The history of the morphology and electrophysiology of the neuroglia, which was the historical term used for what are now termed astroglia or astrocytes, is briefly reviewed. The interpretation of these data around 1970 was that astroglia in situ represented a homogeneous electrophysiological phenotype with a major function, based on this, in maintaining a constant extracellular concentration of potassium ions ([K⁺]o). It was soon found that astroglia in situ played a major role in the uptake and inactivation of the synaptically released amino acid transmitters glutamate and γ-aminobutyric acid. Subsequent studies in isolated systems, such as primary astrocyte cultures, greatly expanded this view to a more protean cell, with much wider properties in terms of transmitter uptake systems and release, a variety of voltage-dependent ion channels and varying membrane potentials and electrophysiological behaviour, and receptors for a large number of neurotransmitters.
Thus, it seemed quite reasonable that the astroglia would form a functionally as well as morphologically heterogeneous population, with far more varied properties reflective of different roles in different brain regions and sub-regions. However, our recent in situ data has suggested, at least for astrocytes in the stratum radiatum of the hippocampus of the adult rat, that the original homogeneous electrophysiological phenotype for mature astrocytes is likely correct, or at minimum provides a characteristic signature for mature astrocytes because of several interpretative problems inherent in applying the whole-cell voltage-clamp technique to these low resistance cells, which are discussed. It remains to be seen whether such cells represent the true mature astrocyte population in other brain regions, but if so then these electrophysiologically defined astroglia can be systematically examined as a function of region for a number of other important characteristics to accurately determine the degree of heterogeneity within this defined cell population in situ.

1.1 The Classification Problem

1.1.1 Morphology and Classification

The discovery of a non-neuronal element, the neuroglia, in the central nervous system (CNS) is generally attributed to Rudolph Virchow around 1850, but in reality should be attributed to anatomists such as Golgi, Ramón y Cajal and others who applied the Golgi potassium dichromate/silver staining method (reazione nera) (Golgi, 1985) to brain tissue in the last two decades of the nineteenth century (reviewed in Kettenmann and Ransom (2005), Somjen (1988) and Kimelberg (2004)). This staining revealed a considerable morphological heterogeneity among these neuroglia as illustrated for the mammalian cerebellum in Fig. 1.1 and for the cerebral cortex in Fig. 1.2a. Two decades later other glial classes, the oligodendroglia and microglia, were identified and the astrogliia with oligodendroglia were classified as the macroglia (reviewed in Kettenmann and Ransom (2005), Somjen (1988) and Kimelberg (2004)). All the cells illustrated in Figs. 1.1 and 1.2a and the green glial fibrillary acidic protein (GFAP)(+) cells in Fig. 1.2b are now referred to as astrogliia based on the fancy that the morphology of the most dominant types, classically referred to as protoplasmic (grey matter) and fibrous (white matter) resembled stars seen in the night sky, whereas before 1920 they were more usually referred to as neuroglia, although the term astroglia had been used sporadically since around 1890 (reviewed in Kettenmann and Ransom (2005)). Why some of these cells with an obviously different morphology, namely the Bergmann glia (or originally the Bergmann fibres plus Golgi epithelial cells) are also included as astrogliia is treated in detail in other reviews (Kettenmann and Ransom, 2005; Somjen, 1988; Reichenbach and Wolburg, 2005), but is often used to support morphological heterogeneity. This is logically an unacceptable circular argument, unless there are other criteria that define these cells as astrogliia.
Fig. 1.1  Golgi staining of astroglia in the human cerebellum. M, molecular layer; P, Purkinje-cell layer; G, granule-cell layer; W, white matter. From Ramón y Cajal (1913).

Fig. 1.2  Glial cells in the human and rat brains are morphologically heterogeneous. (a) Astrocytes in the cerebral cortex of a 2-month-old infant stained with the Golgi method. (A–D) are cells in the first cortical lamina and (E–H) are cells in the second and third lamina. (I–J) are cells with end-feet contacting blood vessels. V, blood vessel. From Ramon y Cajal (1913). (b) Staining of cerebral cortex from adult rat. Top is surface of cortex showing intense GFAP (green) staining due to the glia limitans. Red is for NG2, which stains both NG2(+) cells and blood vessels. Scale bar, 100 µm. Unpublished work of G. Schools. (See Color Plates)
To break this circular reasoning one needs to know how other more functionally related biochemical and physiological properties define the astroglia, but up to the present there is an insufficient body of systematic work leading to a resolution of this question. There was a gap of around 40 years from 1920 before any physiological studies on glia were done to flesh out the morphological studies, and these were in the amphibian optic nerve where the only penetratable cell bodies were glia (Kuffler et al., 1966). This was due to methodological limitations for studying CNS tissue of other regions of vertebrates as these nervous systems are an extremely intricate mosaic of neurons and glia and their processes. Apart from histology there was little more that could be done on a cellular basis, for the current cell-specific methods of antibody staining, imaging of dye-filled cells and electrophysiological methods for small cells in a complex tissue mosaic were yet to be developed. Parenthetically, when these more advanced imaging techniques were applied they confirmed in greater detail what was apparent from the original Golgi staining; that the radiating processes form an extremely complex framework of processes that ends in finer and finer extensions, as shown in Fig. 1.3b, to compare with older Golgi staining shown in Figs. 1.1, 1.2 and 1.3a. It was also known that the end of these processes surrounded many synapses and surrounded all blood vessels in the mammalian CNS, which early on led to hypotheses of function, such as taking up transmitters (Lugaro, 1907), affecting synaptic activity (see Dierig, 1994) and bringing nutrients from the blood to neurons (Golgi, 1885); themes echoed today but now with more accurate details and the essential experimental support.

Rather than drawing up detailed competing balance sheets showing how different properties vary between astroglia in different experimental systems I will concern myself with major issues and techniques that bear on this question. I do not
consider heterogeneity or homogeneity in regard to developmental changes within the astrocyte population, i.e. I am excluding immature and developing astrocytes, since this is a separate issue. Also cells positive for NG2 (see red cells in Fig. 1.2b), which morphologically resemble astroglia to the extent that they were initially termed smooth protoplasmic astrocytes (Levine and Card, 1987) but are not now considered astrocytes as they do not show a number of defining properties of astrocytes such as excitatory amino acid (EAA) transporters and are not gap-junction-coupled (Nishiyama et al., 2005) (also see below).

