Development of GLAST(+) Astrocytes and NG2(+) Glia in Rat Hippocampus CA1: Mature Astrocytes Are Electrophysiologically Passive

Min Zhou, Gary P. Schools, and Harold K. Kimelberg
Neural and Vascular Biology, Ordway Research Institute, Albany, New York

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Zhou, Min, Gary P. Schools, and Harold K. Kimelberg. Development of GLAST(+) astrocytes and NG2(+) glia in rat hippocampus CA1: mature astrocytes are electrophysiologically passive. J Neurophysiol 95: 134–143, 2006. First published August 10, 2005; doi:10.1152/jn.00570.2005. Glia show marked heterogeneity in terms of electrophysiology in the developing brain, and two major types can be identified based on GFAP or NG2 expression. However, it is associated to be determined if such an electrophysiological diversity holds for the adult brain and how GFAP and NG2 lineage glia are associated with different electrophysiological phenotypes during the course of development. To address these fundamental questions, we performed in situ whole cell recording from morphologically identified glia from the rat hippocampal CA1 region from postnatal (P) days 1–106 and double-stained postrecorded cells with GLAST and NG2 antibodies. We found glia express mostly voltage-gated outward K+ currents and also have inward Na+ currents in the newborn (P1–P3), but these are no longer present after P22. They consist equally of GLAST(+) cells in the newborn, but are mainly NG2(+) glia in juvenile animals (P4–P21). Glia showing voltage-gated outward and inward K+ currents are also present at P1, peak at P5 and decline to a stationary level of ~10% in the adult. They are GLAST(+) astrocytes from newborn to juvenile but NG2(+) glia in the adult. Electrophysiologically passive glia first appear at P4 and increase to 91% in adults, of which 85% are GLAST(+). These results indicate that glial electrophysiological diversity occurs predominantly in the developing brain. While most glia in the NG2 lineage preserve a certain amount of voltage-gated ion conductances, mature GLAST(+) astrocytes are electrophysiologically passive.

INTRODUCTION

Astrocytes distinguished by different ion channel expression have been described for both freshly isolated astrocytes (Zhou and Kimelberg 2000) and astrocytes in slices (Chvatal et al. 1995; D’Ambrosio et al. 1998; Steinhauser et al. 1992). The concept of functional heterogeneity among astrocytes has been further supported by findings that show a segregated expression of ionotropic glutamate receptor AMPA subtypes and functional glutamate transporters by two or three electrophysiologically distinctive astrocyte phenotypes (Grass et al. 2004; Matthias et al. 2003; Zhou and Kimelberg 2001). Such differences contradict the homogenous view of astrocytes based on their appearance under infrared differential interference contrast (IR-DIC) microscopy and their nonexcitability by in situ whole cell recording in the rat hippocampal CA1 region from newborn through adult [postnatal days 1–106 (P1–P106)]. To correlate electrophysiological glial phenotypes with the astrocytic and NG2 glial lineages, we loaded biocytin into the recorded cells and performed postrecording GLAST/NG2 double staining. We confined our studies to the stratum radiatum (SR) and stratum lacunosum moleculare (SLM) to exclude those glia in the dentate gyrus (DG) retaining neurogenetic potential in the adult hippocampus (Rietze et al. 2000).

METHODS

Hippocampal slice preparation

Hippocampal slices were prepared from P1 to P106 rats. The procedure was performed in accordance with a protocol approved by the Wadsworth Center, New York State Department of Health Institutional Animal Care and Use Committee. Animals were anesthetized in a combination of ketamine (100 mg/kg, injected intraperitoneally) and xylazine (10 mg/kg, injected intraperitoneally).

Address for reprint requests and other correspondence: M. Zhou, Neural and Vascular Biology, Ordway Research Inst., 150 New Scotland Ave., Albany, NY 12208 (E-mail: mzhou@ordwayresearch.org).
with 100% CO₂ before decapitation, and their brains were removed from the skull and placed in an ice-cold, oxygenated (5% CO₂, 95% O₂, pH = 7.4) slice preparation solution containing (in mM) 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 10 MgCl₂, 10 glucose, 0.5 CaCl₂, and 240 sucrose. Final osmolarity was 350 ± 2 mOsm. Coronal slices of 300 µm were cut with a Vibratome (Pelco 1500) and transferred to a nylon-basket slice holder in 20–22°C artificial cerebral spinal fluid (aCSF) containing (in mM) 125 NaCl, 25 NaHCO₃, 10 glucose, 3.5 KCl, 1.25 NaH₂PO₄, 2.0 CaCl₂, and 1 MgCl₂ (osmolarity, 295 ± 5 mOsm). The slices were allowed to recover in aCSF with continuous oxygenation for ≥60 min before recording.

