Published in final edited form as:

Neurochem Res. 2012 November ; 37(11): 2569–2588. doi:10.1007/s11064-012-0868-0.

Primary cultures of astrocytes: Their value in understanding astrocytes in health and disease

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Abstract

During the past decades of astrocyte research it has become increasingly clear that astrocytes have taken a central position in all central nervous system activities. Much of our new understanding of astrocytes has been derived from studies conducted with primary cultures of astrocytes. Such cultures have been an invaluable tool for studying roles of astrocytes in physiological and pathological states. Many central astrocytic functions in metabolism, amino acid neurotransmission and calcium signaling were discovered using this tissue culture preparation and most of these observations were subsequently found *in vivo*. Nevertheless, primary cultures of astrocytes are an *in vitro* model that does not fully mimic the complex events occurring *in vivo*. Here we present an overview of the numerous contributions generated by the use of primary astrocyte cultures to uncover the diverse functions of astrocytes. Many of these discoveries would not have been possible to achieve without the use of astrocyte cultures. Additionally, we address and discuss the concerns that have been raised regarding the use of primary cultures of astrocytes as an experimental model system.

INTRODUCTION

Historically the term neuroglia (“nervenkitt”) dates back to Virchow in 1856 (1) and the word was related to the notion that the function of these cells primarily is to provide a connective scaffolding for the nerve cells; in other words, the function of neuroglia was thought to be of a passive nature filling up space not occupied by neurons. We now know after one and a half centuries that this passive role of glia is far from being true and it appears that glial cells are actively involved in all aspects of nervous tissue activity. The present review will discuss astrocytic functions and provide evidence that primary cultures of astrocytes have been instrumental as an experimental model system that has facilitated progress attained about the multifaceted repertoire of astrocytic properties that enable these cells to monitor and regulate synaptic events (for references, see (2-4)). Furthermore, this system has made a major contribution in our understanding of the role of astrocytes in many

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neurological conditions. At the same time it will be acknowledged that in spite of the fact that cultured astrocyte preparations have been extremely useful, there are limitations in using this system and that observations originating from these preparations must ultimately be verified *in vivo*.

THE USE OF PRIMARY ASTROCYTE CULTURES

In vivo and in vitro astrocytes

The possibilities for studying astrocytes in a physiological setting have faced issues of resolution, distinction between cell types accounting for the observed effects and lack of sufficiently sophisticated *in vivo* techniques. Such issues have been overcome by the use of primary astrocyte cultures, where detailed studies can be carried out, e.g. employing electrophysiological, biochemical, molecular and genetic tools. Manipulation of cultured astrocytes such as the knockdown of a specific gene with siRNA, where the roles of a single gene can be studied in detail, is a valuable tool to gain detailed knowledge of specific mechanisms in astrocytes. In addition, functional studies of receptors expressed by astrocytes and their responses can be studied in real time in astrocyte cultures, giving indications of their functional roles that *in vivo* techniques are presently unable to accomplish.

A major constraint encountered when investigating the functions of primary astrocyte cultures is to which degree results obtained in culture can be translated to astrocyte functions *in vivo*. In recent years, several articles highlighting this issue have emerged (5-8). Astrocytes show great morphological and functional diversity, thus defining an astrocyte is not straightforward (reviewed in (9)). Gliogenesis (astrocyte maturation) takes place in mammals from late embryonic development and continues through the neonatal and postnatal periods, during which time astrocytes continuously divide. Different astrocyte lineages give rise to protoplasmic astrocytes of the gray matter, fibrous astrocytes of the white matter and Bergmann glia of the cerebellum (9, 10). Additionally, astrocytes differ in properties from different brain regions (11, 12) and one has to be careful not to generalize observations generated at one site to all astrocytes. The extracellular milieu during gliogenesis defines the regulation of transcription factors and gene expression, creating a further level of differentiation (13). Mature astrocytes cease to divide and acquire their highly complex and heterogeneous morphology; protoplasmic astrocytes possess many branching processes that envelope synapses and whose endfeet cover blood vessels, while fibrous astrocytes have long, thin unbranched processes whose endfeet envelope nodes of Ranvier (9). Astrocytes in contact with blood vessels and synapses display a highly polarized morphology and constitute spatially exclusive microdomains as observed in Bergmann glia and hippocampal astrocytes with electron microscopy (14, 15). *In vivo*, astrocytes may have in excess of 100,000 processes (14), signifying their highly diverse integrative functions in the brain. Such a highly intricate 3-dimensional network is not created *in vitro*, where primary astrocytes are grown in a contact-inhibited monolayer, creating epitheloid-like cells devoid of synaptic contact and vascular elements.

