The Type-I Astrocyte


Ion Channel Expression by White Matter Glia: The Type-1 Astrocyte

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Introduction

Glial cells are generally defined as passive support cells that are not integral components of neural circuits. This concept, first proposed on the basis of anatomical observations in the late 19th century, was supported by electrophysiological studies in the mid sixties. Perhaps most influential were the pioneering studies of Kuffler and his colleagues (Kuffler et al., 1966; Orkand et al., 1966; Kuffler, 1967), which showed that CNS glial cells of the lower vertebrate Necturus had membrane properties in situ that fundamentally differed from those in neurons: the cells had "passive" electrophysiological properties in that the current-voltage relationships were linear. They thus concluded that glial cells lacked voltage-dependent ionic currents and had mainly a potassium-selective leakage conductance.

More recently, the passive nature of glia has been questioned: glial cells in vitro express a variety of voltage-dependent ion channel types (for review see Barres et al., 1990b). This channel expression could be an artifact of tissue culture, or it may represent the phenotype in vivo. Consistent with this latter possibility, voltage-dependent ionic currents have been observed in acutely isolated mammalian Schwann cells (Chiu, 1987). The presence of voltage-dependent ion channels in glia in vivo would suggest that glia are less passive than has been thought and that they could be involved in signaling processes. This paper addresses three issues: Do mammalian CNS glial cells express voltage-dependent ion channels in vivo? How does channel expression in vitro compare with that in vivo? Do the electrophysiological properties of glial cells in vivo provide any clues to glial function?

The accurate measurement of ion channels in glia presents some problems. On the one hand, in situ or slice preparations do not usually provide exposed membrane surfaces for patch-clamp recording, or adequate accessibility for antibodies used for cell identification. Moreover, glia are extensively coupled by gap junctions, and the electrical properties of this syncytium prevent adequate control of membrane voltage. On the other hand, acute dissociation of cells often shears off the fine processes that may uniquely contain certain channel types. In culture, cells may regrow these processes, but there is no assurance that they express the same channels.

Thus for these studies, we have developed a new "tissue print" technique that produces dissociated cells still bearing processes. The cells have good visibility and accessibility for antibody and patch-clamp procedures and are expected to retain their full ion channel phenotype. We have applied this procedure to the postnatal rat optic nerve preparation developed by Martin Raff and colleagues (for review see Miller et al., 1989a; Raff, 1989). The optic nerve contains three glial cell types—type-1 astrocytes, type-2 astrocytes, and oligodendrocytes—each identified by a distinct antigenic phenotype (Raff et al., 1983a, 1983b, 1984; Miller and Raff, 1984). Here the tissue print procedure was used to characterize the properties of optic nerve glia, particularly type-1 astrocytes, under three conditions: during development, in the adult, and after optic nerve transection.

Results

Voltage-Dependent Currents in Type-1 Astrocytes in Culture

Type-1 astrocytes in cultures prepared from optic nerve (Figure 1; see Experimental Procedures) were examined using the whole-cell, patch-clamp technique with a series of isolation solutions designed to reveal ionic currents carried by sodium, calcium, potassium, or chloride ions. Voltage-dependent potassium current was detected in all cells (Figure 2, first row). Two types of potassium currents were present: a delayed rectifying potassium current, IK, which was present in 100% of cells and which averaged about 470 pA, and a smaller inwardly rectifying potassium current, IKir, which was present in 85% of cells and averaged...
Table 1. Average Currents in Type-1 Astrocytes

<table>
<thead>
<tr>
<th>Culture</th>
<th>P2 Print</th>
<th>P10 Print</th>
<th>P10 Print, P3 Trans.</th>
<th>P20 Print, P12 Trans.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacitance (pF)</td>
<td>24 ± 9</td>
<td>21 ± 5</td>
<td>42 ± 11</td>
<td>25 ± 7</td>
</tr>
<tr>
<td>K&lt;sub&gt;0&lt;/sub&gt; Current (pA)</td>
<td>470 ± 350</td>
<td>2300 ± 770</td>
<td>3800 ± 1100</td>
<td>480 ± 380</td>
</tr>
<tr>
<td>Density (nS/pF)</td>
<td>0.19 ± 0.13</td>
<td>0.95 ± 0.6</td>
<td>0.87 ± 0.22</td>
<td>0.35 ± 0.19</td>
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<tr>
<td>Percentage of cells</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>K&lt;sub&gt;0&lt;/sub&gt; Current (pA)</td>
<td>133 ± 76</td>
<td>0</td>
<td>4100 ± 1100</td>
<td>6</td>
</tr>
<tr>
<td>Density (nS/pF)</td>
<td>0.09 ± 0.07</td>
<td>1.3 ± 0.4</td>
<td>0.04 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Percentage of cells</td>
<td>85</td>
<td>100</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Na&lt;sub&gt;0&lt;/sub&gt; Current (pA)</td>
<td>510 ± 267</td>
<td>310 ± 533</td>
<td>59 ± 20</td>
<td>70 ± 9</td>
</tr>
<tr>
<td>Density (nA/pf)</td>
<td>12 ± 10</td>
<td>7.6 ± 13</td>
<td>2 ± 1</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Percentage of cells</td>
<td>21</td>
<td>100</td>
<td>31</td>
<td>14</td>
</tr>
<tr>
<td>Ca&lt;sub&gt;0&lt;/sub&gt; Current (pA)</td>
<td>Variable&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42 ± 18</td>
<td>32 ± 21</td>
<td>0</td>
</tr>
<tr>
<td>Density (nA/pf)</td>
<td>1.8 ± 0.4</td>
<td>0.69 ± 0.38</td>
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<td></td>
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<tr>
<td>Percentage of cells</td>
<td>100</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca&lt;sub&gt;0&lt;/sub&gt; Current (pA)</td>
<td>0</td>
<td>24 ± 10</td>
<td>25 ± 12</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>Density (nA/pf)</td>
<td>1.0 ± 0.4</td>
<td>0.68 ± 0.44</td>
<td>0.43 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Percentage of cells</td>
<td>100</td>
<td>76</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

All values represent the mean of 10-20 cells and are expressed as mean ± SD. Each current component was recorded with the specific ion isolation solutions given in Experimental Procedures. Averages include only cells expressing the current. Conductances for potassium currents were calculated at +60 mV for K<sub>0</sub> and at -120 mV for K<sub>0</sub> and were not corrected for residual uncompensated series resistance, which was about 10 MΩ. This resulted in an underestimate of conductance, which was significant only in the case of the large potassium currents at P10: for K<sub>0</sub> the conductance is underestimated by about 30%, and for K<sub>0</sub> the conductance is underestimated by at least 100%. Trans: transection.

<sup>a</sup>Transient calcium currents, Ca<sub>0</sub>, are variably present depending on the lot of fetal calf serum used to culture the cells (Barres et al., 1989a).

About 130 pA. Both the potassium currents were blocked by extracellular barium (5 mM; lower concentrations not tested) and were not blocked by charybdotoxin (30 nM), a blocker of some calcium-dependent potassium channels (Miller et al., 1985a).

Sodium currents were found in only about 20% of cells in culture (Table 1); when present, the peak currents averaged 510 ± 270 pA (at -10 mV). This current was blocked by tetrodotoxin (10 μM; lower concentrations not tested). The properties of these sodium channels were similar to those found in several other glial cell types and dissimilar to those found in neurons. They have previously been described in detail (Barres et al., 1989b). Calcium currents of the transient type, Ca<sub>0</sub>, were variably present, depending on the lot of fetal calf serum used to culture the cells (see Barres et al., 1989a). Calcium currents of the long-lasting type, Ca<sub>0</sub>, were absent but could be induced by cAMP (Barres et al., 1989a).

The types of current present and their densities did not vary with the culture conditions (serum-containing or serum-free), nor did they change significantly with time up to 8 days after the initial plating.

Although no whole-cell chloride currents were observed in type-1 astrocytes in culture, it has been reported that chloride currents are active in type-1-like cortical astrocytes after a sustained period of intracellular dialysis, suggesting the presence of chloride currents that are normally inhibited (Gray and Ritchie, 1986). Similarly, single-channel chloride currents are not found in cell-attached patches in type-2 astrocytes and oligodendrocytes, but can be observed after patch excision (Barres et al., 1988a). Thus, cell-attached and excised patches in type-1 astrocytes were examined in isolation solutions designed to reveal only chloride channels: the bath and pipette each contained tetramethylammonium chloride (TMA; see Experimental Procedures).