Electrophysiology

For in situ recording, individual hippocampal slices were placed in the recording chamber that was constantly perfused with oxygenated aCSF (2.5 ml/min). Glia located in the CA1 region were identified using IR-DIC video microscopy (Olympus BX51W1) with a ×40 water immersion objective. The image was detected with an IR-sensitive CCD camera and displayed on a monitor. Whole cell membrane currents were amplified by a MultiClamp 700A amplifier, sampled by a DIGIDADA 1322A Interface, and data acquisition was controlled by PCamp 9.0 software (Axon Instruments, Foster City, CA) installed on a Dell personal computer. Low resistance patch pipettes (3–6 MΩ) were fabricated from borosilicate capillaries (OD: 1.5 mm; Warner Instrument Corporation, Hamden, CT) using a Flaming/Brown Micropipette Puller (Model P-87, Sutter Instrument Co., Novato, CA). Membrane potential was read in I = 0 mode, and the variation during the 20- to 30-min whole cell recording was always less than ±5 mV. Capacitance and series resistances (Rs) were measured using the “membrane test” protocol built into the PCamp 9.0. Patch pipettes were filled with a solution containing (in mM) 140 KCl, 0.5 CaCl₂, MgCl₂, 5 EGTA, 10 HEPES, 3 Mg-ATP, and 0.3 Na-GTP (pH 7.3, 280 ± 5 mOsm). Biocytin (0.1–0.5%) was added into electrode solution to detect the recorded astrocyte and show its full morphology and coupling to other cells. All the experiments were conducted at room temperature (22–24°C).

Confocal immunocytochemical studies

After electrophysiological recording, the hippocampal slices containing the biocytin-filled cells were fixed for 45–60 min in 4% formaldehyde in phosphate-buffered saline (PBS; pH 7.4). After washing with PBS, the slices were permeabilized with 1.0% Triton X-100 in PBS (1 h). To visualize the biocytin-filled cells, the slices were treated with 1:1,200 Cy2-streptavidin in PBS for 4 h, followed by washing in PBS. Three percent normal goat serum in PBS (4 h) was used to block nonspecific binding of primary antibodies. Antibodies recognizing the GFAP (rabbit, Dako), GLAST (guinea pig, Chemicon) and NG2 (rabbit, Chemicon) antigens were diluted in 3% normal goat serum plus 0.1% Triton X-100 (NGS/TX). The slices were incubated for 18 h at room temperature with gentle shaking. Slices were washed successively in NGS/TX, 0.1% Triton X-100 in PBS, and PBS. Cy3- and Cy5-conjugated secondary antibodies were selectively used based on the minimal emission wavelength overlap. Three percent normal goat serum was used again to block nonspecific binding before incubation in the diluted secondary antibodies overnight. The specificities of all the secondary antibodies were tested with samples not exposed to primary antibodies, and little or no signal was seen. After the final washes, the slices were mounted on slides. Slides were viewed by confocal microscopy.

We used an inverted Zeiss LSM510 META confocal microscope with ×10/0.3 NA, ×25/0.8 NA, and ×63/1.4 NA objectives. The recorded cell was initially found using a 50-W mercury arc lamp with a ×10 objective and a filter set for Cy2. The intensity of the Cy2 signal and the location of the mark made by the recording electrode were used to identify the recorded cell from other cells coupled to this cell. In the laser scanning mode, the extent of the syncytium in the z-axis was determined, and detector gain for each fluorochrome in the sample was set to minimize saturated and black pixels. Each fluorochrome was excited, and its emission was acquired individually to minimize bleed through from channel to channel. A DIC image of each z-plane was also acquired.