1.1.2 Functional Properties and Classification

The intricate process-bearing structure of astroglia cannot per se be taken to indicate functional complexity, in the sense for the CNS of involvement in information processing. Certainly morphology can give clues for basic physiological processes and the speculations of Golgi and Lugaro noted above have been borne out by later more defined hypotheses and experiments (Magistretti et al., 1999; Berl et al., 1961; Rothstein et al., 1996; Danbolt et al., 1992). One clear feature from Figs. 1.1–1.3 is that astrocytes have massive arborizations of finer and finer processes. Thus when one considers the question of heterogeneity or homogeneity it is actually far from clear whether there is a greater heterogeneity within cells in regard to varying properties among the multitudinous processes of single astrocytes compared with the aggregate properties of individual astrocytes, and therefore is it really meaningful to speak of aggregate properties? There could well be spatial segregation between different processes or between the processes and the soma, so that this variation is greater than the differences of aggregate properties between different astrocytes. One of the drawbacks of cell-selective patch-clamp electrophysiology is that it will mainly record the membrane electrophysiological properties of the cell soma as the command voltage and dependent currents likely will not penetrate far and rapidly enough into the processes for the electrophysiological properties of the process tips to be measured. This is still a major technical drawback and we cannot be confident that electrophysiology can see the processes, although recently effective cell–cell current transfer has been reported for mature astrocytes in situ (D’Ascenzo et al., 2007). On the other hand, fluorescent imaging can now discern differences in the Ca$^{2+}$ responses in different processes of Bergmann glia to stimulation of the parallel fibres, and these have been referred to as functional microdomains (Grosche et al., 1999).

1.2 Neurons and Glia

Of course the most fundamental cellular classification in the CNS is into neurons and glia. The true structure of neurons is due to the work of Ramón y Cajal and others in the last decades of the nineteenth century, again using Golgi’s stain.
The attribution to their axons of excitability was an extension to these cells (see Katz (1966)) of studies of the injury potentials of nerve tracts by du Bois-Reymond, Bernstein and others from 1850 to 1900, and the elucidation of the ionic basis of the action potentials worked out for the large axons of the giant squid by Hodgkin and Huxley in 1939. This of course has been amply justified by the large body of data acquired since then, and it seems rationally unchallengeable, i.e. beyond reasonable doubt, that the regenerative passage of the polarity change of the axonal membrane potential of neurons (the “action potential”) is the fundamental currency of brain information processing. But this “information processing” is so varied, from control of motor function and processing of the activity of our sensory apparatus, through emotions to abstract thinking and “consciousness,” that real understanding of how this neuronal electrical activity forms a general substrate for the higher brain functions eludes us (Koch, 2004). Much of cellular neuroscience related to this topic is devoted to how neuronal electrical activity is controlled by the action of transmitters at the circuits’ switches, the synapses, to activate or inhibit the switches and thereby control the existence or frequency of trains of action potentials.

1.3 Some Basic Principles of Astroglial Classification

Within the context of the last two sections how can we approach classification and the roles of the astroglia, which bears on the question of homogeneity or heterogeneity? First, we must absolutely distinguish between their roles in the embryological and postnatal development of the nervous system and their roles in the mature nervous system. Then, how can we experimentally explore the functional properties of astroglia, identified morphologically and by markers. Finally, how do we use these properties to reasonably classify these cells so that we will all be talking about the same entities? What number of properties is sufficient to define a cell as astrocytic will be unclear until we study their properties and, because of the empirical nature of the scientific method, will always be a work in progress because, simply put, it depends on observations. This process should be no different from classical classification systems for plants and animals and perhaps we are simply in the early days of our observations. But those classifications are for macroscopic, unmodified characteristics. For cellular classification we always have to select and amplify the characteristics we will use. We generally start with staining or filling for morphology and try to correlate this with selected proteins, which we hope are specific markers and with cellular physiological measurements to arrive at some idea of function. For individual organisms we also have the guiding principle of evolution that all their characteristics have evolved towards survival and procreation. For individual cells this is the larger objective but their specific tasks are to enable the tissue community of which they are members to function optimally so that the organism of which the tissue is a part can survive and procreate.

The issue of the characteristics needed to define astrocytes can be illustrated in the form of a Venn diagram (see Fig. 1.4). The area A is the total of the basic
properties that are needed to define a cell as an astrocyte and will be shared by all astrocytes. For convenience I depict three different subclasses of astrocytes but these could be more numerous. The partially overlapping parts depict properties that are shared by some astrocytes and the non-overlapping areas depict properties that are unique to only one subclass of astrocytes. At the start there has to be agreement whether we wish to define the class of astrocytes in this way or as a class of cells that all share the same properties. I propose the former because I think it is more realistic. Thus if all the cells shown in Figs. 1.1 and 1.2 are classified as astroglia they can be divided, rather crudely, into different subclasses based on morphology.

Astrocytes are also often defined on a biochemical basis as expressing an astrocyte-specific protein such as (to date) GFAP, glutamine synthetase (GS), the astrocyte specific EAA transporters GLAST or GLT-1 and the calcium binding protein S100β (also see Reichenbach and Wolburg (2005)). These are very practical because one can use immunocytochemistry to identify the cell more precisely. But even long-used markers such as GFAP are not shared by all cells (Bignami and Dahl, 1974), that would otherwise on the basis of morphology and that their processes abut blood vessels and other distinctive relationships, be characterized as astroglia (Walz, 2000). Also immunohistochemical identification of a cell as being positive is a matter of subjective visual judgment and the sensitivity of the technique, such as whether one uses an amplified or non-amplified antibody-based detection system. Now use of these proteins is being extended to use of DNA constructs artificially incorporated into the cell’s genome, which include presumed cell-specific promoters for these proteins linked to a gene expressing a fluorescent protein to define and to mark living cells for further study. Further

**Fig. 1.4** Venn diagram of the astrocyte properties. “A” represents the core astrocyte properties. See text for details.
extension of such genetic engineering includes promoter-specific knockin and knockout of proteins to determine their functions in astrocytes. See Slezak et al. (2007), Djukic et al. (2007) and Chap. 14 for recent references on these topics, which are also beginning to uncover to-be-expected problems in the outcomes of these complex genome-altering procedures.