Under these conditions, chloride channels were observed in excised but not cell-attached patches and were identical to those previously reported in type-2 astrocytes and oligodendrocytes (Figure 3A). They were found in 3 of 10 excised patches. The single-channel currents reversed at zero, as expected for symmetric chloride solutions, and were outwardly rectifying (Figures 3A and 3B). At negative potentials the slope conductance was about 25 pS, but increased to 60 pS at positive potentials.

The Tissue Print Dissociation Technique

Type-1 astrocytes, acutely isolated at P7 by trituration (see Experimental Procedures), expressed the same basic channel types as seen in culture (data not shown). However, most of these acutely isolated type-1 astrocytes did not have processes; apparently these were shorn off during the dissociation procedure (see Fig-
Electrophysiological Properties of Type-1 Astrocytes

Figure 1. Type-1 and Type-2 Astrocytes in Culture
Nomarski micrograph of a type-1 astrocyte (left) and a type-2 astrocyte (right) in culture. The cells are shown live (unfixed). Bar, 15 μm.

Figure 2. Voltage-Sensitive Currents in Type-1 Astrocytes
Whole-cell ionic currents elicited in type-1 astrocytes are demonstrated in culture (top row), P2 prints (second row), P10 prints (third row), P20 prints after transection of the optic nerve at P12 (fourth row), and co-cultured with neurons for 36 hr (fifth row). Currents are plotted as average current densities (see Table I), so that amplitudes of currents in each column (but not between columns) can be directly compared. The capacitive transients have been blanked. The voltage step protocols used for each current are shown in the bottom row. Bath and pipette solutions differed for each type of ionic current (see Experimental Procedures). Only the major current components found in >65% of cells are shown; absent or minor current components are depicted as a horizontal trace.

It was possible that specific channel types might be localized to the (lost) processes (Newman, 1984; Brew et al., 1986); this is of particular concern since in vivo most of the surface area of type-1 astrocytes is found in their processes. Thus we developed a procedure to isolate viable type-1 astrocytes still bearing many processes.

Tissue adheres tightly to nitrocellulose paper (Seshi, 1986; Cassab and Varner, 1987; Shepherd et al., 1989). To exploit this adhesion for cell isolation, the tissue was treated with papain and touched gently to a piece of nitrocellulose paper (see Experimental Protocols). Examination of tissue prints revealed that a thin layer of tissue consisting of cells, many of which were process-bearing, remained adherent. Type-1 astrocytes bearing processes were found in prints prepared this way, either by hematoxylin and eosin staining (Figure 4A), by glial fibrillary acidic protein (GFAP) labeling and immunofluorescence (Figure 4B), or by detection of the GFAP with horseradish peroxidase (HRP)-conjugated antibodies (data not shown). This procedure also

K_D
K_R
Na_G

CULTURE

P2 PRINT

P10 PRINT

P20 PRINT AFTER P12 TRANSECTION

CO-CULTURE WITH NEURONS N.D.

25 pA/pF
25 pA/pF
7 pA/pF

20 ms
12.5 ms
2.5 ms

Figure 1 in Barres et al., 1990c. This figure demonstrates that specific channel types might be localized to the (lost) processes.
allows isolation of cells from other neural and non-
neural tissues (data not shown).

For physiological recording, glass coverslips were
coated with a variety of sticky transparent substances
and tested for adhesivity and toxicity. Two substances
worked reasonably well: high molecular weight poly-
lysine and a 1% nitrocellulose solution in amyl acetate.
The nitrocellulose coating was used for the rest of the
tissue print experiments involving electrophysiology.
Although it was not quite as adhesive as the poly-
lysine, cells remained rounded (Figures 5A and 5B)
without flattening onto the coverslip as they did on the
poly-lysine, and it was not toxic.

With traditional dissociation methods, it is increas-
ingly difficult to isolate viable cells from the optic
nerve after P11. Large numbers of viable, process-
bearing cells could be obtained using the tissue print
protocol, even from adult animals, if prints were pre-
pared from vibratome sections of 50-100 μm thickness
(Figures 5C and 5D).

Tissue printing onto nitrocellulose-coated cover-
slips yielded many process-bearing and viable cells,
which excluded trypan blue (some cells did become
injured in the printing process and failed to exclude
trypan blue; however, these cells were visibly dam-
aged and could thus be reliably avoided without try-
pan blue labeling). Cells adhered sufficiently tightly
to permit immunohistochemical labeling of surface
antigens prior to electrophysiological recording.

**Voltage-Dependent Currents in Type-1 Astrocytes**
in P2 Tissue Prints

Type-1 astrocytes were antigenically identified as
GFAP+, RAN-2+ (data not shown), and A2B5- (data not
shown) cells. They characteristically had multiple long
processes: all cells with at least 3 long processes were
identified as type-1 astrocytes by this phenotype.
Thus, for most recordings from type-1 astrocytes, im-
munohistochemical identification was not necessary,
although it was always used to identify O-2A progeni-
Electrophysiological Properties of Type-1 Astrocytes

Figure 5. Optic Nerve Tissue Prints on Nitrocellulose-Coated Glass Coverslips
Cells were prepared and printed as described in Experimental Procedures. They are shown live and as they appear prior to electrophysiological recording. (A and B) P7 prints. (C and D) Adult prints. Bar, 20 μm (A); 10 μm (B, C, and D).

tors and oligodendrocytes accurately (see Barres et al., 1990c; see also Figure 10).

Whole-cell currents of type-1 astrocytes in P2 tissue prints were compared with those in vitro. In contrast to the cells in culture, sodium currents and KIR were not present. K0 was found in all cells, but its density was 4-5 times greater than that in culture (Figure 2, second row; Table 1). In addition, two types of calcium current were observed that were not found in culture without pretreatment to increase intracellular cAMP (Figure 6): a transient calcium current, CaT, and a sustained or long-lasting calcium current, CaL (nomenclature of Nowycky et al., 1985).

Voltage-Dependent Currents in Type-1 Astrocytes in P10 Tissue Prints
The optic nerve changes rapidly during postnatal development: about 50% of the retinal ganglion cells die (Perry et al., 1983); myelination begins at about P6 (Foster et al., 1982), and O-2A progenitor cells may be migrating into the nerve (Small et al., 1987). Moreover, in the second postnatal week the nerve becomes capable of buffering potassium (Ransom et al., 1985). Thus we looked at the properties of type-1 astrocytes at P10.

In contrast to the P2 cells, 100% of cells expressed sodium currents, and these were of the "glial" form, as judged by their slow kinetics and their more negative voltage dependence of activation and inactivation. Sodium currents reached 5% of peak current at

Figure 6. Calcium Currents in Type-1 Astrocytes in P2 Tissue Prints
Two components of calcium current were present in most type-1 astrocytes at P2: a CaT current that had a voltage-dependent inactivation (A) and a CaL current that remained sustained during the voltage step (B). Currents are plotted as average current densities.
third row; Table 1). At PIO, CaT could be found in only the cells expressed KIR at a high density (Figure 2, peak current averaged -80 mV. In addition, 100% of the cells expressed this current. By PIO, observed in most cells at densities similar to those found at P2 (Table 1).

To determine more exactly when this change occurred, we followed the time course of expression of the inwardly rectifying potassium channel, which undergoes a marked transition with age. At P2-P3, no cells expressed KIR. By P5 and P8, 40% and 30%, respectively, of the cells expressed this current. By P10, 100% of cells expressed it. Thus the change in phenotype occurred progressively over about a week. Interestingly, when cells exhibited KIR at all during the transition, the current density approximated that seen at P10 rather than a transitional amount. The transition to expression of KIR must occur rapidly.

Certain whole-cell current types were not found in type-1 astrocytes at any age: these included voltage-dependent chloride current, sodium current of the "neuronal" form, and a transient outward potassium current, Kd.

The resting potential of P10 type-1 astrocytes in prints was measured in the current-clamp configuration with bath and pipette solutions containing 5 and 120 mM potassium, respectively (see outwardly rectifying potassium solutions given in Experimental Procedures). In more than 90% of the cells, the resting potential was within several millivolts of the equilibrium potential for potassium, -85 mV. This provided further confirmation that the cell membranes were not damaged during the printing procedure.

**Voltage-Dependent Currents in Type-1 Astrocytes in Adults**

Examination of type-1 astrocytes in tissue prints prepared from adult optic nerves (about 60 days of age) indicated that the same channel types present at P10 were present in the adults, although average densities were not determined. However, at P30, the densities of sodium current and Kd were more than double those found at P10. At P30, no cells with CaL could be found, and CaL, when present, occurred at a density similar to that at P10, although the proportion of cells with CaL had fallen to 37%.