Two-dimensional projections of the image stacks and three-dimensional reconstructions of the recorded and coupled cells were made using the LSM510 program. Colocalization of fluorescent labels within individual cells were displayed using the National Institutes of Health ImageJ program (Rasband 1997) with the Colocalization plugin (Bourdoncle 2003).

Statistics

All the data are given as means ± SD.

RESULTS

Identification of astroglia in situ

The glia soma visualized under IR-DIC microscopy can be described as either round or irregular (Fig. 1, A and B) as reported before (Lin and Bergles 2004). We examined whether each shape correlates with GFAP and NG2 glial lineages. However, GFAP immunoreactivity could not be reliably detected from the recorded cell, although it readily detected in glia coupled to the recorded cell (see Supplementary Fig. 1). We alternatively used the glutamate transporter isoform GLAST as a marker for GFAP lineage astrocytes because it stained reliably after whole cell recording. Possibly this is because it is expressed as an astrocyte membrane protein and thus should be less perturbed by the electrode solution during whole cell recording compared with GFAP. The fact that GLAST expression begins in the newborn and persists throughout life (Furuta et al. 1997), and GLAST expression is linked to a functional role of astrocytes in glutamate homeostasis, also makes it a good marker for astrocyte identification. In addition, GFAP/GLAST double-staining experiments in unre-corded cells showed an equal percentage of GFAP(+) glia among irregular and round soma shape cells (71%; Fig. 1E). However, GFAP/GLAST double-staining experiments in unre-corded cells showed an equal percentage of GFAP(+) glia among irregular and round soma shape cells (71%; Fig. 1E). Also, GLAST immunoreactivity was detected nearly identi-cal-ly from irregular (85%) and round soma (84%) glia (Fig. 1E). Except for one round soma glia, all the GFAP(+) glia of both soma shapes were GLAST(+) (n = 107); however, GFAP immunoreactivity was absent in 14% of GLAST(+) glia of both soma shapes (n = 107; Fig. 1E). These results indicate that, although the number of GLAST(+) glia are slightly higher than GFAP(+) glia, GLAST(+) overlaps excellently with GFAP(+) glia and therefore is an equivalent marker for GFAP lineage glia.

We next sought to answer if NG2 corresponds with a certain soma shape by GLAST/NG2 double staining, and we found that 72 and 76% of all irregular and round soma glia were GLAST(+) glia, respectively, and NG2 immunoreactivity was equally detected from irregular and round soma shaped glia in ~20% of all the cells studied (Fig. 1, E and F). Importantly, GLAST and NG2 immunoreactivity were never colocalized in the same cell, regardless of soma shapes. The quantitations in Fig. 1, E and F, were calculated from P10 rats, and the same

1 The Supplementary Material for this article (a Figure) is available online at http://jn.physiology.org/cgi/content/full/00570-2005/DC1.
percentages were observed for P35 rats (data not shown). The identification of soma shapes by DIC and the correlation of GLAST and GFAP immunoreactivities with these DIC soma shapes images were performed by different individuals in a blinded fashion. Therefore we conclude that 1) the variation in glial soma shapes observed with two-dimensional IR-DIC cannot be applied, at least in the CA1, to discriminate GFAP/GLAST from NG2 lineage glia; and 2) GLAST and NG2 are specific markers for GFAP and NG2 lineage glia identification. Based on these findings, glial cells having both round and irregular soma shapes were included in the following experiments, as specified.

Diverse electrophysiological astroglial phenotypes identified by whole cell recording

To understand the developmental changes in glial electrophysiological phenotypes, 561 whole cell recordings were taken from glial cells in the hippocampal CA1, SR, and SLM regions from P1 to P106. Three current profiles in voltage-clamp mode were distinguished (Fig. 2, A–C). Two of them resemble what has been described from freshly isolated hippocampal astrocytes (Zhou and Kimelberg 2000) and from hippocampal slices (D’Ambrosio et al. 1998; Steinhauser et al. 1992). Figure 2A corresponds to what we termed (Zhou and Kimelberg 2000) an outwardly rectifying astrocyte (ORA), characterized by a depolarization-induced voltage-gated inward Na\(^+\) (\(I_{Na}\)), outward transient K\(^+\) (\(I_{Ka}\)), and delayed rectifier K\(^+\) (\(I_{Kdr}\)) channel currents. Figure 2B corresponds to what we termed a variably rectifying astrocyte (VRA) with expression of both inward and outward K\(^+\) currents, no inward Na\(^+\) currents, and with multiple rectification points in its voltage-current relationship. However, because postrecording staining showed that these cells actually represented both GLAST(+) and NG2(+) glia, we will now term the electrophysiological phenotypes without postrecording identification outwardly rectifying glia (ORG) and variably rectifying glia (VRG). The third electrophysiological glial phenotype that we found (Fig. 2C) is distinguished by a linear current-voltage relationship in whole cell recording, which has been previously