Likely, morphology nor markers will be sufficient to characterize A in Fig. 1.4. A list was drawn up by the organizer and audience at the 2006 American Society of Neurochemistry in a workshop organized by Dr. Steven Levinson, which I reproduce in Table 1.1, as best I can from my notes with some additions (see also Table 2.1 in Reichenbach and Wolburg (2005)). It also compares immature and mature astrocytes and also mature NG2(+) cells with which astrocytes are still sometimes confused. Obviously, neurons, oligodendroglia and microglia are so different that there would seem to be no useful purpose in including them. Logically we need at least one characteristic and preferably more in region A (Fig. 1.4) to define astrocytes; otherwise if the class of astrocytes is heterogeneous what is it that makes them all members of the astrocyte class? Some might say that it would comprise all

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Young astrocytes</th>
<th>Mature astrocytes</th>
<th>Mature NG2(+) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Property</td>
<td>Varied ( V_m )</td>
<td>( V_m = E_{K^+} )</td>
<td>( V_m ) always &lt; ( E_{K^+} )</td>
</tr>
<tr>
<td></td>
<td>VDCs</td>
<td>VDCs not apparent; linear ( I-V ) plot</td>
<td>VDCs</td>
</tr>
<tr>
<td></td>
<td>Varied input resistances</td>
<td>Very low input resistance</td>
<td>High input resistances</td>
</tr>
<tr>
<td></td>
<td>Varied degree of cell–cell coupling</td>
<td>Extensive cell–cell coupling</td>
<td>No cell–cell coupling</td>
</tr>
<tr>
<td>A) Electrophysiology</td>
<td>AOAs</td>
<td>AOAs</td>
<td>AOAs not excessive</td>
</tr>
<tr>
<td></td>
<td>No Glutathione transport</td>
<td>Glutathione transport</td>
<td>No Glutathione transport</td>
</tr>
<tr>
<td></td>
<td>No D-serine racemase</td>
<td>D-serine racemase</td>
<td>No D-serine racemase</td>
</tr>
<tr>
<td></td>
<td>EAA transporters (e.g., GLAST)</td>
<td>EAA transporters</td>
<td>No EAA transporters</td>
</tr>
<tr>
<td></td>
<td>GFAP sometimes</td>
<td>Some strongly GFAP (+)</td>
<td>No GFAP</td>
</tr>
<tr>
<td></td>
<td>GS</td>
<td>GS</td>
<td>No GS</td>
</tr>
<tr>
<td>B) Markers</td>
<td>Extensive processes</td>
<td>More extensive processes and arborizations</td>
<td>Extensive, but less-branched processes</td>
</tr>
<tr>
<td></td>
<td>Processes contact blood vessels and partition the CNS, e.g., glial limitans, or parts thereof</td>
<td>More extensive processes and arborizations and also contact mature synapses</td>
<td>Processes directly contact nodes of Ranvier</td>
</tr>
<tr>
<td></td>
<td>Ionotropic and metabotropic receptors for EAs and other transmitters</td>
<td>Mainly metabotropic receptors</td>
<td>As for young astrocytes</td>
</tr>
<tr>
<td></td>
<td>No direct synaptic inputs</td>
<td>No direct synaptic inputs</td>
<td>Receives direct synaptic inputs</td>
</tr>
</tbody>
</table>

AOs antioxidants, \( E_{K^+} \) Equilibrium potential for \( K^+ \), GFAP glial fibrillary acidic protein, VDCs voltage-dependent channels, \( V_m \) membrane potential, EAA excitatory amino acid, GS glutamine synthetase
cells that are not neurons, oligodendroglia, microglia or ependyma. But such a definition by exclusion is not satisfactory and not acceptable to taxonomists. The first problem is to identify the core characteristic or characteristics that represent A. A combination of properties is safer from Bayesian logic, as the author pointed out in a previous publication (Kimelberg, 2004). For example if there are two characteristics instead of one, each with an independent 95% probability of being expressed in astrocytes in a population of cells of which the astrocytes represent 25%, this raises the probability that the dual stained cell is an astrocyte from 86.4% for each of the single characteristics, to 99.2% for both characteristics. The increase in probability becomes greater as astrocytes represent a progressively smaller proportion of the total cell population; if the astrocytes represent 10% of the total cell population the probability that expression denotes an astrocyte is 67.8% with one marker vs. 97.6% for two markers.

A multi-properties definition, as an example three groupings from Table 1.1, groups A, B and C, could be used as a current working definition of a cell as an astrocyte. The morphology would be that some of the processes contact blood vessels and the extensive arborization of processes is contained within a limited volume of tissue; i.e., no projections beyond this volume termed the domain of each cell (Bushong et al., 2002; but see Oberheim et al. (2006) and references therein for extradomain projection of some processes in the human brain). Electrophysiology would be a low membrane resistance and a linear current–voltage (I–V) relationship (see section 1.4.2.3 for a discussion of what this means for low resistance astrocytes). Markers to date would include GFAP, GS, GLAST and serine racemase. However, these markers will be expanded or modified by the new emerging microarray work on freshly isolated astrocytes and other neural cells, which for example, has unexpectedly shown that message for an aldehyde dehydrogenase 1 family, member L1 (Aldh1L1), is one of the messages most widely expressed in astrocytes (Cahoy et al., 2008).