**Calcium Sensitivity of Outward Potassium Current in Type-1 Astrocytes**

In cultured type-1-like astrocytes derived from cortex, potassium channels that carry a sustained outward current have been reported to be sensitive to intracellular calcium. To determine whether this is true for type-1 astrocytes in prints, we measured the average density of Kd with high and low intracellular calcium (see Experimental Procedures). With 1 μM calcium in the pipette, the average conductance density of Kd in P9 astrocytes in prints was 0.84 ± 0.30 nS/pF (18 cells). With 10 nM calcium in the pipette, the density fell to 0.42 ± 0.14 nS/pF (15 cells), suggesting that a large portion of the outward current was sensitive to intracellular calcium. Charybdoxin (30 nM), a blocker of some types of calcium-dependent potassium channels, did not block this current.

**Potassium Inward Rectification in Type-1 Astrocytes**

Potassium inward rectifiers may mediate potassium buffering by glia (for discussion see Barres et al., 1990b). This was further suggested by the temporal correlation of their occurrence in type-1 astrocytes in prints with the ability of the nerve to buffer potassium. In P10 type-1 astrocytes, this current was purely potassium-selective, as it reversed at the potassium equilibrium potential of -45 mV when external potassium was 20 mM (Figures 7A and 7D) and at -90 mV when external potassium was 3 mM (internal potassium was 120 mM). It was specifically blocked by the addition of 5 mM cesium to the bath solution (Figure 7B). Although this conductance predominantly carried inward current, the cesium blockade revealed some outward current positive to the reversal potential (Figure 7C).

4-Aminopyridine (4-AP) has recently been shown to block inwardly rectifying channels in Schwann cells (Wilson and Chiu, 1990), although it was reported not to block potassium inward rectifiers in oligodendrocytes in culture (Barres et al., 1988a). Similarly, no effect of 5 mM 4-AP was found on KIR in type-1 astrocytes in tissue prints (Figure 7E), although it blocked all the outwardly rectifying potassium current (figure 7F).

Recently, inwardly rectifying potassium channels have been reported to interact with intracellular divalent cations (e.g., Vandenberg, 1987). Thus we tested the effect of charybdoxin on this channel. Charybdoxin (50 nM) did not block Kd.

**Calculation of the Astrocyte Electrical Space Constant**

Because Kd is the only voltage-dependent ion channel active at the resting potential of astrocytes (Figure 7) and because of its large density, it must underlie the resting potential in type-1 astrocytes, as it does in other types of glial cells (Barres et al., 1988a, 1990c). It has been suggested that astrocytes regulate extracellular potassium by rapidly shunting it away from a region of excess along their processes and through the astrocyte syncytium. This hypothesis is called "spatial buffering" (Orkand et al., 1966). In order for this mechanism to account for a significant portion of potassium buffering, the space constant of the astrocyte process must be long relative to the path the potassium must travel, i.e., relative to the length of one or more astrocyte processes. The astrocyte space constant in white matter has not been directly measured before (but see Trachtenberg and Pollen, 1970, for an estimate in gray matter glia), but can be estimated from the inwardly rectifying potassium conductance of the astrocyte at rest. The potassium con-
Electrophysiological Properties of Type-1 Astrocytes

A CONTROL

B. AFTER Cs+

C. CURRENT BLOCKED BY Cs +

D CONTROL

E. AFTER 4-AP

F. CURRENT BLOCKED BY 4-AP

Figure 7. Pharmacological Blockade of Potassium Currents in Type-1 Astrocytes

Current-voltage relationships determined by a slow ramp voltage command are plotted for a type-1 astrocyte in a P8 tissue print before (A) and after (B) addition of 5 mM cesium to the bath solution. KIK (C) was blocked by cesium and was determined by subtracting the current in (B) from that in (A). The current-voltage relationships are also plotted for another type-1 astrocyte in a P8 tissue print before (D) and after (E) addition of 5 mM 4-AP to the bath solution. Ko (F) was blocked by 4-AP and was determined by subtracting the current in (E) from that in (D). Linear leakage currents were not subtracted from any of the traces shown in this figure.

The conductance of the type-1 astrocyte was measured at its resting potential, in a bath solution containing the normal amount of extracellular potassium (3 mM) found in the CNS extracellular space. Only data from cells that lacked significant seal leakage, having potassium reversal potentials within 5 mV of Ek, were used for this analysis.

At P10, the input resistance of type-1 astrocytes at Ek (-90 mV) was about 100 ± 30 MΩ (8 cells). However, by P50 the input resistance had fallen (as expected from the increasing density of the inwardly rectifying conductance, see above) to 30 ± 10 MΩ (7 cells).

In order to use these measurements to calculate the electrical space constant, it is also necessary to know the average diameter of the astrocyte processes. In tissue prints, astrocyte processes are several micrometers in diameter in early postnatal life and appear to decrease progressively from P2 to P50. Astrocyte processes in electron micrographs of adult rat optic nerve have a diameter of about 0.25 μm (Peters et al., 1976), close to the diameter of processes we measured in P50 type-1 astrocytes in optic nerve tissue prints.

For a cylinder, the space constant is equal to the $\sqrt{0.5aR_m/R}$, where a is the axon radius, $R_m$ is the specific membrane resistance, and R is the internal specific resistivity of the astrocyte process (Jack et al., 1975). The values for specific membrane capacitance, $C_{m}$, of 1 μF/cm² and R of 60 Ω cm have been assumed (Aidley, 1971). At P10, the space constant was thus estimated to be 408 μm (Figure 8A); by P50, because of the decrease in average process diameter and the increase in inwardly rectifying potassium conductance, the calculated space constant had fallen to 104 μm (Figure 8B).

How does this compare with the length of astrocyte processes in the optic nerve? Good measurements are only now beginning to be obtained, by injections of HRP and Lucifer yellow into astrocytes in the optic nerve (Butt and Ransom, 1989; Barbara Fulton and Julia Burne, unpublished data). In animals that are about P14, the length of processes in astrocytes is generally between 100 and 200 μm, but may be as long as 500 μm (Butt and Ransom, 1989). However, between P14 and adult, the average length of astrocyte processes increases significantly as the animal grows (Barbara Fulton, personal communication). Type-1 astrocytes are found in two orientations in the nerve, predominantly radial or predominantly longitudinal, but these do not appear to differ in average process length (Barbara Fulton, personal communication). Thus in the adult, most type-1 astrocyte processes are greater than 200 μm, although the average length has not yet been determined.

Therefore, at P10 the space constant is about twice as long as the length of an astrocyte, whereas at P50 (adulthood) the space constant is at least 4 times shorter than the length of an astrocyte.

The Effect of Neurons on Channel Expression by Type-1 Astrocytes: Transection

The rapid change in ion channel phenotype of type-1 astrocytes between P2 and P10 could be caused by an intrinsic timing mechanism in astrocytes, by extrinsic factors coming from neurons or other cell types, or by both. To determine whether the change was purely intrinsic to astrocyte aging, P2 type-1 astrocytes were cultured for 10 days and then studied electrophysiologically. No change in ion channel phenotype was observed: the old type-1 astrocytes in culture con-
Figure 8. Comparison of the Electrical Space Constant to the Length of Type-1 Astrocytes

The fall off along the length of an astrocyte of the spatial membrane potential gradient generated by a local increase in potassium at the left-most point of the astrocyte is plotted for P10 astrocytes (A) and for adult (P50) astrocytes (B). The membrane potential is plotted as the percent decay of the original potential at a distance of X μm from the left side of the astrocyte.

continued to express mostly a delayed rectifying potassium current (K_o) and a very small inwardly rectifying potassium current (K_I), and about 20% of cells had sodium current.

This suggested that extrinsic factors are involved in the maturation of channel phenotype in type-1 astrocytes. To test this possibility, optic nerves were transected at P3 and type-1 astrocytes from transected nerves were studied in tissue prints at age P10. The phenotype of P10 cells from transected nerve was like that of normal P2 cells: sodium currents could be found in only 30% of cells, and their average density was 10-fold lower than that in untransected controls of the same age (average includes cells with zero current; Table 1). In addition, K_I could not be found in any cells, whereas it was present at high density in all control cells (Table 1). K_o continued to be present in all cells, although its density was half of control, approaching that found in type-1 astrocytes in culture (Table 1). C_a was still not found in most cells, and the density of C_a was not significantly different from that of the control nerves (Table 1).