The culture protocol factor

Most protocols for culturing astrocytes are derived from the early work by Booher and Sensenbrenner (16) or the later modification by McCarthy and de Vellis (17). A wealth of different modifications to these protocols has resulted in virtually every laboratory using its own protocol (summarized in (18), additional file 1). Culturing conditions can affect gene expression (6) and receptor expression (19), thereby influencing the interpretations of data obtained with astrocyte cultures. During maturation astrocyte functions change, and the developmental stage of an astrocyte influences the transcriptome as well as the proteome, both *in vivo* and *in vitro*. Astrocytes in culture undergo a considerable maturational process with regard to the expression of a number of important enzymes such as glutamine synthetase (GS) (20). Newborn mice or rats are generally used for culturing astrocytes, at an age when gliogenesis is not complete, and the immature astrocytes continue to divide *in vitro* until confluence, where contact inhibition prevents further cell division. Maturation of astrocytes in culture is achieved by several approaches, such as supplementing the culture medium with dibutyryl cyclic AMP (dbcAMP) and fetal bovine serum (FBS). How this artificial maturation and the culture protocol factor influences gene expression in relation to *in vivo* astrocytes is unknown, as investigating gene expression of “*in vivo*” astrocytes requires isolation steps that change the astrocyte environment. Therefore, a direct comparison between gene expression of *in vivo* and *in vitro* astrocytes is currently not feasible.

Factors differing between culture protocols include medium composition, culture plate coating, subculturing and days *in vitro* (DIV) (18). The influence of such culturing conditions on astrocyte gene expression and morphology is often neglected in data obtained from these cultures, when culturing conditions may actually play an important role. To the extent possible, astrocytes should be cultured in conditions mimicking the physiological environment as much as possible, as this will increase the validity of results obtained from these cultures.

As a response to injury, astrocytes become reactive (reactive astrogliosis) and attain functions different from their natural physiological response. Reactive astrocytes can be recognized *in vivo* by their morphological appearance (hypertrophy). Reactive astrogliosis is characterized by rapid glial fibrillary acidic protein (GFAP) synthesis (21), an intermediate filament of astrocytes supplying structural stability for astrocytic processes and providing modulation of motility. Due to the physical stress associated with the initial preparation of astrocyte cultures, it has been proposed that astrocytes in culture are of a reactive nature. Different culturing protocols affects the expression of GFAP in astrocytes, thus signifying that astrocytes are sensitive to both subculturing and shaking (done to remove oligodendrocytes and microglia from culture) (22). However, definitive data addressing the nature of cultured astrocyte phenotypes compared to reactive *in vivo* astrocytes is not available.

Protocols used to culture astrocytes should both create an optimal environment for astrocyte proliferation and maturation, in addition to minimizing the potential for contaminating cell types to proliferate. A major advantage of using astrocytes in culture is the absence of other

cell types, providing a simplified model in which to study astrocyte functions in detail. Subpopulations of other cell types in astrocyte cultures may create difficulties ensuring that the effects observed in culture are indeed astrocyte specific (18). Astrocyte purity has often been estimated by the percentage of GFAP-expressing cells; however this approach may not be preferable. As previously mentioned, GFAP expression is increased in reactive astrogliosis, thus an increase in GFAP may not characterize a purer astrocyte culture. White matter astrocytes show a more prominent expression of GFAP than grey matter astrocytes (Allen Brain Atlas, <http://mouse.brain-map.org>), and therefore investigators generally attempt to only remove cortical grey matter for culture preparation which is straightforward in rodents which have relatively little white matter. In addition to astrocytes, small populations of neuronal stem cells also express GFAP, and these have the capability to differentiate into neurons upon a change to neurogenic conditions (23). Neuronal stem cells tend to be confined to the periventricular area, thus this is not a major issue for preparation of astrocytes from other brain regions. In view of the above, investigations of GFAP expression can be supplemented with other astrocyte-specific markers such as GS (20), pyruvate carboxylase (PC) (24, 25), excitatory amino acid transporter 2 (GLT-1) (26) or aldehyde dehydrogenase 1 family member L1 (Aldh1L1)(6).

Astrocyte microenvironment

The different cell types living in close proximity in brain modulate each other in numerous ways. Astrocytes play roles in controlling local blood flow via secretion of vasodilators and constrictors (27-29); influence the polarized distribution of blood-brain barrier proteins (30); and secrete factors that regulate neurogenesis (31-33). In addition, astrocytes release signals influencing synapse formation and maturation (34, 35), as well as eliminating redundant synapses during development (36). Since these functions all require the presence of other cell types, a monotypic *in vitro* culture system does not provide a milieu where interactions and adaptations to the surrounding environment are required. A means of addressing the lack of the dynamic microenvironment *in vitro* while still maintaining the advantages of monotypic astrocyte cultures is by co-culturing. Astrocytes have been co-cultured with neurons from either cerebral cortex or cerebellum by seeding the neurons isolated from embryonic or early post-natal brain tissue on a preformed confluent layer of astrocytes (37, 38). Recently, an alternative co-culture system for cortical neurons and astrocytes was developed based on seeding cells isolated from embryonic cerebral cortex in Petri dishes and subsequently culturing the cells for a week without addition of cytosine arabinoside (39). Regardless of the methodology, such cultures exhibit a pronounced exchange of neuroactive amino acids and their precursors, as well as a metabolic activity resembling that in brain *in vivo* (37-41). Alternatively, astrocytes can be cultured in plates, where inserts with neurons (42), oligodendrocytes (43), neuronal stem cells (44), microglia (45) or endothelial cells (46-48) are added, so that they share the same medium, but are grown in separate layers. A more dynamic *in vivo*-like environment is thus created for the cells, enabling the researcher to investigate cell-type specific effects separately. While many attempts can be made to create the optimal culturing conditions for astrocytes, they will never provide the dynamic extracellular milieu astrocytes are exposed to under normal physiological conditions.