FIG. 1. Glial soma shape in 2-dimensional infrared differential interference contrast (IR-DIC) do not distinguish GFAP and NG2 lineage glia. A and B: DIC images of irregular and round soma glia, respectively, located in the CA1 region. C and D: representative GFAP/GLAST and GLAST/NG2 double-stained images for the irregular and round soma glia as indicated. Images from left to right for C are DIC, anti-GLAST, anti-GFAP, and color merge; for D, they are DIC, anti-GLAST, anti-NG2, and color merge. Anti-GLAST in C and D are red, and anti-GFAP in C and anti-NG2 in D are green. Color merge shows the colocalization of different soma shapes with the selective marker expression. Bar graphs in E and F are summaries of the GFAP/GLAST and GLAST/NG2 double-staining results. In E, the left bar graph shows that all the GFAP(+) glia were also GLAST(+) glia; the middle bar graph labeled as GLAST(+)/GFAP(−) also represents 14% of GLAST(+) glia that were GFAP(−) while the rest were GLAST(+)/GFAP(+). Right bar graph: remaining glia that were GFAP(−)/GLAST(−). Images in A and B and all the quantitative data for C–F were obtained from acute hippocampal slices prepared from P10 rats.
Inward ion conductances yield a strong outward and transient outward $K^+$ capacitance current subtraction and displayed in expanded scale in the inset of their corresponding voltage commands. The whole cell membrane potential (60 mV) was taken from P7 and $H_{11001}$ are distinguished by their predominant leak-type $K^+$ currents, which results in multiple $K_{\text{dr}}$ currents after the withdrawal of voltage steps (Chvatal et al. 1995; Steinhauser et al. 1992) (data not shown). No cells that might correspond to microglia (Boucsein et al. 2003) were observed.

Voltage-clamp measurements were always followed by measurements in the current-clamp mode to check for excitability and possible postsynaptic electric events (Fig. 3, A–C). Only ORGs from newborn rats (P1–P3) showed some immature spiking when positive current was injected. These were described in in situ hippocampal glial studies from P10 to P30 rat hippocampus (D’Ambrosio et al. 1998; Steinhauser et al. 1992).

### Table 1. Passive membrane properties of electrophysiologically identified glia and interneurons in situ

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Membrane Potential, mV</th>
<th>Membrane Capacitance, pF</th>
<th>Membrane Resistance, MΩ</th>
</tr>
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<tbody>
<tr>
<td>ORG</td>
<td>$-45.9 \pm 17.2$ (111)</td>
<td>$24.5 \pm 15.4$ (111)</td>
<td>$870.3 \pm 705.7$ (111)</td>
</tr>
<tr>
<td>VRG</td>
<td>$-71.8 \pm 12.7$ (134)</td>
<td>$74.4 \pm 54.3$ (134)</td>
<td>$114.6 \pm 112.4$ (134)</td>
</tr>
<tr>
<td>PG</td>
<td>$-72.8 \pm 8.3$ (275)</td>
<td>$2897.7 \pm 13041.0$ (275)</td>
<td>$6.3 \pm 12.2$ (216)</td>
</tr>
<tr>
<td>Neuron</td>
<td>$-53.8 \pm 8.2$ (15)</td>
<td>$34.1 \pm 25.4$ (10)</td>
<td>$147.2 \pm 170.0$ (10)</td>
</tr>
</tbody>
</table>

The values in each column represent means ± SD and the numbers in parentheses are the cell numbers used to calculate each value.