These results indicated that an extrinsic factor, most likely emanating from or dependent on neurons, is required for the change in astrocyte phenotype. To determine whether this factor is also required to maintain the change once it has taken place, transections were performed at P12 and type-1 astrocytes were studied 8 days later. At P20, sodium currents were then found in only 14% of cells at only 2% of the density found in untransected P10 nerves (Table 1; Figure 2, fourth row; cells with zero current were included in the average). Similarly, K_I was found in 66% of cells, at less than 2% of the density seen in untransected P10 nerves. K_o was also diminished: it continued to be present in 100% of cells, at a density only 20% of the P10 control density (Figure 2, fourth row; Table 1). Calcium currents were almost entirely absent. The type-1 astrocytes did not decrease in size after transection: the average capacitance of cells at P12 was 42 ± 11 pF; the average capacitance at P20 was 58 ± 11 pF. Thus, the P20 cells after a P12 transection appeared nearly identical to those in culture and were similar to the early postnatal astrocytes in prints in that expression of K_o, K_I, and Nao were greatly decreased. Some neuronal (or neuronally dependent) factor appears necessary for the maintenance of the phenotype.

The loss of expression of ion channel types after transection was specific to type-1 astrocytes; O-2A progenitors continued to express voltage-dependent sodium currents and the potassium currents, K_o and K_I.

The Effect of Neurons on Channel Expression by Type-1 Astrocytes: Co-Culture

To test directly whether neurons could up-regulate expression of glial ion channel types, purified retinal ganglion cells were co-cultured with type-1 astrocytes. Retinal ganglion cells were purified from P10 retinas using a two-step panning procedure that yields nearly pure ganglion cells (Barres et al., 1988b; and see Experimental Procedures). These cells were added to type-1 astrocyte cultures of P10 equivalent age (see Experimental Procedures). Retinal ganglion cells are difficult to culture and survived for only 24-36 hr.

After just 1-2 days of culture, sodium currents were found in 25% of cells in the control, neuron-free cultures, but were found in 95% of cells in the co-cultures (Figure 2, fifth row). The average density of sodium current in co-cultured astrocytes was 8.1 ± 9.6 pA/pF, a value nearly identical to that found in P10 astrocytes in tissue prints (Table 1).

The density of K_I in type-1 astrocytes also significantly increased in cocultures, almost 6-fold from 0.07 ± 0.06 pA/pF (16 cells, includes zero current cells) to 0.41 ± 0.3 pA/pF (11 cells; K_I was found in 100% of cells compared with 85% in controls [Figure 2, fifth row]). The density of K_o in the co-cultured astrocytes
was still about 3-fold lower than the density of KIR in P10 prints (Table 1).

The mechanism of the neuronal inductive and maintenance effects on glial ion channel expression is not known, but one observation suggests it may be contact-mediated: astrocytes that were not directly contacted by neurons lacked both sodium current and KIR (10 cells). The increases described above were from cells touching neurons. We have not tested neuron-conditioned medium for either inductive or maintenance factors.

Glutamate-Gated Currents in Type-1 Astrocytes

It has been reported that type-1-like astrocytes in culture have non-NMDA, glutamate-gated, cation-selective channels (Sontheimer et al., 1988). Conversely, Cull-Candy et al. (1988) observed only an electrogenic current in type-1-like astrocytes in culture. Type-2 astrocytes and their progenitors, O-2A cells, in culture do have glutamate-gated cation channels (Usowicz et al., 1989), and these are also found on acutely isolated O-2A progenitors (Barres et al., 1990c), suggesting they are not an artifact of culture. Thus we next asked whether glutamate-gated channels are present in type-1 astrocytes in vivo.

We used the whole-cell configuration to study type-1 astrocytes in P10 tissue prints, selecting only cells with long processes, since glutamate receptors are thought to be mainly extrasomatic (see Experimental Procedures). The isolation solutions were carefully designed so that only glutamate-gated cation channels would be detected: the bath solution contained lithium rather than sodium to ensure that electrogenic glutamate currents would not be present (e.g., Schwartz and Tachibana, 1990), and the pipette contained cesium, which passes through the glutamate channels but blocks outwardly rectifying, voltage-dependent potassium currents, so that modulatory effects of glutamate on potassium currents would not interfere. The total elimination of potassium from the bath solution both ensured that the large inwardly rectifying potassium conductance in the processes would not be measured and acted to increase substantially the space constant along the astrocyte processes so that more current entering distally would be detected at the soma.

Glutamate-gated current was not detected in 19 out of 19 type-1 astrocytes in P10 tissue prints (Figure 9A). In contrast, glutamate currents continued to be present in O-2A progenitors (2 of 2 cells) in these prints, studied with the same lithium-containing solution (Figure 9B). Glutamate currents in these O-2A progenitors reversed at zero, as previously reported (Barres et al., 1990c), and were cation-selective, since they remained when chloride was replaced with methanesulfonate on both sides of the membrane.

Oligodendrocytes

Optic nerve oligodendrocytes in culture express three components of voltage-dependent potassium current—KIR, KA, and K0—and they lack other voltage-dependent channels (Barres et al., 1988a, 1990c). To determine the phenotype in vivo, oligodendrocytes were studied in tissue prints (Figure 10). Prior to P9, all oligodendrocytes expressed three components of potassium current (20 of 20 cells): inwardly rectifying potassium current, KIR (Figure 10A), and two components of outwardly rectifying potassium current, K0 and KA (Figure 10B). These currents appeared identical in their voltage dependence and kinetics to those found in oligodendrocytes in culture and were not studied further. Oligodendrocytes older than P9 (Figure 10C) were not studied because of difficulty obtaining the whole-cell configuration.

Type-2 Astrocytes

Type-2 astrocytes are found in cultures from many brain regions and can be distinguished from type-1 astrocytes by their surface antigens and morphology (Miller and Raff, 1984). We looked for cells in adult optic nerve tissue prints that had the antigenic phenotype of type-2 astrocytes in culture—both A2B5+ (or LB1*) and GFAP+. (We could not use the antibod-
Figure 10. Oligodendrocytes in Tissue Prints
Oligodendrocytes in P7 tissue prints on nitrocellulose-coated coverslips were identified by labeling with indirect immunofluorescence prior to recording (A and B). Two oligodendrocytes are present in the right side of (A), and both are labeled by antibodies to galactocerebroside (B) (see Experimental Procedures). In (C), an oligodendrocyte in a P40 optic nerve tissue print is shown still attached to the fiber it is myelinating. Bar, 15 μm.

Figure 11. Voltage-Dependent Ionic Currents in Oligodendrocytes in P7 Optic Nerve Tissue Prints
(A) Inwardly rectifying current, KIR, and (B) Outward potassium currents, K+, and KD, are present in most oligodendrocytes. The presence of KA can also be detected in the tail current shown in (A), since it is activated upon stepping back to a depolarizing prepulse after the hyperpolarizing test step.

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sequently isolated: rather than shearing the tissue apart by passage through a syringe needle or pipette, a "touch prep" is prepared by gently touching the tissue to a sticky, nontoxic surface. Several other procedures similar to the one we developed have been previously reported. For instance, Cassab and Varnar (1987) isolated and immunocytochemically studied soybean seed coats using nitrocellulose paper; because of this similarity, we have adopted the name of their procedure, tissue printing. Unlike these previous methods, this tissue print procedure allows isolation of viable cells still bearing processes for further study. Here we have used tissue prints for electrophysiological recording, however, we have also used the procedure for other purposes, including isolation of cells for culture, scanning electron microscopy, and immunohistochemistry.

An advantage of tissue prints is that they allow easy access of antibodies to cell surfaces, which is often a problem when staining fixed tissue sections. Separation of cells can be advantageous in other ways: in brain sections it can sometimes be difficult to discern at the light microscopic level whether apparent membrane labeling corresponds to neuronal membranes or to closely apposed glial processes. Thus tissue printing offers a way of correlating surface antigenic phenotype with the presence of intracellular markers often used to identify cell type, such as GFAP or neurofilaments.

**Comparison of Type-1 Astrocyte Properties In Vitro and in Prints**

The pure grade of papain we used to isolate type-1 astrocytes apparently did not destroy any of the main types of voltage-dependent ion channels when applied extracellularly: channel types not found in acutely isolated type-1 astrocytes were found in other types of glial cells or in retinal ganglion cells isolated identically (this paper; Barres et al., 1989b, 1990c). Because the cells studied in prints retained many of their processes, loss of specific channel types should not have been a problem. Thus, it is likely that type-1 astrocytes in tissue prints have many of the properties present in vivo.