Astrocytes in culture are most often derived from newborn mice or rats, when astrocytes are still in an immature state and continuously divide. Culturing astrocytes from older animals when astrocytic development is complete does not result in more mature astrocytes in culture; instead, the cultured astrocytes appear to be derived from a small population of immature astrocyte stem cells that remain in brain throughout life (49). *In vivo* astrocytes form syncytia with other astrocytes and create highly intricate networks between several types of cells, including endothelial cells and neurons. This highly complex 3D network is not mimicked *in vitro* where they exhibit a flat, epitheloid morphology and due to the nature of mono-culturing they only form syncytia with other astrocytes (50). A recent addition to the possibilities for culturing cells is the emergence of 3-dimensional plates where cells are grown on a polystyrene scaffold, allowing the cells to form contacts in three dimensions. This creates the possibility of restoring some of the *in vivo* astrocyte morphology, although this will not fully recreate the intricate networks found amongst astrocytes and other cell types *in vivo* (Milos Pekny, personal communication).

Culturing medium composition

The astrocyte culturing medium composition is of paramount importance for primary cultured astrocytes, affecting culture purity, gene expression profile and response to starvation (51). The most frequently used media are DMEM, MEM or F12, often supplemented with glucose, glutamine, NaHCO₃ or a combination of these (additional file in (18)). In addition, astrocyte cultures are often supplemented with 10-20% FBS. FBS contains growth factors and fulfills many of the metabolic requirements of cultured cells, but the complete composition is difficult to determine, and inter-vendor and inter-batch variability is often found. This causes different growth conditions for the astrocytes potentially creating variability in results obtained with these cultures (51). In addition, many of the proteins found in serum are unable to cross the blood-brain barrier and would therefore not be part of the extracellular milieu normally encountered *in vivo* (5). The presence of serum in the culturing medium affects the gene expression of astrocytes, but this does not fully account for the difference between immunopanned astrocytes (isolated by a series of panning steps to deplete other cell types and enrich the population of astrocytes (5)) from astrocytes cultured in the presence of serum (5, 6). Therefore, other culturing factors or isolation procedures must also influence the gene expression differences observed. Due to concerns regarding the use of serum for mammalian cell cultures, effort has been placed into developing chemically defined medium, where the complete composition is known and reproducible (52). This type of medium was shown to increase expression levels of astrocyte specific proteins in rat spinal cord astrocytes, when compared to medium containing serum from three different sources (51). However, chemically defined medium is currently not widely used and its utility requires further investigation prior to implementation.

To provide astrocytes in culture with some of the growth factors, metabolites and extracellular matrix proteins they are exposed to *in vivo*, one possibility is the use of conditioned medium (CM). CM can be obtained from various cultured neural cells, and the medium added to another cell type. The use of this strategy showed that addition of CM from cultured cerebral cortical or cerebellar neurons to cultured astrocytes resulted in up-regulation of expression of transporters for glutamate and GABA (53, 54). This approach

has the advantage of more closely mimicking the *in vivo* environment of astrocytes and is currently underutilized.

An often used approach to create more mature astrocytes in culture is addition of the cell permeable cAMP analogue dibutyl cAMP (dbcAMP) in the final week of astrocyte culturing. After a few days in culture, the addition of dbcAMP produces morphological and functional changes to the astrocytes (55, 56). Numerous processes are observed creating a highly complex network amongst the astrocytes. Functionally, dbcAMP increases the expression of astrocyte-specific proteins GS and GLT-1, as well as almost doubling the uptake of D-aspartate, used as a non-metabolizable substitute for glutamate (57). These changes appear to represent astrocyte differentiation (58). The observation that chronic noradrenaline exposure has the same effect on astrocyte differentiation as dbcAMP indicates that dbcAMP serves as a substitute for the noradrenergic stimuli that astrocytes are exposed to *in vivo* (59).

Most culturing protocols use a glucose concentration close to 6 mM which is rapidly taken up by astrocytes in culture. Once the culturing medium is depleted of glucose, the astrocytes start using lactate as a substrate for oxidative metabolism (60). Due to rapid depletion of glucose in the medium, some investigators have instead used high glucose concentrations of 20-25 mM. However, this approach has a number of drawbacks. Raising the glucose concentration in culture induces experimental diabetes, which decreases gap junctional communication (61) and increases the level of multiple inflammatory cytokines and mediators linked to the pathogenesis of diabetes in the CNS (62). Exposure to high glucose levels also increases the production of reactive oxygen species (ROS) in astrocytes (62), and activates the pentose phosphate pathway in an attempt to prevent ROS elevation (63). Exposure to 15 mM glucose has been shown to morphologically alter astrocytes *in vitro* including increases in GFAP and vimentin expression, and very high glucose (30 mM) was shown to induce apoptosis (62). In contrast, lowering the glucose concentration to 2 mM caused an increased capacity for oxidizing glucose and lactate (64).