1.4 Experiments Relevant to Heterogeneity or Non-Heterogeneity

To answer the question “are astrocytes heterogeneous,” the first order of business is to determine what properties should be considered common or homogeneous to all astrocytes, which is no means a simple task as just discussed. Then what other properties will we examine to see if they vary enough to conclude that the class is heterogeneous, and can be divided into subclasses. This is the basic issue in the logic of the method of classification, as illustrated in the Venn diagram in Fig. 1.4. As noted in the preceding sections both issues and the defining characteristics are still being debated and therefore ongoing; so here, as an example, I discuss mainly how the electrophysiological properties of astrocytes have been shown to vary. These were the first studies that were not purely morphological and were the first used to attempt to define astroglia on other than morphological grounds.
1.4.1 Early In Situ Electrophysiology Studies

These first studies were those of Kuffler and colleagues on glial cell bodies in the amphibian optic nerve (Kuffler et al., 1966; Orkand et al., 1966). This was followed by work using sharp electrode impalements in living mammalian brains as shown in Fig. 1.5 (Picker et al., 1981), and here the cells were post-stained using an injection of horseradish peroxidase from the pipette and visualizing with benzidine, an important step as you can check the morphology of the cell that you recorded. It was therefore hypothesized that the following properties were basic characteristics of all astrocytes. Namely, that all these cells were electrically non-excitatory with no capacity for generating action potentials upon injection of positive current, but rather showed a linear relationship between injected current and the change in membrane potential. This was likely due to $K^+$ channels as a Nernstian relationship between the measured membrane potential and changes in $[K^+]_e$, in both amphibian and mammalian tissues, was found (see Fig. 1.5 for mammals) and the membrane potential measured at zero current was close to the $K^+$ equilibrium (Nernst) potential. This was a considerable advance. Further blind impalements of glia in the cortex of anesthetized mammals, which were defined by the general criterion of lack of electrical excitability, also responded to the limited range of endogenous $[K^+]$ increases due to neuronal stimulation, by a Nernstian relation (Somjen, 1995).

Fig. 1.5 (A) Typical appearance of bushy protoplasmic astrocytes. Cells were visualized after electrophysiological recordings (B) by injecting horseradish peroxidase from the electrode and subsequent histochemistry. Arrow indicates an astrocyte process touching a capillary. (B) A Nernst plot of the changes in membrane potential in millivolts (y axis) plotted against the logarithm of imposed $K^+$ concentrations in the bath solution, in astroglia in normal or epileptic (reactive) human biopsy, and guinea pig cerebrocortical, slices. For this type of plot a slope of 60 shows that the changes in membrane potential can be completely explained by only $K^+$ carrying the transmembrane currents according to the Nernst equation $V = 60 \text{ mV} \times \log ([K^+]/[K^+]_i)$. Also when $V = 0 \text{ mV}$ external $[K^+]_e = internal [K^+]$. The slopes from normal human and guinea pig tissue were ~60 with $[K^+]_i = 120–130 \text{ mM}$. Recordings from epileptic tissue showed a smaller slope (~40) indicating some permeability to other ions. From Picker et al. (1981).
Because post-staining was usually not done these cells can only be referred to as “glia.” On these bases the K⁺ channels were termed leak channels, showing no voltage and time-dependent changes, and showing a linear voltage response to injected current. Therefore these neuroglia, which were presumably often astroglia, were homogeneous by electrophysiology but there was always the problem of selection since cells with a membrane potential equal to –50 mV or less were excluded, as there was no independent way of establishing that this was not due to a low electrode seal resistance or even other damage. These were, however, reported to be a minority of the cells sampled, and so damage and/or imperfect seals were a reasonable explanation for such low potential cells. When separate electrodes were used for injecting current and measuring voltage a contribution of the high resistance of the sharp microelectrodes to the linearity of the membrane voltage change was not a factor. I will discuss this issue later in relation to current injection using a single low resistance patch electrode where it is a problem because the membrane voltage change in response to injected current is measured at the top of the electrode and the voltage drop is therefore across both the electrode and membrane resistance (Sontheimer, 1995).

Other defining characteristics soon followed, and since in these microscope-based studies the morphology was checked, they could more safely be referred to as astroglia. Astroglia were found to be a major site of uptake of synaptically released glutamate and its conversion to glutamine based on GS being astrocyte-specific (Martinez-Hernandez et al., 1977). Also that they were extensively linked by gap junctions (Massa and Mugnaini, 1982), which prima facie seemed to fit Kuffler and colleagues’ (Orkand et al., 1966) hypothesis of K⁺ spatial buffering. However, the short space constant of the cells (=60 to 200 µm) because of their low membrane resistances was always considered to limit the process to only localized increases in extracellular K⁺ for most astrocytes, and spatial buffering over limited distances over which K⁺ can be transferred of only a few hundred micrometers (Newman, 1995; Gardner-Medwin, 1983). However, regional localization of K⁺ channels and the thin planar structure of the retina allowed a form of spatial buffering localized to operate in the more cylindrical and less-branched retinal Muller cells (Newman, 1984). This again is a topic that needs further clarification and may be better resolved when the K⁺ channels of astrocytes and their spatial segregations within the cell body and its processes are fully resolved. Other characteristics of the astrocytic processes is that they surround synapses and collections of synapses (glomeruli), and blood vessels and form limiting interfaces, such as the glial limitans that is the last region between the CNS and the meninges, as has already been mentioned.