However, P10 type-1 astrocytes in prints had an ion channel phenotype that was strikingly different from the phenotype of cells of equivalent age in culture (Table 2). Both the types and densities of currents were decreased in cultured cells: whereas Na\textsubscript{v}	extsuperscript{1}j, Ca\textsuperscript{2+}, K\textsubscript{IR} and K\textsubscript{O} were found in most acutely isolated cells, in culture K\textsubscript{O} was the main current found, and it was at 4-fold lower density.

Type-1 astrocytes in vitro appear morphologically primitive, since they lack processes, and are often thought to be in a dedifferentiated state. Two methods of inducing apparent type-1 astrocyte differentiation in culture have been reported. First, forskolin induces rapid process formation (Lim et al., 1973; Wu and deVellis, 1983) and induces expression of the voltage-dependent calcium current, Ca\textsubscript{v} (Barres et al., 1989a).

It is not yet known whether forskolin can also induce the other channel types found in vivo but not in vitro. Second, co-culture with neurons induces formation of astrocyte processes (Hatten, 1985; Miller et al., 1989b). Similarly, we found that co-culture with neurons induces expression of ion channel types found in differentiated astrocytes in vivo.

Most of the ion channel types not present in culture may be preferentially localized to processes in vivo: at P8, sodium currents can be found only in 20% of acutely isolated type-1 astrocytes lacking processes, but are present in 100% of process-bearing type-1 astrocytes in prints. Similarly, calcium currents were not detected (even in the presence of BAYK-8644, unpublished data), and K\textsubscript{IR} was either absent or small in type-1 astrocytes lacking processes. Thus the expression of processes and of most of the ion channel types appears connected in some way, although it is not yet clear whether this link is causal.

In contrast to the type-1 astrocytes, the glial cells of the O-2A lineage expressed similar channels in culture and in prints: First, oligodendrocytes express the

### Table 2. Comparison of Currents in Type-1 and Type-2 Astrocytes

<table>
<thead>
<tr>
<th>Type</th>
<th>Type-1 Astrocyte Culture</th>
<th>Type-2 Astrocyte Culture</th>
<th>Type-1 Astrocyte P10 Print</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na\textsubscript{i}</td>
<td>Density (pA/pF)</td>
<td>0.19 ± 0.13</td>
<td>0.24 ± 0.16</td>
</tr>
<tr>
<td>K\textsubscript{i}</td>
<td>Density (nS/pF)</td>
<td>0.66 ± 0.55</td>
<td>1.30 ± 0.40</td>
</tr>
<tr>
<td>K\textsubscript{O}</td>
<td>Density (nS/pF)</td>
<td>0.31 ± 0.20</td>
<td>0.63 ± 0.38</td>
</tr>
<tr>
<td>Na\textsubscript{v}</td>
<td>Density (pA/pF)</td>
<td>17.00 ± 10</td>
<td>14.00 ± 2</td>
</tr>
<tr>
<td>Ca\textsubscript{v}</td>
<td>Density (pA/pF)</td>
<td>2.00 ± 1.6</td>
<td>1.70 ± 0.9</td>
</tr>
<tr>
<td>Ca\textsubscript{2+}</td>
<td>Density (pA/pF)</td>
<td>4.30 ± 4.0</td>
<td>0.68 ± 0.44</td>
</tr>
</tbody>
</table>

All values represent the mean of 10-20 cells and are expressed as mean ± SD. Only the major current types found in at least 66% of cells are shown. Values for type-2 astrocytes are from Barres et al. (1988a, 1990c).

* Two figures are given: the top one was measured in type-2 astrocytes in serum-containing cultures (Barres et al., 1988a); the bottom one, in type-2 astrocytes that developed in serum-free medium (Barres et al., 1990c).

* Na\textsubscript{v} forms about 15% of sodium current in type-2 astrocytes in serum-containing cultures (Barres et al., 1988b), but is not found in type-2 astrocytes that develop in serum-free medium (Barres et al., 1990c).
same three types of potassium channels in prints and in culture (compare results in this paper with those in Barres et al., 1988a, 1990c). Second, O-2A progenitors themselves express the same five voltage-dependent currents in prints and in culture.

Comparison of Type-1 and Type-2 Astrocyte Properties

In optic nerve cultures, type-1 astrocyte properties differ markedly from those of type-2 astrocytes (Table 2). Type-2 astrocytes express a complex ion channel phenotype, with at least seven different components of whole-cell current: $\text{Na}_c$, $\text{Na}_a$, $\text{Ca}_r$, $\text{Ca}_t$, $\text{K}_r$, $\text{K}_o$, and $\text{K}_a$ (Barres et al., 1988a, 1990c). Because several of these channel types had previously been thought to be unique to neurons (particularly $\text{Ca}_t$, $\text{Ca}_l$, $\text{Na}_a$, and $\text{K}_a$) and because glutamate-gated currents are present, the type-2 astrocyte has been suggested to have a "neuronal" phenotype (Bevan and Raff, 1985; Barres et al., 1988a; Usowicz et al., 1989). In contrast, the type-1 astrocyte has a much simpler phenotype in culture: it expresses none of the neuronal properties found in type-2 astrocytes and instead expresses mainly $\text{K}_o$. A further difference is the presence of $\text{K}_a$ at a 8 times higher density in type-2 astrocytes (about 0.7 versus 0.09 nS/pF). Sodium currents were found in only about 20% of type-1 astrocytes in culture, whereas, they were found in all type-2 astrocytes. When present in type-1 astrocytes, sodium currents are of the glial and not the neuronal form, although both forms are present in type-2 astrocytes, the neuronal form predominates (Barres et al., 1989b).

However, type-1 astrocytes in culture appear to be in a dedifferentiated state, as discussed above. What is the comparison between these cells in vivo? Since few if any type-2 astrocytes were isolated from optic nerve (see below), the best comparison may be between type-1 astrocytes in P10 prints and type-2 astrocytes in culture, as cells of the O-2A lineage seem to maintain their in vivo properties in culture. Compared this way, the differences between type-1 and type-2 astrocytes become a little less pronounced: each expresses $\text{K}_o$, $\text{K}_r$, $\text{Ca}_t$, and $\text{Na}_a$ currents (Table 2). However, the type-2 astrocyte continues to express channel types not found in the type-1 astrocyte: $\text{Na}_c$ and $\text{K}_a$.

Up Regulation of Type-1 Astrocyte Channels by Neurons

Neurons have previously been shown to promote glial differentiation. They signal oligodendrocytes whether and when to myelinate and cause astrocytes in culture to form processes and to stop dividing (Bunge and Bunge, 1984; Hatten, 1985). Thus, it was not surprising that neurons up-regulated expression of type-1 astrocyte ion channels. Up regulation of several different channels — $\text{K}_r$, $\text{Na}_c$, and $\text{K}_o$ — was observed. Since sodium currents were present in all adult type-1 astrocytes in adults, this up regulatory effect may account for the previous observation that astrocytes in transected optic nerves lack sodium channels (Tang et al., 1979; Pellegri and Ritchie, 1984).

How do neurons influence the differentiation of astrocyte channels, and is the signal for type-1 astrocytes the same as or different from that affecting oligodendrocytes? The effect could be mediated either by contact, by a soluble factor, or by both. In our co-culture experiments, only astrocytes directly contacting neurons had up regulation of specific ion channels. Thus, the neuronal effect is probably contact-mediated at least in part. It is possible that the neuronal signals to oligodendrocytes and to astrocytes are the same, since during development, myelination begins to occur at the same time as the change in astrocyte channels. cAMP might mediate, at least in part, both effects. Neurons up-regulate myelin-specific proteins in Schwann cells, and this effect can be partly mimicked by increasing Schwann cell cAMP (Sobue et al., 1986; Shuman et al., 1988). The ability of cAMP to induce astrocyte morphological differentiation has already been discussed (see above); similarly, it will be interesting to determine whether sodium currents in inwardly rectifying potassium currents in type-1 astrocytes (in culture or early postnatal prints) can be induced by forskolin.

Is the timing of this regulatory effect on glial channel expression intrinsic to the neurons, the astrocytes, or both? Although both axons and oligodendrocytes are present in the optic nerve at birth, myelination does not begin until P6, similarly, the change in astrocyte channel phenotype does not begin until about P6, although astrocytes are present at birth. Our data are consistent with two possibilities: the timing could be entirely intrinsic to neurons, or it could be intrinsic to the astrocytes but require the presence of neurons. These possibilities could be sorted out by coculturing type-1 astrocytes of different intrinsic ages with retinal ganglion cells of different intrinsic ages.

