to acknowledge the contribution of Hermes Yeh, who not only allowed us to use his unpublished technique for isolating retinal layers, but coached us by telephone in all the important procedural details. We also thank him for a critical reading of our manuscript.

This work was supported by National Institutes of Health grants F32 NS-07970 (to B. A. B.), NS-22059 (to D. P. C.), and NS-21269 and NS-16367 (to L. L. Y. C.), and by the Howard Hughes Medical Institute.

Received August 10, 1988; revised September 9, 1988.

**References**


Avieus, J., Willis, A. C., and Williams, A. F. (1986). MRC OX-45 an-