### Figures

**Fig. 2.** Whole cell current profiles of glia in hippocampal CA1 region. D: voltage commands for whole cell voltage-clamp recording with the cell held at $-70$ mV under resting conditions. To maximally activate $Na^+$ ($I_{Na}$) and transient outward $K^+$ currents ($I_{K_{\text{dr}}}$), a 300-ms preconditioning pulse at $-110$ mV was delivered to the recorded cell before voltage steps; voltage steps for the current induction were 50-ms pulses from $-180$ to $+60$ mV with 10 mV increments. A–C: whole cell current profiles recorded from morphologically identified astroglia as shown in Fig. 1, A–C. Outwardly rectifying glia (ORG; A) are characterized by successive activation of inward $I_{Na}$ (see inset) and outward transient ($I_{K_{\text{dr}}}$) and delayed rectifying ($I_{K_{\text{dr}}}$) $K^+$ currents all induced by depolarization pulses. Combined expression of these voltage-gated ion conductances yields a strong outward rectification in the $I-V$ relationship (E). $I_{Na}$ are disclosed after leak and capacitance current subtraction and displayed in expanded scale in the inset in A. Variably rectifying glia (VRG; B) show a combined expression of outward $I_{K_{\text{dr}}}$, $I_{K_{\text{dr}}}$, and inward $K^+$ currents, which results in multiple rectification points in the whole cell $I-V$ relationship (F). Passive glia (PG; C) are distinguished by their predominant leak-type $K^+$ channel current expression, which yields linear $I-V$ relationship in whole cell current profile (G). To construct the $I-V$ curves, the current amplitudes at 5 ms (indicated by the arrows) of the voltage pulse onsets were taken and plotted against their corresponding voltage commands. The whole cell membrane potentials of A–C were $-45$, $-82$, and $-80$ mV, respectively. A and B were taken from P7 and C was taken from P35 rats.
characterized by a slow rise but no overshoot (Fig. 3B). At later postnatal and adult stages, we never saw immature spikes in current clamp for any of the three electrophysiological glial phenotypes. Although, in current-clamp mode, some ORGs in the early developmental stage show fluctuations in their basal membrane potential, no clear postsynaptic events were measurable (Fig. 3C). Although interneurons are distributed throughout the same brain region and some of them are similar in soma shape and size to glia, they always showed miniature excitatory postsynaptic potential (mEPSP) and spontaneous electric spikes (Fig. 3F). Thus differences in electrical excitability are one essential criterion discriminating glia from interneurons.

Developmental changes in ion current profiles

We systematically examined the existence and distribution of the electrophysiological phenotypes recorded in voltage-clamp mode, in this case from irregular soma shaped glia, in slices from newborn to adult rat (Fig. 4A). In light of the dramatic changes we found occurring in the first postnatal week, we studied cells on each successive day. For each day, ≥25 glial cells were recorded to determine the relative ratio among the different electrophysiological glial phenotypes. In an additional study (Fig. 4B), recordings from round soma glia were taken from P2, P7, and P38. The electrophysiological developmental profile of round soma clearly follows the pattern for irregular soma glia, further supporting the view that irregular and round soma represent the same glial population.

In the newborn rat, which we define as P1–P3, a total of 102 whole cell recordings showed that glia identified by an irregular soma were comprised of ORGs and VRGs. ORG was the dominant phenotype in the newborn, and the ratio of ORG/VRG declined with age (Fig. 4A). It is noteworthy that none of the glial cells recorded in the newborn period were electrophysiologically PGs (n = 102). Starting from P4 and continuing to P21, PGs, ORGs, and VRGs were found to coexist (Fig. 4A). We define this developmental time period as juvenile. After P7, we pooled our recording data week by week, and in each week, ≥25 recordings were taken.

During the juvenile period, ORGs continually decreased in number and completely disappeared at the end of the juvenile period. Concomitantly, the proportion of VRGs increased and peaked at P5 and declined gradually to a stationary level at the end of the juvenile period (~10% of total cells examined). After the first appearance of PG at P4, the number of PG increased and rapidly became the dominant glial type at the end of the juvenile period.

For the round shape soma glia (Fig. 4B), the electrophysiological glial phenotypes followed the same pattern that we found for irregular soma glia, which is consistent with the conclusion from the staining results that both round or irregular soma equally represent GLAST and NG2 lineage glia, and therefore the same cell population (Fig. 1).