Why is ion channel expression by type-1 astrocytes so plastic? One possibility is that the channels arise, temporally and spatially, in response to neuronal requirements. For instance, the inwardly rectifying potassium channel implicated in potassium homeostasis develops in type-1 astrocytes at about the same time as the nerve develops the ability to buffer neuronally released potassium (Ransom et al., 1985). A question raised by these observations is whether other cell types in the optic nerve, such as endothelial cells, might also regulate ion channel expression by type-1 astrocytes.

How Do Astrocytes Help Regulate Potassium in White Matter?

Type-1-like astrocytes are believed to play an important role in the regulation of extracellular potassium. Two alternative mechanisms of potassium homeostasis involving passive flux of ions through channels, rather than uptake by cotransporters or pumps, have been hypothesized. "Spatial buffering" is postulated to allow rapid shunting of potassium away from sites
of excess through the glial syncytium (Orkand et al., 1966) and would require only a potassium permeability. In contrast, "potassium accumulation" would store potassium, chloride, and water locally and would require both potassium and chloride conductances.

**Spatial Buffering**

If spatial buffering is to play any significant role in potassium regulation, then it is necessary that potassium can be shunted along the glial syncytium without leaking back out through a resting potassium permeability; the astrocyte space constant must be long relative to the length of its processes. We have estimated the value of the space constant for type-1 astrocytes from our measurements of resting potassium conductance and found that in adult optic nerve it is significantly less than the length of an astrocyte: the space constant is 100 μm, whereas the length of an adult astrocyte is at least 400 μm.

According to the spatial buffer hypothesis, potassium is shunted away from a region of excess by a spatial voltage gradient. Local potassium elevation produces a depolarization relative to the more negative resting potential present distally. The electrical space constant provides a measure of how rapidly this voltage gradient falls off over distance along the astrocyte process: it falls as an exponential function of the space constant \( V_0 / \lambda \cdot \exp(-X/\lambda) \), where \( V_0 \) is the magnitude of the original depolarization, and \( V_0 / \lambda \) is the membrane potential at a distance \( X \) along the process. Therefore, at a value of \( X \) equal to the space constant, the gradient has dropped to \( 1/e \) (see Figure 8). In order for the potassium to be shunted effectively, it must travel at least the length of one astrocyte, 400 μm, which is 4 times the length of the space constant we have estimated. Thus, because the astrocyte processes are so leaky to potassium, the potential gradient postulated to drive potassium current flow will have fallen off long before the potassium can move the length of one process to the soma (let alone to another astrocyte or blood vessel; Figure 8).

It could be argued that the value of 100 μm represents a "least spread" estimate, since the calculation assumed that the potassium conductance was uniformly distributed. In Müller cells, 90% of the potassium conductance is found localized to the endfeet (Newman, 1984, 1986). However, while endfoot-like structures were frequently observed in young postnatal prints (e.g., Figure 5A), they were only infrequently observed in older prints (e.g., Figure 5D), most likely because the increasing length and decreasing diameter of astrocyte processes made it more likely for them to be shorn away during the print process. On the other hand, the short space constant will prevent voltage clamp of distal processes, leaving open the possibility that distal processes have a space constant that is different from that of proximal processes.

In contrast to the adult rat optic nerve, several factors suggest that spatial buffering in early postnatal rats, prior to myelination, could be an effective mechanism of potassium regulation: astrocyte processes are many times thicker, the inwardly rectifying conductance is lower, and astrocyte processes are shorter. Thus the space constant is longer, about 400 μm, than the astrocyte processes (100 μm or less; Figure 8).

However, in the optic nerve the spatial buffering model suffers from the additional difficulty that it is hard to see how either the longitudinal or radial membrane potential gradient could be formed: electrical activity will be uniform longitudinally and to some extent radially, resulting in spatially homogeneous extracellular elevation of potassium. Thus it is not clear whether a sufficient spatial gradient of membrane potential along the glial syncytium could ever develop postnatally or in the adult (see also Barres et al., 1988a, 1990b).

However, some spatial buffering must occur if a spatial membrane potential gradient is generated, since astrocyte membranes are potassium-permeable. Even if the space constant is short, potassium may not need to be shunted far for effective potassium buffering. Thus an important question is not whether spatial buffering actually occurs, but whether it predominates, or whether other mechanisms, particularly potassium accumulation (see below), make significant contributions to potassium homeostasis. For instance, strong evidence now supports a spatial buffer process in vertebrate retina (Kawolski et al., 1989), but the possible contribution of a simultaneous potassium accumulation process has not yet been addressed. It has been suggested that the relative contributions of potassium accumulation and spatial buffering may differ in gray and white matter (Barres et al., 1990b).

**Potassium Accumulation**

The ion channel phenotype of type-2 astrocytes and oligodendrocytes suggested a specific mechanism of potassium accumulation, termed the "modulated Boyle and Conway" hypothesis (Barres et al., 1988a, 1990c). According to this hypothesis, glial membranes are potassium-permeable and chloride-impermeable at rest. During impulse activity, a glial chloride conductance, found to be present but normally inhibited, is postulated to become active by activity-dependent liberation of a factor from neurons. Potassium influx into glia could then be followed by chloride and then water. The activation of a glial chloride conductance would thus allow local storage of potassium, chloride, and water in glia. This hypothesis makes several predictions that are fundamentally different from those of the spatial buffer hypothesis, and some of these are supported by new evidence from a variety of preparations (for discussion of these predictions see Barres et al., 1990b).

Our recordings indicate that type-1 astrocytes have the same conductances as that found in type-2 astrocytes and oligodendrocytes and postulated to be involved in potassium accumulation: the inwardly...
rectifying potassium conductance and a chloride conductance inhibited in resting cells. Thus type-1 astrocytes may be capable of potassium accumulation mediated by a modulated Boyle and Conway mechanism. Type-1 astrocytes contribute to the perinodal astrocyte processes in optic nerve (see Suarez and Raff, 1989) and thus may participate in potassium regulation at nodes of Ranvier.

However, there are at least two essential pieces of evidence for the modulated Boyle and Conway hypothesis still lacking. First, a chloride conductance in glial cells in vivo must be demonstrated. Thus far, all the evidence for a chloride conductance in glial cells has been derived from studies of cultured cells (we have not yet looked for the presence of these chloride channels in glia in tissue prints). Second, the presence of molecules present in the optic nerve capable of activating the glial chloride conductance needs to be demonstrated.

**The Existence of Type-2 Astrocytes in the Optic Nerve In Vivo**

Astrocytes with the antigenic phenotype of type-2 astrocytes in culture (A2B5+ and GFAP+) were absent or rare in optic nerve tissue prints. Astrocytes with the electrophysiological properties of type-2 astrocytes were also rare: although many astrocytes were examined, none with K_\text{IR} were found, and only one with a Na_\text{r} was found. Three possible explanations can account for these observations: in vivo type-2 astrocytes have antigenic and electrophysiological properties that differ from those in vitro, type-2 astrocytes are rare or absent in optic nerve in vivo, or type-2 astrocytes are not isolated in high yield by the tissue print protocol.

Although we cannot distinguish among these possibilities, it is possible that type-2 astrocytes have altered properties in vivo. A2B5 immunoreactivity on astrocytes is lost on type-2 astrocytes with age in culture (Lillien and Raff, 1990) and is not found on putative type-2 astrocyte perinodal processes in vivo that do label with other type-2 astrocyte markers, including NSP-4 and HNK-1 (ffrench-Constant and Raff, 1986).

It is also possible that the tissue print protocol failed to isolate type-2 astrocytes or that our failure to identify type-2 astrocytes in prints could represent a systematic morphological bias in cell type identification: A2B5+ cells with small somata and few processes were assumed to be O-2A progenitors; however, a small percentage of these cells are also GFAP+ (Miller et al., 1983b; Martin Raff, personal communication). Our observations show only that in tissue prints obtained from P7 to adult optic nerves, cells with the morphology of type-1 astrocytes that were A2B5+ and GFAP+ could not be found. Furthermore, only one cell with an astrocyte morphology was found to have the neuronal form of the sodium channel.

**Other Possible Functions of Ion Channels in Type-1 Astrocytes**

1. **Transfer of Channels to Neurons**
   Astrocytes have been postulated to be local sodium channel synthetic factories for axons (Chiu et al., 1984; Bevan et al., 1985). Because type-1 astrocytes may form the bulk of perinodal astrocytes, our data do not appear to be easily consistent with this hypothesis, since type-1 astrocytes synthesize the glial form of the sodium channel (Barres et al., 1989a). Moreover, the inwardly rectifying potassium channels that have been recently described in axons have properties which are different from those of the inwardly rectifying potassium channels found in type-1 astrocytes; for instance, the channels in axons are barium-resistant and may also be permeable to sodium (Baker et al., 1987; Eng et al., 1990).