To ensure an adequate supply of glucose to meet astrocyte needs, while avoiding low or elevated glucose levels, we suggest that glucose levels should not be raised, but rather that the cultures be given low doses of glucose daily so as to maintain glucose levels in a physiological range. This will supply the astrocytes with continuous access to this important energy substrate.

Contaminating cell types

A major advantage of astrocytes in culture is that a single cell type is studied and any effects observed with these cultures are therefore astrocyte-specific. This underscores the importance of culture purity, as the presence of other cell types in astrocyte cultures may result in observations that cannot be attributed to astrocyte functions. Cultured astrocytes most often derive from brain tissue of mice or rats, where astrocytes are enriched by either mechanical or enzymatic methods. Each brain region consists of a number of cell types, amongst these are neurons, oligodendrocytes, endothelial cells, neuronal stem cells and microglia, all of which have the potential of contaminating the astrocyte cultures. However, differences in their culturing requirements and vulnerability ensure the loss of neurons due

to mechanical passaging through a mesh, the absence of culture plate coating and frequent medium changes. Oligodendrocytes can be removed by shaking. Microglia are the principal contaminating cells in astrocyte cultures (18), and estimating their proportion is important when evaluating the cell-type specificity of results observed *in vitro*, especially in studies involving inflammatory events (65). The presence of contaminating microglia is a concern when studying gene expression profiles of astrocyte preparations, as even a small contribution of microglial mRNA may lead to erroneous conclusions regarding genes not normally expressed in astrocytes. In addition, microglia secrete and take up components of the culture medium, changing the extracellular milieu for the astrocytes. Several approaches to estimate microglial contamination are mentioned by Saura (18), as well as approaches to minimize microglial content. Strategies employed for enriching astrocytes should be carefully evaluated as procedures such as shaking may change protein expression (22, 49). While including a microglial toxin such as cytosine arabinoside may not eliminate astrocytes, less obvious changes, e.g. in gene expression could occur. To summarize, every effort should be made to confirm the astrocyte-specificity of any observations found in astrocyte cultures.

Astrocyte gene expression profiling

Molecular biology has undergone a major transformation with the emergence of techniques such as microarray that have provided large quantities of information about gene expression in different cell types and under different experimental conditions (6, 66, 67). Expression profiling studies are useful for providing information about cell functions by identifying the expression of genes involved in these functions. In addition, information on cell type specific markers can be gained by comparing different cell types from whole tissue. To investigate differences in gene expression associated with anaerobic and oxidative glycolytic metabolism between astrocytes and neurons, Lovatt et al. (66) published the first systematic genomic analysis of “acutely isolated” adult astrocytes. The term “acutely isolated” indicates the absence of culturing prior to their experimental use; i.e., the astrocytes are isolated and used for experiments immediately following the isolation procedure. Two astrocyte populations were purified with fluorescence activated cell sorting (FACS); one was based on GFAP promoter-driven green fluorescent protein (GFP) reporter expression in a transgenic mouse, while the other astrocyte population was defined by surface expression of the astrocyte-specific transporter GLT-1 and the absence of GFAP expression. The two astrocyte pools, defined as GLT1⁺/GFAP⁺ and GLT1⁺/GFAP⁻, as well as a neuronal pool were subjected to microarray analysis to investigate the expression of genes involved in anaerobic and glycolytic metabolism. All enzymes of glycolysis and oxidative metabolism were found in both astrocytic and neuronal cell populations, indicating self-sufficiency in glucose metabolism by both cell types. In light of the ongoing discussion concerning the ability of astrocytes to perform oxidative metabolism of glucose rather than glycolytic conversion of glucose to lactate (see, (68)), it was surprising that most enzymes of the tricarboxylic acid (TCA) cycle were expressed at higher levels in astrocytes relative to neurons, indicating an extensive oxidative metabolism of glucose in astrocytes, which was also confirmed by metabolic mapping using ¹³C glucose and mass spectrometry analysis (66). Compartmentalization of lactate metabolism has been shown in studies using astrocyte cultures (69) and was supported by a high capacity for lactate synthesis at the transcript level

and a high lactate production by acutely isolated astrocytes (66). Altogether, this shows that astrocytes possess the capacity for oxidative as well as non-oxidative metabolism of glucose.

To gain a better understanding of gene expression in various cell types in brain and under different developmental stages, a transcriptome database for acutely isolated central nervous system cell types was reported by Cahoy et al. (6). Astrocytes were isolated from a transgenic mouse line expressing enhanced GFP under the control of an S100 β promoter using immunopanning and FACS. Gene expression profiles were analyzed for acutely isolated astrocytes from transgenic P1-P30 as well as for cultured astrocytes, and hierarchical gene clustering revealed a closer clustering between these astrocytes as compared to neurons or oligodendrocytes. Nevertheless, of the 12,416 CNS expressed genes investigated, 2103 were found to be enriched in cultured astroglia and 2819 were enriched in the acutely isolated astrocytes. The authors speculated that cultured astrocytes may represent an immature stage of the astrocyte lineage, or may be a reactive astrocyte phenotype (6), however they do not address the issue of using transgenic mice for the study of “*in vivo*” astrocytes, which may also influence astrocyte gene expression. In the final analysis, which astrocyte preparation closer resembles *in vivo* astrocytes still remains to be determined.