Interestingly, 70% of VRGs (n = 19) after the third postnatal week expressed INa (Fig. 5), which was never seen in VRA-type isolated astrocytes, and has been used by us as one of the diagnostic features discriminating ORA from VRA (Zhou and Kimelberg 2000). The cellular identity of VRGs

FIG. 3. Comparison of excitability between glia and interneurons. A and D: voltage-clamp recordings from an ORG and interneuron, respectively, in the CA1 stratum radiatum. Although the small INa in ORG (A) can be subtracted after leak and capacitance currents subtraction (as shown in Fig. 2A, inset), it can be easily distinguished from the large INa from a small-sized interneuron (D) located in the same CA1 region by the magnitude and repetitive activation of INa during the 30-ms depolarization voltage pulse. Voltage command for A and D was the same as used in Fig. 2. B and E: current-clamp recordings from the same ORG (A) and interneuron (D), respectively. For the neuron, positive current injection (100 pA) induces repetitive spikes that reach an overshoot potential value more positive than +20 mV (E). For the ORG, the more positive current injection could only induce an immature single spike that is characterized by a slow rise and never exceeded 0 mV (B). C and F: traces of basal potential membrane potential without current injection for the ORG and interneuron, respectively. For the interneuron, spontaneous miniature excitatory postsynaptic potentials (mEPSPs) are clearly measurable and a spontaneous action potential (SAP) can be seen (F). Although ORG shows fluctuations in membrane potential, no clear postsynaptic events were distinguishable and SAPs were never seen (C). ORG and the interneuron were taken from P3 and P35 rats, respectively.

J Neurophysiol • VOL 95 • JANUARY 2006 • www.jn.org
after the third postnatal week was further explored with post-recording GLAST/NG2 double-staining studies, as described in the next section.

The ratio of glial electrophysiological phenotypes therefore became stabilized after the third postnatal week, which suggests that the maturation of the hippocampus starts from the fourth postnatal week.

Together, the analysis of glial electrophysiological phenotypes as a function of animal age led to the conclusions that 1) electrophysiological glial heterogeneity occurs mainly within the first 3 postnatal wk in the hippocampal CA1; 2) PGs originate postnatally, and in the adult brain, starting from the fourth postnatal week, PG becomes the dominant electrophysiological glial phenotype that amounts to 92% of the total cells examined from P22 to P106; and 3) the apparent VRG-like glia in the adult hippocampal CA1 region exhibits features similar to ORGs and therefore appears as a variant of the ORGs seen in the earlier developmental stages (see Fig. 2A), but additionally with inward K⁺ channel currents. We never saw any ORGs after the third postnatal week, and the VRG-like glia were always <10% of the total cells in every week after the third postnatal week.

Immunocytochemical identification of different electrophysiological glial phenotypes

To determine the association between the different electrophysiological glial phenotypes and the GLAST and NG2 glia lineages as a function of age, postrecording double immunofluorescent staining was done.

To identify recorded cells after postrecording immunocytochemistry, 0.1–0.5% biocytin was included in the electrode solution so that recorded cells could be located based on Cy2-streptavidin labeling of biocytin. The hippocampal slice was fixed immediately in 4% formaldehyde and subjected to immunocytochemistry, and the resulting staining was viewed by laser scanning confocal microscopy (see METHODS). Representative GLAST/NG2 double-staining results are shown in Fig. 6, A–D, and the quantitative data for all the results are shown in Fig. 6, E–G.

For ORGs (Fig. 6E), we found a similar percentage of GLAST(+) (55%) and NG2(+) (45%) cells in the newborn rat (n = 14). In the juvenile period, 76% of the ORGs were found to be NG2(+), with the remaining 24% being GLAST(+) (n = 13).

All the VRGs (Fig. 6F) from the newborn and juvenile period were GLAST(+) glia. In the adult, however, the VRGs were exclusively NG2(+) glia. Given that VRGs in the adult also
observed in slices from mature rats. The association between the electrophysiological phenotypes, GLAST and NG2 lineage, and the degree of coupling as a function of age will be the subject of a separate paper (unpublished data).