2. **Maintenance of the Resting Potential**
   The only ionic current that we observed to be active at the resting potential of type-1 astrocytes is K_\text{IR}; thus K_\text{IR} is likely to underlie the resting potential. No evidence of a “leakage” potassium conductance in type-1 astrocytes was found.

3. **Excitability**
   Because the glial sodium channel has more negative voltage dependence than neuronal channels, it appears to be functionally specialized for a role in astrocytes (Barres et al., 1989a). Here we have found further evidence for this possibility. All type-1 astrocytes in P10 or older prints express sodium current, and at least some of these express a high density of sodium channels with 1–2 nA of peak current. In the absence of extracellular potassium, these cells were capable of firing single regenerative potentials. This did not occur when potassium was present because of the large inwardly rectifying potassium conductance, which lowered the input resistance. Since ionic currents are often subject to modulation by transmitters, it remains possible that type-1 astrocytes are excitable under certain conditions in vivo.

**Experimental Procedures**

1. **Preparation of Cell Suspensions**
   Optic nerves from postnatal Long-Evans rats were dissected from just posterior to the optic foramen to, but excluding, the optic chiasm. This tissue was then dissociated enzymatically to make a suspension of single cells, essentially as described by Huettner and Baughman (1986). Briefly, the tissue was minced and incubated at 37°C for 75 min in a papain solution (30 U/ml; Worthington) equilibrated with 95% O_2 and 5% CO_2. This solution also contained Earle’s balanced salts (EBSS), calcium, magnesium, EDTA, sodium bicarbonate, glucose, and L-cysteine, as described (Huettner and Baughman, 1986). The tissue was then triturated sequentially with #21 and #23 gauge needles in a solution containing ovomucoid (2 mg/ml; Calbiochem-Behring) and bovine serum albumin (BSA; 1 mg/ml; Sigma) to yield a suspension of single cells. The cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM).

2. **Preparation of Tissue Prints**
   *Enzymatic Treatment of Tissue*
   Optic nerves were dissected and placed at room temperature in

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**Neuron** 540
EBSS containing calcium and magnesium (pH 7.4) in a 35-mm petri dish. For adult tissue and tissue older than P12, 50-100 μm thick vibratome sections were prepared.

A papain solution was prepared as described above except 10 mM HEPES was substituted for the bicarbonate buffer in most experiments. For tissue older than about P7, the EDTA can be increased to 2 mM and the calcium and magnesium can be removed to increase the yield greatly. However, as it is difficult to form stable seals on cells exposed to such low divalent ion concentrations, this method was used only for immunohistochemical experiments in which recordings were not performed. The EBSS was removed, and the papain solution was added. The nerves were treated with papain for 10 min, the leptomeninges were removed, and the enzyme incubation was continued for 20 min (P2 nerves) to 75 min (P12 nerves or adult vibratome sections). The papain solution was removed, and 3 ml of an ovomucoid inhibitor solution in L15 medium containing Dnase (as above) was added.

Printing

Depending on the experiment, for immunohistochemical labeling of fixed cells, tissue was printed onto nitrocellulose paper or onto glass coverslips coated with poly-L-lysine or nitrocellulose. For electrophysiological or immunohistochemical experiments on live cells, tissue was printed onto glass coverslips coated with nitrocellulose. These were prepared as follows:

- Nitrocellulose paper (Schleicher and Schuell, 0.4 μm pore size) was prewetted by placing it on the surface of PBS or EBSS for a few seconds and then immersed. The paper was cut into small pieces, about 5-8 mm in diameter, and left in EBSS until use.
- Poly-L-lysine-coated glass coverslips: 13-mm glass coverslips were prewetted with 70% ethanol for several hours and rinsed with water. These were then incubated in poly-L-lysine (MW > 300,000; Sigma) at 1 mg/ml for at least 10 min and rinsed in water 3 times before use.
- Nitrocellulose-coated coverslips: Glass coverslips were cleaned as described above and air dried completely. These coverslips were dipped into 1% nitrocellulose in amyl acetate (Ladd or Ted Pella), placed on a piece of filter paper to air dry overnight, and collected the next day into a large petri dish keeping the “good” side face up. Slips were handled gently with forceps at the edges only, to avoid scratching or tearing the nitrocellulose film, and were transferred through air-liquid interfaces edge first to avoid lifting off the film.

The tissue was printed under a dissecting scope in a 35-mm petri dish in a normal saline solution containing 5 mM HEPES, 100 μM calcium, and 5 μM potassium. Enzymatically treated optic nerve tissue was gently touched or pressed against the nitrocellulose paper or nitrocellulose-coated glass coverslip. This was accomplished by first stabilizing the tissue on a piece of nitrocellulose paper with the side of the tissue to be blotted face up. Slips were handled gently with forceps at the edges only, to avoid scratching or tearing the nitrocellulose film, and were transferred through air-liquid interfaces edge first to avoid lifting off the film.

Tissue sections were then placed into fixative or the recording bath solutions, depending on the experiment. For recording, optic nerves were left in L15 medium containing 0.1% BSA (Sigma) for 5 min to 3 hr before printing and recording.

Immunohistochemistry of Tissue Prints on Nitrocellulose Paper

Tissue prints were stained with standard immunofluorescence and immunoenzymatic procedures. Tissue was fixed with paraformaldehyde in a normal saline solution containing 5 mM HEPES, 100 μM calcium, and 5 μM potassium. Enzymatically treated optic nerve tissue was gently touched or pressed against the nitrocellulose paper or nitrocellulose-coated glass coverslip. This was accomplished by first stabilizing the tissue on a piece of nitrocellulose paper (with the side of the tissue to be blotted face up) and then inverting this over the coverslip. The tissue was then lifted from the coverslip and discarded. The print was then placed in fixative or the recording bath solutions, depending on the experiment. For recording, optic nerves were left in L15 medium containing 0.1% BSA (Sigma) for 5 min to 3 hr before printing and recording.

After immunoenzymatic labeling, the tissue was dehydrated in graded t-butyl alcohol at 70%, 95%, 100%, and 100% for 3-5 min each. The nitrocellulose was rendered transparent by immersion in xylene for several min (see also Seshi, 1986). The print was mounted in a xylene-based mountant, such as Permount. (Dehydration could not be accomplished with ethanol, since this dissolved the nitrocellulose, nor with isopropanol, since this tended to dissolve the diaminoenzidine reaction product. t-Butyl alcohol avoided these difficulties and was miscible with both water and xylene. The black alkaline phosphatase reaction product is also compatible with this technique.) A more detailed protocol is available on request.

Preparation of Cultures

Type-1 Astrocytes

 Cultures of optic nerve type-1 astrocytes were prepared according to the procedures of Raff and coworkers (Raff et al., 1983a, Miller and Raff, 1984). Briefly, cultures were prepared from cell suspensions of optic nerves from P0 to P8 rats. Cells were cultured in DMEM containing 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. In some cases, cells were cultured in a modified Bottenstein-Sato serum-free medium (Raff et al., 1983b). The cells were plated at a density of 15,000 cells per cm² on 13-mm round glass coverslips that had been precoated with poly-L-lysine, and were cultured at 37°C in a humified atmosphere of 5% CO₂ and 95% air.

Retinal Ganglion Cell and Type-1 Astrocyte Co-Cultures

Purified retinal ganglion cells, prepared from P2 or P10 rat retinas, were added to cultures of type-1 astrocytes prepared from P7 rats and grown for 3 days in a medium containing DMEM, fetal calf serum, P-S, and glutamine as described above. The medium on the astrocytes was changed 1 day prior to addition of the neurons to an L15-based medium.

Ganglion cells were purified to near homogeneity using the two-step panning procedure described previously (Barres et al., 1988b). In brief, retinal cell suspensions were prepared using papain. Cells were incubated in a rabbit anti-rat ganglion antigen that bound to both macrophages and endothelial cells and then incubated on the first panning dish, which was coated with anti-rabbit IgG. Nonadherent cells were transferred from this plate to the second panning dish, which was coated with Thy-1 antibodies. Nonadherent cells were washed away, and the adherent cells, which represented pure retinal ganglion cells, were released by treatment with trypsin. The purified neurons were then plated onto the type-1 astrocyte cultures at a density of 50,000 cells per coverslip.