1.4.2 Studies in Different Astroglia Cell Preparations Subsequent to the Early In Situ Studies

1.4.2.1 Cultured Astroglia

Because of the methodological difficulties inherent to work in tissue, the small field of “astrocytology” from the late 1970s, adopted the use of primary cultures prepared from 1- to 2-day-old rodent brains for more detailed electrophysiological,
imaging and biochemistry studies. Such cultures grow as monolayers that express GFAP, show glutamate uptake and GS activity, and still have predominantly K⁺-based membrane potentials. With studies such as transmitter-receptor effects the omnipresent and vexing problem of indirect effects via neurons was neatly side-stepped. They quickly became the major experimental model for studying astrocyte properties (Kimelberg, 1983, 2001). In contrast to the earlier in situ data these astrocytic primary cultures showed a quite different electrophysiological phenotype, expressing voltage-gated K⁺ channels, Ca²⁺ channels, Na⁺ channels and Cl⁻ channels among others (Barres et al. (1990a); Barres (1991b); also see Table 9.1 in Olsen and Sontheimer (2005)). The presence of voltage-gated Na⁺ channels in these astrocytes was particularly confusing since they are always found to be electrically non-excitable. The reviews just mentioned can be consulted for the original papers, where full details of the preparations and techniques are given. The clear discrepancy between the newer and the older in situ data was suggested to be due to limitations with the older electrophysiological techniques (Barres, 1991a). That this does not appear to be the case will be argued in the following sections, so that much of the discrepancy is likely due to modified gene expression in the cultures. This is not surprising since a basic biological principle is that gene expression is plastic and of course varies with development and responds to environmental cues via receptors affecting transcription factors. Even the properties of the cultures that were correct in principle, i.e. such as the expression of a number of transmitter uptake systems and ionotropic and metabotropic receptors, were wrong in some of the details (Kimelberg, 2001). Note added in proof: a recent microarray study of cultured and isolated astrocytes (Cahoy et al., 2008) has confirmed this principle. A more focused microarray study of gene expression in isolated cells (Lovatt et al., 2007) makes the same point.

1.4.2.2 Astrocytes Freshly Isolated from Brain Slices

It seemed possible that the atypical gene expression problem of primary cultures, yet their amenability to precise experimental control and measurements, could be combined by using acutely isolated astrocytes. This had been done for biochemical studies as early as 1965 using density gradient centrifugation but these preparations were impure and appeared quite damaged and were never examined electrophysiologically to see if they were even viable (see Hamberger et al. (1975) and references therein).

A method that better preserved the cells’ integrity was simply to triturate an enzymatically softened tissue, or in some cases mechanically dissociated to avoid enzymatic degradation of exposed surface proteins, and examine the cells individually by electrophysiology and fluorescence indicators for dynamic measurements, or autoradiography for uptake, and then immunocytochemistry for identification. Patch clamp electrophysiological studies on these cells also showed a heterogeneity of electrophysiological phenotypes and the isolated cells never showed the characteristic linear I–V curves of astroglia in situ (Steinhauser, 1993; Steinhauser et al., 1994; Verkhratsky
and Steinhauser, 2000; Barres et al., 1990b; Zhou and Kimelberg, 2000; Zhou et al., 2000). Figure 1.6a, b shows the two different types found in isolated cells and termed by Zhou and Kimelberg (2000) as outwardly rectifying (a) and variably rectifying (b) astrocytes, respectively. These were also termed glutamate receptor and transporter astrocytes (Glu-R and Glu-T, respectively) on the basis that the former showed α-amino-3-hydroxy-5-methyl-isoxazole propionate (AMPA)-type currents and not transporter currents, and the latter the converse. This was taken as evidence of heterogeneity but, as I will argue in the next section, mature hippocampal astrocytes in situ are always Glu-T, and the Glu-Rs are likely to be NG2(+) cells in mature tissue. Freshly isolated cells were also used to show the presence of metabotropic receptors, especially metabotropic glutamate receptors by measuring changes in intracellular Ca²⁺ concentration using fluorescent probes, and there was some degree of heterogeneity between cells in their responses (Kimelberg et al., 2000). Thus a picture of emerging heterogeneity based mainly on the expression of voltage-dependent currents representing different ion channels and glutamate receptor and transporter currents emerged from the studies on the acutely isolated cells.

1.4.2.3 Recordings from Astrocytes in Brain Slices

The next question was obviously whether even the isolated cells reflected the cells present in situ. No cells with a purely “passive” (i.e. linear $I–V$ curves) electrophysiological phenotype (see Fig. 1.6c) were reported in acutely isolated cells, but there had been reports of such cells in astrocytes recorded in freshly cut slices (Steinhauser et al., 1994; Wallraff et al., 2004; D’Ambrosio, 2004). A dye-filled cell in situ from which a recording as shown in Fig. 1.6c would be obtained is shown in Fig. 1.6i, which also shows its dye coupling to other astrocytes. There had been comments that such cells appeared to be more frequent in slices from older animals (Matthias et al., 2003). However, the only systematic development study in slices up to the year 2003 excluded such cells on the basis that they could not be adequately voltage clamped because of their very low input resistances ($R_i$) of 10–20 MΩ (Bordey and Sontheimer, 1997), and obviously came to different conclusions than if the passive cells, which have $R_i$ values in the range just mentioned, were included. This is the inherent limitation of the scientific method, which only deals with doable observations and was illustrated in a fable by the well-regarded astrophysicist and relativist Sir Arthur Eddington (1882–1944). Namely, that an ichthyologist seeking to classify fishes caught his samples in a net as a scientist would start off doing, and analyzing them concluded that all fish have gills but none were less than 2 inches long (Taylor, 1949)!