DISCUSSION

Development of GLAST and NG2 lineage glia and electrophysiological phenotypes

As noted in the introduction, the relationships between the three different electrophysiological glial phenotypes and the GFAP and NG2 lineages are currently unclear. For example, the ORG phenotype has been shown to be exhibited by GFAP(+) astrocytes (Bordey and Sontheimer 1997; Zhou and Kimelberg 2000), GFAP mRNA(+) “complex glia” (Zhou et al. 2000), and NG2(+) glia (Schools et al. 2003) in the rat hippocampus. Our postrecording GLAST/NG2 double-staining results clarify this issue. For the first time, our studies show that both GLAST (+) and NG2 (+) glia are present at P1, and some of them share the same electrophysiological phenotype, ORG, during the first 3 postnatal weeks but not at a latter time (Fig. 7). We show that the progression of GLAST lineage glia follow the electrophysiological phenotype order of ORG, VRG, and PG during development (Fig. 7). This leads to the inference that the electrophysiologically passive glial phenotype is the mature form of classically defined GFAP(+) astrocytes, similar to the early studies on amphibian optic nerve (Kuffler et al. 1966) and later in mammalian glia (Picker et al. 1981; Somjen 1979).

Because GFAP and NG2 lineage glia show no cross-lineage relationship in the postnatal brain (Bergles et al. 2000; Butt et al. 2002; Peters 2004), the postrecording immunostaining results also indicate that the development of NG2 lineage glia follows the electrophysiological phenotype order of ORG in the newborn and juvenile brain and VRG-like in the adult (Fig. 7). However, although 100% of VRG cells are NG2(+) glia in the adult, 15% of PGs (n = 13) were also NG2(+) in the adult
brain, showing that PG also represents NG2 glia but to a much lesser extent. Why NG2 glia persistently express voltage-gated K\(^+\) and Na\(^+\) currents in the adult brain, or alternatively why some of them are electrophysiologically passive, remains to be determined. NG2 glia give rise to interneurons and other glia postnatally (Aguirre and Gallo 2004), and the persistence of voltage-gated ion channels may be involved in this proliferative capacity.

Other groups studying astrocyte heterogeneity have used a transgenic mouse line that expresses enhanced green fluorescent protein (GFP) under the control of the human GFAP promoter. This facilitates selection of astrocytes for in situ patch-clamp recordings. However, the GFP signal is not limited to astrocytes but also labels NG2\(^+\) cells (Grass et al. 2004; Matthias et al. 2003) and a varying number of GFAP\(^-\) cells throughout the brain (Nolte et al. 2001). In this study, we chose to identify cell lineages, after initial morphological identification, by performing postrecording immunohistochemistry. With the mutually exclusive nature of GLAST and NG2 labeling in the rat and mouse, there is little chance for ambiguous results. Thus our postrecording GLAST/NG2 double staining allows us to correlate glia lineage and electrophysiological phenotypes with good confidence.

Relative ratio of GLAST\(^+\) versus NG2\(^+\) glia in CA1 region

Although NG2\(^+\) glia are considered to be numerous in the brain, no quantitative data are currently available. We show that from P22 on, 100% of VRGs and 15.4% of PGs are NG2\(^+\) glia in the CA1 region (Fig. 6). From P22 on, 212 PGs and 19 VRGs were identified (Fig. 4A). Based on this, the total number of NG2\(^+\) glia should be 52, which amounts to 22% of the total glia in the CA1 region. This is reasonably close to a preliminary analysis on the relative numbers of GFAP\(^+\) and NG2\(^+\) cells in slices from P31 rat. From five images of the CA1 region from two slices, we counted a total of 63 GFAP\(^+\) and 21 NG2\(^+\) cells, or a ratio of 75/25% GFAP\(^+\)/NG2\(^+\) (data not shown).

Developmental regulation of K\(^+\) and Na\(^+\) channel expression

Our systematic analysis of the astroglial electrophysiological phenotypes clearly shows a developmental switch of current profiles in both GLAST and NG2 lineage glia. Specifically, in the newborn and regardless of GLAST or NG2 lineage, glia expresses the voltage-gated K\(^+\) channel currents, \(I_{KdR}\) and \(I_{Cl} \). Additionally, ORGs also express voltage-gated \(I_{Na}\). Our study precisely shows that the first appearance of cells expressing predominant leak type K\(^+\) currents starts at P4. We refer to these as electrophysiologically passive glia or PG, and the number of PGs increases rapidly to become the predominant glia at the end of the third postnatal week. Given that astroglia in CA1, SR, and SLM are postmitotic at birth (Rietze et al. 2000), the switch in ion channel currents could be reasonably inferred as an intrinsic transition of ion channel expression among the same population of glia instead of different glia proliferating from a common precursor.