Immunohistochemical Labeling of Cells in Culture or in Prints

Cells in culture, suspensions, or tissue prints were labeled by incubation with monoclonal antibody supernatants; antibodies to galactocerebroside were generously provided by B. Ranscht (Ranscht et al., 1982), and antibodies to A2B5 (Eisenbarth et al., 1979) were obtained from the American type Culture Collection. For labeling of prints on nitrocellulose-coated glass coverslips, the slips were incubated in 1% BSA after printing and prior to antibody incubations. For labeling of surface antigens, cells were incubated in the primary antibody for 15 min, washed, and incubated in a 1:100 dilution of the secondary antibody. The secondary antibodies (Accurate) were fluorescein- or rhodamine-coupled goat anti-mouse IgG. L15 medium containing 0.1% BSA was used in all antibody incubations and washes to decrease nonspecific binding. Controls, with the primary antibody omitted and replaced with fresh hybridoma growth medium or a control monoclonal antibody, revealed negligible background. Cells in culture, labeled by indirect immunofluorescence as described above, were identified by viewing with a Zeiss microscope equipped with differential interference contrast optics and epifluorescence illumination. The verification of cell type identity by both morphology and antigenic phenotype in our cultures has previously been described (Barres et al., 1988a).

For A2B5 and GFAP double labeling, tissue prints were first fixed in 4% paraformaldehyde for 15 min, blocked with 5% BSA for 30 min, and incubated in A2B5 supernatant at 1:2 for 30 min.
followed by an affinity-purified Texas red-conjugated goat anti-mouse antiserum (Accurate at 1:100). Prints were then incubated in acetone at -20°C for 5 min, washed, and incubated in 5% BSA again. Finally, they were incubated in a rabbit antiserum to cow GFAP (Dako) at 1:200. Followed by an affinity-purified FITC-conjugated goat anti-rabbit antiserum. Control experiments demonstrated that the secondary antibodies each bound only to the correct primary antibody, and the FITC and Texas red fluorescence of GFAP and A2B5 single labels could not be detected by Texas red and FITC filter sets, respectively.

Identification of Cell Types

Optic Nerve Cultures

Type-1 astrocytes were identified with RAN-2 and GFAP double labeling. As previously reported, in neuron-free cultures these cells did not bear processes and thus could be distinguished from type-2 astrocytes and oligodendrocytes. About 18% of non-process-bearing cells in our cultures were RAN-2+ and GFAP- and were identified as meningeal cells (Raff et al., 1979; Bartlett et al., 1988). RAN-2 could not be used to distinguish type-1 astrocytes from meningeval cells, since they were both positive and most of the meningeval cells did not label with fibronectin antibodies; thus surface labeling could not be used to record from the meningeval cells. However, GFAP labeling demonstrated that the meningeval cells were distinguishable on morphological grounds, since they were much thinner than type-1 astrocytes and were sheet-like and often almost transparent.

Optic Nerve Co-Cultures with Retinal Ganglion Cells

Since the astrocyte cultures were prepared from P1 optic nerves, they contained few O-2A lineage cells (Lillien and Raff, 1990). Therefore RAN-2 was used to label the type-1 astrocytes prior to recording; positive cells were either type-1 astrocytes or meningeval cells, and these could be distinguished by morphology (see above), particularly since the type-1 astrocytes formed processes in the presence of neurons (Miller et al., 1989b), whereas the meningeval cells did not.

Optic Nerve Tissue Prints

Oligodendrocytes were always identified prior to recording by labeling with antibodies to galactocerebroside (see below), and O-2A progenitors were always identified by labeling with A2B5 antibodies. All A2B5+ cells were O-2A progenitors, since no A2B5- cells were labeled by GFAP antibodies. Type-1 astrocytes were identified by RAN-2 labeling and GFAP labeling. All cells with multiple long processes (more than three) had the antigenic phenotype of type-1 astrocytes (RAN-2+, GFAP+, and A2B5+), and thus these cells were identified in most experiments using these morphological criteria.

Electrophysiological Recording

Gigapohm Seal Recording

A piece of glass coverslip with cultured cells was placed in the recording chamber, which contained the appropriate bath solution (volume 500-750 μl). Standard procedures for preparing pipettes, 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 40–45 (corresponding to an internal tip diameter of about 1.2 μm; Corey and Stevens, 1983). Pipette capacitance and series resistance were electronically compensated. All experiments were done at room temperature, approximately 24°C.

Data Acquisition and Analysis

Voltage stimuli were generated and responses were recorded with a PDP 11/73 computer (INDEC). A Yale Mark V patch clamp was used. Analog signals were filtered with an 8-pole, low-pass Bessel filter before being digitized and recorded by the computer. In each experiment, linear capacitative 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 and Current Isolation

The solutions for the bath and those for the pipette (which replace diffusible constituents of cytoplasm) were designed in each case to isolate current carried by a specific ion through a specific channel type. All solutions contained 5 mM HEPES, which were adjusted to pH 7.4, and had junction potentials of less than 1-2 mV. The pipette solutions were diluted by 15% after preparation, to prevent dilation of the cell during recording. In many experiments, 0.2% BSA was added to the bath solution. This significantly improved the quality of seals and the length of time prints could be recorded from prior to cell deterioration.

- Glutamate-activated currents. External: 140 mM LiCl, or 140 mM LiCH₃SO₄H, 2 mM CaCl₂, 0 mM MgCl₂, Internal: 140 mM CsCl, 1 mM MgCl₂, Ca²⁺ buffered to 10⁻⁵ M with 2 mM EGTA.
- Sodium currents. External: 140 mM NaCH₃SO₄H, 2 mM CaCl₂, 0.1 mM CdCl₂, Internal: 140 mM CsCH₃SO₄H, 1 mM MgCl₂, Ca²⁺ buffered to 10⁻⁵ M with 10 mM EGTA.
- Calcium currents. External: 10 mM BaCl₂, 135 mM NaCl, 10 μM tetrodotoxin. Internal: 140 mM CsCl, 1 mM MgCl₂, Ca²⁺ buffered to 10⁻⁵ M with 10 mM EGTA.
- Chloride currents (whole-cell experiments). External: 140 mM NaCl, 2 mM CaCl₂, 5 mM KCl (or 0 mM if K⁺ present). Internal: 140 mM CsCl, 1 mM MgCl₂, Ca²⁺ buffered to 10⁻⁵ M with 10 mM EGTA.
- Chloride currents (single-channel experiments). External (pipette): 130 mM TMA-Cl, 0.5 mM CaCl₂, 5 mM HEPES. Internal (bath): 130 mM TMA-Cl, 10 mM EGTA, 1 mM MgCl₂, Ca²⁺ buffered to 10⁻⁵ with 5 mM HEPES.
- Outwardly rectifying potassium currents. External: 140 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl. Internal: 140 mM KCl, 1 mM MgCl₂, Ca²⁺ buffered to 10⁻⁵ M with 10 mM EGTA.
- Irreversibly rectifying potassium currents. External: 120 mM NaCl, 20 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂. Internal: 140 mM KCl, 1 mM MgCl₂, Ca²⁺ buffered to 10⁻⁵ M with 10 mM EGTA.

In addition, two alternative solutions were used to study outward potassium currents when an inward rectifier was present, or to identify an inward rectifier by its elimination. In one, 3 mM cesium was added to the bath; in the other, potassium was entirely eliminated from the bath. In the latter, elimination of potassium from the bath caused the rapid depletion of intracellular potassium. In that case, we used a new piece of coverslip for each cell recorded and dialyzed cells with the potassium-containing internal solution for at least 5 min prior to measuring currents.

Current densities (pA/pF) were calculated for inward current components as peak inward currents divided by the cell capacitance. Conductance densities (nS/pF) were calculated for outward potassium currents by dividing the chord conductance by the cell capacitance. Chord conductance was calculated as (V₅₀ - Vₑₜ) / (Vₑₜ - Vₑᵣ), where the equilibrium potential for potassium (Vₑₜ) was -50 mV when the bath potassium was 20 mM and -85 mV when the bath potassium was 5. When bath potassium was omitted, a value for Vₑᵣ of -100 mV was used to allow an approximation of conductance for comparison with the other calculations, since current-clamp measurements showed the resting potential to be about -110 to -100 mV in these cells. V₅₀ was +60 mV for K⁺ and -120 mV for Kᵣ (see Barres et al., 1988a).

Application of Drugs to Whole Cells

Drugs were applied by pressure ejection through double-barreled pipettes with tips of 5 μm diameter separated by 50 μm. During application of drugs, the recording chamber was continuously perfused with fresh bath solution at a rate of 1 mL/min. The large tip separation prevented cross-contamination of the barrels. Phenol red was used at a concentration of 0.01% to allow visualization of the ejected solutions.

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