Microarray is a good exploratory tool to generate data for further investigations using more detailed techniques, but it is neither quantitative nor does it provide functional information about protein expression and functionality. Therefore, it should be kept in mind when interpreting microarray data that mRNA expression does not necessarily translate to functional protein expression. In addition, the mRNA levels observed only represent a snapshot of a particular developmental and experimental time point, and will be influenced by the culturing or preparation steps carried out prior to RNA extraction. Sensitivity of the microarray technique is also important for obtaining a complete transcriptomic analysis. Doyle et al. (67) used a BACarray to create transcriptome data for 24 CNS cell populations and detected more than 4000 additional probesets as compared to a microarray analysis from whole mouse cerebellum. Many of the probes not detected with the microarray analysis were cell-type specific, highlighting the importance of supplementing microarray data with more sensitive analyses if information about specific genes is desired.

There is much to be learned by studying the transcriptional profile of astrocytes under different culturing and purification conditions as well as in different stages of maturation. As long as studies are designed to address relevant scientific questions and limitations of the methods kept in mind, transcriptome analyses will likely contribute to uncovering new functions of astrocytes, as well as enhance our understanding of existing astrocyte functions.

Acute isolation of astrocytes

A number of alternative ways of obtaining astrocytes for *in vitro* studies exist, and these methods have different advantages and limitations compared to primary astrocyte cultures (7). Direct isolation of astrocytes from rodent brains has been done using FACS (6, 66) and immunopanning (5); other approaches include differentiating immature stem cell populations into astrocytes *in vitro* (70). Advantages being highlighted include that these methods avoid the culturing procedure and can be directly used upon isolation. They may

also be used as a source to yield purer astrocyte cultures and thereby avoid microglial contamination. However, the precise effects of the isolation procedure with regard to gene and protein expression, as well as morphological characteristics of the astrocytes remain to be determined. Any interpretation made by comparing acutely isolated astrocytes and those in culture must, however, be made with caution.

Using the immunopanning technique or FACS purification, astrocytes are directly isolated in one day, and are then used for experiments immediately. As for any purely astrocytic preparation, these astrocytes have undergone isolation steps altering both their morphology and microenvironment compared to their native *in vivo* environment. Preparation of astrocytes for immunopanning and FACS includes decapitation and dissociation of the brain, naturally causing trauma to the brain tissue. Hereafter the cells are treated with DNase and incubated on panning plates with antibodies to deplete microglia and another panning plate to deplete oligodendrocytes (5, 6). After the panning steps, the cells are centrifuged and washed several times before they are subjected to FACS (66). Being highly plastic, astrocytes are given to respond to the physical stress associated with isolation and it is unlikely that the “acutely isolated” astrocytes resemble *in vivo* astrocytes any more so than do primary cultures of astrocytes. Both are model systems employed to study functions of astrocytes that it is currently not feasible to investigate *in vivo*. Statements that one system is superior to another should be taken with some skepticism.

Expression profiles of secretory organelle proteins change after just 12 h in culture (71), and given the plethora of metabolic and homeostatic functions undertaken by astrocytes (4, 66) they will adapt and adjust their gene expression to new conditions rapidly (72), in ways that should be addressed in further studies. Presently such studies are limited in that preparation steps to obtain pure astrocytes are necessary, and as yet we can only make qualified guesses as to how astrocytes react to such handling. Only well designed *in vivo* studies can establish the exact differences between *in vivo* and *in vitro* astrocytes, which may not happen for some time as techniques to accomplish this are currently not available.

Results obtained with astrocytes isolated by immunopanning and FACS cannot be directly compared to studies published with primary cultured astrocytes, which have routinely been used for studying astrocytes the past decades. On the other hand, other methods of obtaining *in vitro* astrocytes may support previous findings with cultured astrocytes, as well as contributing to expand our current knowledge of astrocytic functions. Whether acutely isolated or cultured, pure astrocytes present a means of identifying potential events, establish mechanisms and pathways that today cannot be achieved *in vivo*, as well as providing hypotheses that can at some point be tested *in vivo*.

DISCOVERIES MADE BY EMPLOYING PRIMARY CULTURED ASTROCYTES TO UNDERSTAND THEIR PHYSIOLOGICAL FUNCTION

Attempts to gain insight into the functional importance of astrocytes in neurotransmission and metabolism in the CNS had for several years relied on the use of either cell lines obtained from astroglial tumors or from astroglial preparations derived from differential centrifugation of brain homogenates, i.e. the ‘bulk-prepared glia’ (for references, see (73)).