It seems reasonable to ask that if the voltage clamp technique has problems for mature, passive, low-resistance astrocytes why use it? In brief, the whole-cell voltage-clamp technique is an excellent way of studying electrophysiological changes in small, high-resistance cells, especially rapidly changing voltage-dependent currents. As is well-known, the techniques involve a relatively large diameter open-tip glass microelectrode, which first requires a high-resistance gigaOhm seal (~10⁹Ω ) to be
Fig. 1.6 (a–c) show the whole-cell recordings in response to the voltage steps (d, -180 to -40 mV in 10 mV increments) shown in (d) for the three types of electrophysiological phenotype using the nomenclature of Zhou et al. (2000, 2006) (also see text). (e–g) show the resultant $I$–$V$ plots. Only (a) and (b) were found in cells isolated from the hippocampus of 1–35 PN rats (Zhou et al., 2000). (a) also shows Na$^+$ channels (inset).
formed on the intact cell surface. Then suction is applied to break this patch of membrane to access the interior of the cell, with a resultant electrode access resistance \( R_a \) of \( \sim 10 \, \text{M} \Omega \). This was first applied to neurons and it showed that action potentials, and even synaptic potentials, could be measured in the cell body, which is the only part of the cell big enough to be routinely recorded from (Neher and Sakmann, 1984). The cell’s membrane resistance \( R_m \) had to be at least \( 100 \, \text{M} \Omega \) for 90% of the changes in potential to be across the cell membrane because it is in series with \( R_a \) of \( \sim 10 \, \text{M} \Omega \) and \( V_c \), the clamp (command) potential, is across the total voltage drop \( (V_t) \) of \( R_a + R_m \) (Sherman-Gold 1993; Sontheimer, 1995). Therefore the voltage drop across \( R_m \) is \( (V_t - V_a) \), where \( V_a \) is the voltage drop across \( R_a \) and \( V_t \) is the total voltage drop. The current pCLAMP 9 program has a membrane test protocol that estimates values for \( R_a \), \( R_m \) and membrane capacitance \( (C_m) \) based on the value for the total charge \( (Q_t) \) delivered to the capacitance, taking into account the offset of the steady-state current, which will rapidly begin to flow across \( R_t = R_a + R_m \), where \( R_t \) is total resistance. \( R_a \) is initially estimated from the time constant of the decay of the capacitance transient, which is small (see Fig. 1.6c) because of the rapidly developing and substantial current flow across the low \( R_a + R_m \). Values for \( R_a \) and \( R_m \) from this analysis have been reported as \( \sim 15 \) and \( \sim 5 \, \text{M} \Omega \), respectively (D’Ascenzo et al., 2007; Djukic et al., 2007), and we find the same values (Zhou et al., in preparation). Since \( V_m = V_a + V_m \), \( V_m \) is considerably less than \( V_a \); but if the channels are not voltage dependent, then \( V_m \) can be calculated from the \( R_a \) estimates. Such analysis needed to parse the continuous single electrode whole cell \( I-V \) data does not preclude some reasonable interpretations. It also adds to the characteristics that are diagnostic of the mature astrocyte and raises the important question of what the extraordinary low \( R_m \) is due to. Potentially, these include a large surface area extended to other cells by gap junctions and/or a high density of leak \( K^+ \) channels. In terms of the former a critical question is how far does the current and voltage changes spread, i.e., the space clamp problem, and in terms of the latter what are the \( K^+ \) channels that could contribute to this extraordinarily low \( R_m \). Continuously open, voltage-independent (leak) potassium channels seems a good bet. Parenthetically, one could also note that freshly isolated GFAP or EAA transporter current positive cells (i.e. characteristics of the passive astrocyte when isolated from older animals) have much higher mean \( R_t \) values of several hundred megaOhms (Lalo et al., 2006; Zhou and Kimelberg, 2000). This means either there is a major loss of processes upon isolation, which seems likely and/or loss of the syncytium if the currents indeed travel that far (D’Ascenzo et al., 2007).

**Fig. 1.6** (continued) The passive cell that gives a linear \( I-V \) plot (g) is only observed when astrocytes are recorded in slices (see Fig. 1.7 for how these different phenotypes change with age of the animal from which the slices were obtained). The lines intercepting the voltage coordinate at 0 current, and therefore called the reversal potential (reverses from inward to outward at 0 current) corresponds to the membrane potential under the ion gradients of the experiment, which are designed to duplicate the physiological ion concentrations. (h) shows an isolated astrocyte, dye-filled from the recording pipette while (i) is a cell in a hippocampal slice showing dye spread to other astrocytes. Scale bar, 10 µm (h).
As already noted sharp electrode recordings, where a high seal resistance is obtained by penetrating the cell, were first used for glial cells (the patch-clamp system had not yet been invented) and they were found to be electrically non-excitable. The patch clamp came up with the same thing but could also record voltage-dependent currents (but not in the mature passive astroglia). It is important to understand the problems of this technique applied to low-resistance cells when one is trying to clamp them at a potential and measure the current required to do that, if anything is to make sense. When the resistance is low it might take more time to deliver the current than the time frame in which the channel activates. More likely, when the resistance at the tip of the electrode is also around 10 MΩ, and so the voltage drop is about equal across both the electrode resistance and the cell membrane, and therefore, $V_c$ is around 2-fold greater than the voltage drop across the cell membrane. Third, it may be only the cell body and proximal processes that are clamped, as the current has to pass through 1,000–10,000 processes (Bushong et al., 2002, 2004) that get smaller and smaller, i.e. their cross-sectional resistance gets larger and larger, which will also, of course, limit spatial buffering. In the current-clamp mode (with $I = 0$) one is using the system as a voltage follower as in sharp electrodes (Purves, 1981), and so there is no problem. Further, if the pCLAMP 9 analysis gives reasonably accurate $R_a$ values for these cells then they can be studied with suitable corrections. The discontinuous single electrode voltage-clamp technique should avoid the $R_a$ problem in measuring $V_m$ (Sherman-Gold, 1993), but is not widely used now. Recordings with two electrodes, one for passing current and the other for measuring $V_m$, are possible but technically difficult given the small size (diameter, ~10 µm) of the astrocyte cell body in situ.

Our group decided to systematically study passive cells by whole-cell voltage clamp, by including all the cell bodies seen with differential interference optics as likely be “glia” from the stratum radiatum in hippocampal slices (cell bodies of ~10-µm diameter) from 1- to 105-day-old animals. Their “glial” nature could then be confirmed by their non-excitability in current clamp passing sufficient current to cause activation of voltage-gated Na$^+$ channels. It turned out that although cells with voltage-dependent currents could be recorded in slices from younger animals some passive cells could also be seen but most significantly, as the age of the animals increased, these represented about 90% of the glial cells in the adult stratum radiatum. The original paper (Zhou et al., 2006) can be consulted for the details and the major results are reproduced in Figs. 1.7 and 1.8. There is clearly a development transition around post-natal day 20 (P20), which corresponds to the completion of synaptogenesis in this region, a reasonable criterion for maturity (see Fig. 1.7). This was supplemented by post-recording staining of a separate and smaller group of cells (Fig. 1.8), which showed that we had recorded a shifting population of GLAST + astrocytes and NG2 + glia (cells) that led us to propose the developmental relationships shown in Fig. 1.9.