Our studies are consistent with an assumption that VRG is an intermediate phenotype between ORG and PG in GLAST\(^+\) astrocyte lineage. The evidence is 1) the decrease in GLAST\(^+\) ORGs in the newborn and juvenile is accompanied by an increase in GLAST\(^-\) VRGs; 2) the decrease in GLAST\(^+\) VRGs also parallels the progressive increase in GLAST\(^-\) PGs; and 3) the GLAST\(^+\) VRGs almost completely disappear after the third postnatal week. The \(\frac{\leq}{\leq}10\%\) of VRG-like glia in the adult brain are actually NG2\(^+\) glia.

Mature astrocytes show electrophysiologically passive phenotypes

We believe that one of the most significant findings derived from our studies is that the electrophysiologically passive glia are the mature form of GLAST\(^+\) astrocytes and are the predominant glia in the adult rat hippocampal CA1 region. Although whole cell current profile of PGs shows a linear \(I-V\) relationship, this does not rule out some contributions of other voltage-gated ion/anion conductance to the apparent overall linear conductance (Barres et al. 1990; Pannicke et al. 2005). The underlying ion/anion species that contribute to the passive conductances are clearly a subject for future work. It is noticeable that the average membrane potential of PGs measured under whole cell conditions (\(-73 \pm 8, n = 275\)) deviates significantly from the theoretical prediction based on [\(K_0\)][\(K_\infty\] = 3.5/140 (\(E_K = -96\) mV), showing that PGs may have contributions from other conductances. Because the Cl\(^-\) concentration was symmetrical in bath and electrode solutions, Cl\(^-\) conductance may contribute to the deviation from \(E_K\). Consistent with this idea, our unpublished observations show that CIC-2–like chloride currents are present in freshly isolated astrocytes (unpublished observations), and a similar finding...
has been reported from an in situ study on astrocytes (Makara et al. 2003).

Comparison with isolated astrocytes

A major difference between these results in slices and our previous data in freshly isolated cells is that passive cells were never seen in the freshly isolated astrocytes, even when the cells were isolated from P21 to P35 (Zhou and Kimelberg 2000) when significant passive cells from the same region are seen in situ. This could be because of removal of passive K⁺ channel–rich regions, the destruction of the astrocytic synctium, or failure of passive cells to survive the isolation procedure. However, a recent study showed that neither the passive current profile nor the membrane resistance of hippocampal PGs was affected by gap junction blockers (Wallraff et al. 2004). This implies that the passive phenotype in situ reflects the intrinsic K⁺ channel properties of PGs instead of syncytial coupling. These different possibilities need further study.

In conclusion, the various reports of astrocyte electrophysiological heterogeneity can now be understood on the basis that the previous studies, even if they were in slices, primarily focused on astroglia from the critical developmental period of 1–3 wk. Our studies show that starting from the fourth postnatal week, all the GLAST(+) astrocytes are passive glia. This amounts to 78% of the total glial population. Coincidently, thrombospondins, which are synthesized by astrocytes to promote neuronal synaptogenesis, are expressed at high levels during the first 3 postnatal weeks and decline in the adult brain (Christopherson et al. 2005), implying only immature astrocytes are involved in synaptogenesis. Other conclusions implied by our studies are 1) that two glial lineages recognized by GLAST or NG2 marker expression, are fate-determined at birth; 2) GLAST lineage astrocytes are always the predominant glial type, whereas NG2-lineage glia constitute a smaller population of 22% of the total glial population in the rat hippocampal CA1 region; 3) during brain maturation, the GLAST lineage astrocytes undergo a progressive loss of voltage-gated ion channels that have been predominantly substituted by leak-type K⁺ conductances; and 4) around 50% of the NG2-lineage glia persistently express voltage-gated K⁺ and Na⁺ channels in the mature brain, implying the potential of NG2-lineage glia for cell proliferation and differentiation.

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