While bulk-prepared glia provided useful information about the function of astroglia in relation to neurotransmission and metabolism (73), the cell lines often generated misleading information, particularly in terms of quantitative aspects of these entities (e.g. (73, 74)). The advent of a procedure for the preparation of primary cultures of astroglial cells from brains of newborn rodents (16) constituted a major event regarding the availability of a model system by which information could be obtained pertinent to the characterization of astrocyte function in brain. However, even with this preparation, one had to be cautious since the cells originated from newborn animals and therefore the developmental stage and maturity of the cultured cells, even after several weeks in culture, could be challenged (75). The work of Leif Hertz and coworkers devoted to the characterization of this culture system ultimately led to the establishment of a reliable model system (76), albeit its ability to precisely reflect the properties of mature astrocytes in brain is still questioned by several investigators (6, 8). The following sections discuss current views on specific aspects of the role of astrocytes in integrating signaling events pertinent to proper synaptic function with regard to maintenance and regulation of amino acid neurotransmission.

Metabolic processes

Detailed studies of glucose and amino acid metabolism in brain performed fifty years ago (for references, see (73)) provided unequivocal proof that different cellular compartments in brain exhibited distinct differences regarding the ability of these compartments to perform certain biochemical processes presumably due to different expression levels of a number of enzymes. The observation that two such enzymes, GS and PC, are expressed only in astrocytes and not in neurons (24, 25, 77) was instrumental for our current view of the role of astrocytes in glucose and glutamate metabolism. Moreover, the use of primary cultures of astrocytes has been important for placing astrocytes as the cell in the brain providing the precursor glutamine for *de novo* biosynthesis of the main neurotransmitters, glutamate and GABA (78, 79). Interestingly, the transcriptomic analysis by Lovatt et al. (66) provided evidence that the above mentioned enzymes are indeed exclusively found in astrocytes and not in neurons. The latter investigation also showed that acutely isolated astrocytes have a high expression of genes for enzymes involved in glycolysis, glycogen metabolism as well as the TCA cycle; all in agreement with results from primary cultures of astrocytes (see (68, 80)). It may therefore be concluded that astrocytes in primary culture can be used as a reliable model system to study basic astrocytic functions regarding metabolic processes in brain.

Ammonia metabolism and the glutamate-glutamine cycle

As noted above, early studies of glucose and glutamate metabolism in brain suggested that glutamate metabolism involved at least two distinct compartments which at the time were thought to represent neuronal and astroglial elements (for references, see (73)). The finding that GS, which plays a prominent role in this context, is expressed almost exclusively in astrocytes, has confirmed the assignment of the small, actively metabolizing glutamate pool forming glutamine to the astrocytic compartment (77, 81). Based on these compartmentation studies it was suggested that glutamate formed in neurons would be transferred to the astroglial compartment where GS would convert glutamate to glutamine that could subsequently be transferred back to the neurons, a metabolic shuttling referred to as the

glutamate-glutamine cycle (82). This metabolic cycling of glutamate and glutamine involves additionally phosphate activated glutaminase (PAG), which was believed to be almost exclusively expressed in neurons (83, 84). It was therefore somewhat controversial that an investigation of PAG activity in cultured astrocytes revealed an activity of this enzyme comparable to that in the brain *in vivo* (85). This finding is, however, in keeping with a high rate of oxidative metabolism of glutamine in cultured astrocytes (86). The failure to detect immunolabeling of PAG in astroglial elements, while it was prominent in neurons in cerebellum (87), has questioned the presence of PAG in astrocytes *in vivo*. It should be noted, however, that the liver isozyme of PAG has been shown by immunostaining to be localized in astrocytes in the brain (88, 89) and in unpublished observations PAG has been identified in astrocytic mitochondria *in vivo* (Rama Rao et al). In addition, the transcriptomic analysis by Lovatt et al. (66) of acutely isolated astrocytes did find PAG mRNA in astrocytes albeit the expression level in neurons was significantly higher. This is in keeping with the operation of the glutamate-glutamine cycle and with the prominent role of glutamine as a precursor for the neurotransmitter pools of both glutamate and GABA (84, 90, 91). Interestingly, such transfer of glutamine between astrocytes and neurons has also been demonstrated in primary co-cultures of neurons and astrocytes using the glia selective metabolic substrate acetate labeled with ^{13}C (39, 40, 92). The results obtained using these culture systems are to a large extent reflective of those obtained after the administration of [^{13}C]-acetate to mice and the subsequent analysis of labeling in GABA, glutamine and glutamate in brain extracts (93-95).

Ammonia plays an important role in the glutamate-glutamine cycle being produced in neurons and consumed in astrocytes (96). Based on studies performed in primary cultures of neurons and astrocytes two groups independently (97, 98) proposed a shuttling mechanism involving lactate and alanine, the latter being responsible for transfer of ammonia nitrogen from neurons to astrocytes (96). A different shuttling mechanism involving branched-chain amino acids as nitrogen carriers was subsequently proposed. In this case, the experimental work was performed using rats measuring metabolic processes *in vivo* in retina as well as the brain (99). This is an example of similar results obtained from studies *in vivo* and in primary cultures of neural cells clearly demonstrating that cultured cells from the CNS including astrocytes can be used as reliable model systems. In line with these findings, subsequent work performed in cultured astrocytes confirmed that astrocytes metabolize branched-chain amino acids and that during glutamate exposure (mimicking neurotransmission activity) valine seems to play a more important role as compared to leucine and isoleucine (100).