Thus the mature protoplasmic astroglia does seem to be homogeneous in terms of their electrophysiological characteristic, with the necessary caveat of what is measured in the CA1 region of mature rats. It is interesting that this corresponds to the original model after a 40-year digression into the electrophysiology of primary cultures, acutely isolated cells and astrocytes recorded in slices from immature rats as
models for the mature protoplasmic astrocyte. Of course a huge number of questions remain, and perhaps instead of me laying these down like some litany, which in any case will be my views, the interested reader can think of them for themselves.

However, an obvious one to start the ball rolling is does this emergence of passive astrocytes upon maturity apply to all brain regions? For the purists, and we should all be purists in scientific studies, this will need to be systematically studied in the different regions. In spite of the interpretative problems, the linear $I$–$V$ plots seen by continuous single electrode voltage clamp are a signature of the mature cells, but the $V_c$ is greater (by a factor of at least 2) than the actual $V_m$, as discussed above. The reversal potential ($E_r$), at $I = 0$ current, will equal the membrane potential (see Fig. 1.6e–f), but the effect of inhibitors on conductance will need to be corrected for the fact that one is measuring both an affected $R_m$ and an unaffected $R_a$ in series. An interesting aspect of this is that if we can identify the channels we can selectively inhibit different ones to get a “clampable” cell because $R_m$ will increase relative to $R_a$. To what extent, if at all, the channels in the end-feet will be measured by an electrode located in the cell soma is unknown, and until this technical question is resolved by measuring directly from the processes we will be restricted to describing what is there by immunocytochemistry, for some time.
Fig. 1.8 Correlation of electrophysiological phenotype with cell type by immunocytochemical identification of recorded cells. This continues the study shown in Fig. 1.7 by post-recording staining a smaller number of cells (given by \( n \) for each case) within the three broad age groups identified in Fig. 1.7. The cells were stained for GLAST to identify astrocytes and NG2 to identify this non-astrocytic glial class. (a)–(d) shows examples of staining in a GLAST(+) (cell 1) and an NG2 (+) cell (cell 2). Green is the filling dye and red represents either antibody staining. White represents colour-coded colocalization. As shown in (e), NG2(+) cells shown as yellow bars represent outwardly rectifying glial cells (ORGs) equally in the newborn stage and predominantly in the juvenile. Red represents GLAST(+) cells. There are no ORGs in the adult. (f) shows that variably rectifying glial cells (VRGs) are all and then predominantly astrocytes, but are only NG2(+) cells in the adult. (g) shows that passive cells are only seen in the juvenile and adult animals and represent mainly astrocytes, although 5–10% are NG2(+) in the adult, but unlike the passive astrocyte these have small \( \text{Na}^+ \) currents (not shown). See Zhou et al. (2006) for further details. (See Color Plates)

Possible relationships between electrophysiological astroglia phenotypes and GLAST and NG2 lineages during development

**NEWBORN** (P1-3) → **ORG NG2(+)** → **ORG NG2(+)** → **VRG GLAST(+)** → **PG GLAST(+)**

**JUVENILE** (P4-21) → **VRG-like NG2(+)** → **PG GLAST(+)**

**ADULT** (2P22 till P106)

Fig. 1.9 Possible relationships between electrophysiological astroglia phenotypes and GLAST and NG2 lineages during development. From Zhou et al. (2006); based on data in Fig. 1.8. (See Color Plates)
A study from our laboratory (Schools et al., 2006) showed that passivity correlated with the extent of cell–cell coupling, and membrane patches pulled from the cell showed a linear $I-V$ relation that corresponded more to the parent cell but a minority of around 30% were variably rectifying (Schools et al., 2006). In an excised patch of several hundred megaOhm resistance $V_m$ essentially equals $V_c$. The issue of different electrophysiological types seems to have been partially solved by the above studies in the hippocampus, where the heterogeneous cells were restricted to earlier development, and mature astrocytes after ~20 days were electrophysiologically passive, but there are at least three characteristics that develop in parallel that could contribute to such behaviour; open K\(^+\) channels, increased syncytium and $R_a > R_m$. Note that the first two also contribute to the third characteristic, and so they are all interrelated. There were also around 20% NG2(+) cells that either showed a variably rectifying or passive electrophysiological phenotype, but with small Na\(^+\) currents that were never seen in passive astrocytes.

The electrophysiologically passive, mature astrocytes can then be individually studied to determine whether they are heterogeneous for transporters and different enzymes and other components that are important for astrocyte functions. To examine these systematically also means defining age, lamina and region from which the cells are obtained. On the basis of such data the field should be reasonably able to answer the question posed in the title of this chapter.

### 1.5 Envoi

For the question posed in the title we do seem to be at the beginning of a journey rather than even well on our way. We need to correlate the well-defined morphological heterogeneity with other properties. With respect to electrophysiology we seem at least to know where we need to go; see if the linear $I-V$ curve correlates with maturity in all astrocytes and what does it represent. For biochemical properties we seem to be restricted to establishing the occurrence by immunocytochemistry as we cannot rely on isolated cells due to likely massive cell process loss plus other still-to-be-defined damage. Genetic engineering techniques linked to specific promoters for astrocytes using so far the GFAP promoter are still in their infancy (e.g., see Pascual et al. (2005), Chap. 14) and will require a large amount of preliminary work with different promoters to understand their specificities (Slezak et al., 2007). A classification could well emerge from these studies of astrocytes defined by a particular promoter-construct activity. Some illustrative and experimentally addressable questions are as follows:

1. Does development of the linear $I-V$ characteristics of mature astrocytes differ in different regions and lamina and what precisely does this linear $I-V$ curve mean?
2. Does morphological or physiological heterogeneity depend on species?
3. Is there spatial segregation of transporters and channels within the astrocyte, much as in epithelial cells? If these are mainly on the ends of the processes that surround blood vessels and synapses, one cannot study these electrophysiologically, at present, but can do so histologically and dynamically by calcium imaging.

The ultimate aim, of course, is to uncover the functions of astrocytes in the brain; support or an integral part of the information-processing system and how one can tell the difference. The latter has been theoretically ruled out by some scientists interested in information processing, consciousness and other such like big questions on grounds that the astroglial responses lack sufficient “specificity and celerity” (Koch, 2004). Whether these objections are valid underlies a lot of current work on astroglia (rather than “glia” in general, a term neuroscientists really should no longer use; see also Cahoy et al. (2008)) and no doubt increasingly in the future.

One message of this chapter is really that we should first adequately define what the term astroglia represents.

1.5.1 Experimental Approaches to Heterogeneity of Mature Astrocytes

What other approaches can we use to study astrocyte heterogeneity? If one catalogues all the gene messages significantly expressed by mature astrocytes (Note added in proof: the first studies in this area have just been published for sorted, isolated astrocytes from different aged animals (Lovatt et al., 2007; Cahoy et al., 2008), and the major mRNAs do correspond to the major proteins known to be expressed by immunostaining of functional studies.), will this enable us to say whether they are heterogeneous, and give us insight to the outstanding questions for astrocytes in the mammalian brain? However, this would be an example of data-gathering, fishing expeditions and all the other pejorative descriptors applied to what was once considered fundamental to scientific inquiry but is currently non-fashionable; the systematic acquisition of data that precedes hypotheses to explain the phenomena observed, an approach Isaac Newton advocated as the “safest method of philosophizing” (Christianson, 1984). But there are also significant methodological problems to this approach. For example the mRNA microarray approach allows one to assess at one time all the mRNAs expressed by the genome. However this requires an amount of RNA that is about 1,000 times that expressed by a single cell. Thus, we would only get an average and this would not then address the question of cellular astrocyte heterogeneity below the microregional level. Nonetheless, with this type of information we would obtain clues about which proteins to look for, and by using immunocytochemistry we could determine cell-to-cell heterogeneity and just as importantly heterogeneity of location within a single astrocyte, but only at present confidently at the electron microscope level. It is quite likely that improvements in techniques, including the use of linear RNA amplification will, hopefully in the not too distant future, make it possible to perform microarray studies at the single-cell level, and this will solve that problem at the message level.
At present then the question raised of whether mature astrocytes are heterogeneous in the sense of Fig. 1.4, that there is a core constellation of properties “A,” which defines astrocytes and then a varying degree of partially overlapping and non-overlapping properties that confers heterogeneity, still needs to be determined. If we ascertain that mature astrocytes in every brain region show linear $I-V$ relations, then using this as a signature, together with positivity for unambiguous astrocyte markers such as GLAST, GFAP and others (Note added in proof: see Cahoy et al. (2008) for other markers disclosed by global gene expression.), we can see whether there is heterogeneity of protein expression and mRNAs between different brain regions. Finally, as I think is likely and partially supported experimentally, there should be clear spatial heterogeneity such as between the astrocyte membranes that surround blood vessels and/or synapses and other regions, at a minimum. One might assume all such processes equivalent and then test that null hypothesis. This can only be disclosed by very precise microscopic studies the very technique, but of course now far more advanced, with which the cellular nature of the neuroglia was revealed by application of the Golgi staining technique over 100 years ago. In the absence of strong evidence to the contrary I would also propose that another and more basic null hypothesis to be disproved for the fundamental function of astrocytes is that it is limited to homeostasis; to provide a controlled environment that allows the information-processing part of the CNS, the different neuronal circuits made from the heterogeneous neuronal populations, to function optimally. However, other current hypotheses concerning astroglial function that they can influence synaptic activity on the basis that they may show exocytotic release of neurotransmitters, supported by the presumed astrocyte-specific elimination of a vesicle fusion protein resulting in suppression of synaptic transmission and increasing the dynamic range of long-term potentiation (Montana et al., 2006; Volterra and Meldolesi, 2005; Haydon and Carmignoto, 2006; Pascual et al., 2005), can be tested further in the sense that the proteins and such-like needed for this process are present in mature astrocytes (Cahoy et al. (2008) did not find mRNA for many of these). Another hypothesis is that the greater complexity of astroglial structure in different lamina of the human cortex, compared with a much simpler pattern for the rat cortex, but the comparable structure of neurons in the two very different mammals, supports an hypothesis that some of the indisputable greater complexity of function of the human brain compared with the rodent may, in part, reside in the astroglia (Oberheim et al., 2006). Here it would be useful to do cross-species studies.

1.5.2 Domain Concept for Mature Astroglia

The morphological complexity that allows one protoplasmic astrocyte to control a wide expanse of territory, the cellular domain concept first put forward by Bushong et al. (2002, 2004), and the independent functioning of individual processes as
shown by calcium imaging (Grosche et al., 1999) can be explained on the basis of cell theory where each part of a cell has to be linked to a cell body containing the cell nucleus. These linkages are the processes emanating from the cell body and target to synapses and blood vessels where they develop systems specialized to sustain, modulate or maintain these targets. When these processes meet other astrocytic processes they form gap junctions, perhaps not so much as a method of communication at all, but as a way of preventing further growth of these processes, defining their boundaries and thus leading to the separate domains (Bushong et al., 2004). On this basis each process ending has to be autonomous and operate independently by simple feedback or feedforward principles. This is further required by there so far being no polarity or clear differences in astrocytic processes, as is known for neurons. More so than other tissues the mammalian brain has limited space, being encased in rigid bone for protection, and the process boundary design prevents the unnecessary multiplication of astrocytic cell bodies beyond what is needed to most parsimoniously perform their functions. Thus we come back (see section 1.1.2) to more heterogeneity within an individual astrocyte than between astrocytes.

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References


**Abbreviations**

\[ C_m \] Membrane capacitance
CNS Central nervous system
EAA Excitatory amino acid
\( E_r \) Reversal potential
GFAP Glial fibrillary acidic protein
GS Glutamine synthetase
\( I-V \) Current–voltage
\( Q_t \) Total charge
\( R_a \) Electrode access resistance
\( R_m \) Membrane resistance
\( R_t \) Total resistance
\( V_a \) Voltage drop across \( R \)
\( V_c \) Clamp (command) potential
\( V_t \) Total voltage drop
\([K^+]_o\) Extracellular concentration of potassium ions
\([K^+]_i\) Intracellular concentration of potassium ions