Inactivation of the neurotransmitters glutamate and GABA by high affinity transport

Studies of glutamate and GABA transport using brain slices or a synaptosomal preparation by Iversen and Neal (101) and Logan and Snyder (102) were fundamental in developing the notion that these amino acids could function as neurotransmitters and that high affinity transport systems likely accounted for their inactivation. It was for many years believed that such transport systems were primarily, if not exclusively, expressed in neurons (74, 103), although this notion was challenged by the demonstration of high affinity uptake of glutamate and GABA in bulk-isolated glial cells (104, 105). The demonstration of efficient, high affinity transporters for these amino acids with a considerable capacity in primary

cultures of astrocytes (20, 106, 107) led, however, to an altered view regarding the role of astrocytes in this context. Particularly in the case of glutamate, it became clear that astrocytic uptake must be of paramount importance, a notion confirmed by cloning the relevant transporters and immunostaining of sections from different brain areas (103, 108). In this context it should be highlighted that the discovery of neuronal-glial signaling mechanisms being responsible for the expression of the glutamate transporters in astroglia (53, 54, 109, 110) was based on the use of primary cultures of astrocytes and neurons. Interestingly, the observation that axotomy was associated with a loss of high affinity glutamate uptake in nerve endings and interpreted as nerve endings being responsible for glutamate uptake (111) likely reflected a decreased astroglial glutamate uptake resulting from a lack of a neuronal factor inducing expression of the transporters in astrocytes.

Contrary to the case for glutamate, high affinity GABA transport is less pronounced in astrocytes compared to that in GABAergic neurons (112). Nevertheless, the glial GABA transport is of considerable pharmacological interest exhibiting characteristics different from the neuronal transport (113, 114). Moreover, as will be elaborated on below, it has been shown that GABA analogs selectively inhibiting the glial GABA transport have interesting properties in relation to protection against seizure activity (115). Again, these findings were to a large extent based on the use of primary cultures of astrocytes.

Calcium transients and the tripartite synapse

In 1990 a revolution in our thinking regarding astroglial “excitability” came about when Cornell-Bell and colleagues (50) reported the development of “calcium waves” in cultured astrocytes derived from rodent brain after the application of glutamate. This essentially brought about a radical change in our thinking about astrocytes as non-excitable cells. While astrocytes are not electrically excitable, they are indeed excitable by elevations in intracellular calcium. This subsequently evolved into the tripartite synapse hypothesis whereby neurons communicate with astrocytes via the excitatory neurotransmitter glutamate (4, 116, 117). This then leads to an elevation in intracellular calcium levels in astrocytes which triggers the formation of “gliotransmitters” (glutamate, D-serine, ATP). These gliotransmitters can then stimulate neurons resulting in a modulation of synaptic activity as well as provide signals to blood vessels resulting in regulation of blood flow (“functional hyperemia”) (118). It would be fair to conclude that these dramatic developments in our contemporary thinking about the function of astrocytes would never have evolved without the use of primary cultures derived from rodent astrocytes.

DISCOVERIES MADE BY EMPLOYING PRIMARY CULTURES TO UNDERSTAND PATHOPHYSIOLOGICAL ASPECTS OF ASTROCYTE FUNCTION

The purpose of this section is not to provide a comprehensive survey of contributions of astrocyte cultures to our understanding of neurological diseases. Rather, the intent is to illustrate the approaches investigators have employed in establishing the role of astrocytes in disease and highlight some of the significant contributions that could not have been obtained *in vivo* or even in brain slice preparations.

Hepatic encephalopathy

Hepatic encephalopathy (HE) is a neuropsychiatric disorder associated with severe liver disease. The disorder may present acutely following fulminant liver failure, or it may evolve chronically when the liver is not as severely damaged. The acute form is principally associated with the development of brain edema, increased intracranial pressure and associated complications (see below). The major finding in brain is marked astrocyte swelling. The chronic form presents with behavioral abnormalities, including forgetfulness, confusion, irritability and abnormal sleep-awake cycles. The histopathology is an astrocytic abnormality referred to as the Alzheimer type II change. No abnormalities are observed in other neural cells, including neurons. These findings strongly suggest that HE represents a primary gliopathy and the symptomatology is due to impairment in astroglial functions (119-122). HE in fact was the first neurological disorder in which astrocytes were proposed to play a primary role in its pathogenesis (119).

The use of ammonia-treated cultured astrocytes as a model for hepatic encephalopathy is highly appropriate since substantial evidence invokes a role of ammonia in the pathogenesis of hepatic encephalopathy (123, 124), and astrocytes are the principal cells affected in this condition (119, 122, 125). Moreover, many of the findings occurring in hepatic encephalopathy *in vivo* are also observed in these cultures, including characteristic morphologic changes, cell swelling, defects in glutamate transport, up-regulation of the peripheral benzodiazepine receptor (recently renamed the 18-kDa translocator protein, involved in neurosteroid biosynthesis), reduction in levels of GFAP and myo-inositol, disturbance in energy metabolism, and evidence of oxidative/nitrosative stress (for review, see (126)). Additionally, activation of signaling factors such as mitogen-activated protein kinases (MAPKs), the nuclear factor kappaB (NF- κ B), and the activation of the ion transporters the Na-K-Cl cotransporter-1 (NKCC1), the ATP-dependent, non-selective cation channel (NCCa-ATP channel), as well as the water channel protein aquaporin-4 (AQP4) have been implicated in this condition (for review, see (126)). Ammonia was also shown to increase intracellular calcium, and to induce the mitochondrial permeability transition (mPT) in cultured astrocytes (for review, see (126)). Additionally, an increase in genes coding for the mouse proline rich protein expressed in brain (PRTB), clusterin, and elongin (127), as well as nitration and phosphorylation of protein tyrosine residues (128), upregulation of heme oxygenase-1 (HO-1) (129), RNA oxidation (130) and increases in free Zn²⁺ and metallothionin mRNA (131) were identified in cultured astrocytes. It should be emphasized that many of these findings have also been observed *in vivo* (126).

Astrocyte swelling (cytotoxic brain edema)

Cytotoxic brain edema, predominantly due to the swelling of astrocytes, is a major form of brain edema which is associated with ischemia, trauma, acute hepatic encephalopathy (acute liver failure, ALF), severe hypothermia, hyponatremia, various intoxications (dinitrophenol, triethyltin, hexachlorophene) and other conditions. While the occurrence of cytotoxic brain edema can be readily detected by non-invasive techniques, including MRI and CT scans, the precise mechanisms involved in the development of cytotoxic brain edema are difficult to investigate *in vivo*. Using primary cultured astrocytes, a better understanding of the mechanisms occurring in cytotoxic edema has evolved.

Ischemia—Oxygen glucose deprivation (OGD) is a well-accepted and characterized *in vitro* model to investigate ischemic changes in various neural cells (132). Employing primary cultures of astrocytes subjected to OGD studies have shown significant astrocyte swelling following the reoxygenation phase (133, 134). This approach has disclosed alterations in the activities/expression of various penultimate factors (ion transport systems, water channels) involved in astrocyte swelling. Thus, increased NKCC1 activity leading to intracellular Na⁺, K⁺, and Cl⁻ accumulation was observed in rat cortical and spinal cord astrocytes as well as in mouse cortical astrocytes (134-138). Additionally, bumetanide, an inhibitor of NKCC was shown to diminish both the intracellular ion accumulation as well as the cell swelling in cultured astrocytes following OGD (137-140). Similar to NKCC, increased expression of AQP4 was reported in cultured astrocytes following OGD (134). These studies suggest that NKCC1 activation (resulting in the build-up of intracellular osmolites), as well as over expression of AQP4 (allowing for water entry), contributes to the cytotoxic brain edema in ischemia. For reviews on NKCC1 in the mechanism of astrocyte swelling/brain edema in ischemia, see Chen and Sun (141) and Kahle et al. (142).

In addition to OGD, exposure of cultured astrocytes to factors identified *in vivo* following cerebral ischemia, was shown to result in astrocyte swelling. Thus, Ringel et al. (143) demonstrated that NKCC contributed to acidosis-induced glial swelling, possibly by altering the sodium-hydrogen exchanger activity, consistent with the well-known ability of acidosis to exacerbate cerebral ischemia (144-146).

Traumatic brain injury—Fluid percussion injury to astrocytes was shown to cause significant cell swelling at 1-6 h after trauma (147), a time course that correlated well with the development of cytotoxic edema in the early phase following traumatic brain injury (TBI) (148). Similar to OGD (see above) increased activity of NKCC (149) as well as the upregulation of AQP4 was shown in cultured astrocytes following fluid percussion injury (150).

Acute hepatic encephalopathy—Acute hepatic encephalopathy usually occurs following viral-mediated hepatitis, acetaminophen toxicity, and exposure to other hepatotoxins (151). It often presents with the abrupt onset of delirium, seizures and coma and has a high mortality rate (80-90%) (152). A major component of ALF is the development of brain edema leading to increased intracranial pressure and brain herniation, ultimately resulting in death (153). The brain edema associated with ALF is cytotoxic due to astrocyte swelling (154-157). A major etiological factor contributing to the cytotoxic brain edema/astrocyte swelling is ammonia (158), which is primarily detoxified in astrocytes by GS (77, 155). Cultured astrocytes exposed to a pathophysiological concentration of ammonia have demonstrated cell swelling (159, 160).

While ammonia continues to represent a major factor in the development of the brain edema in ALF, there is evidence that proinflammatory cytokines likely derived from liver necrosis and/or sepsis, the latter a common complication in ALF, play important roles in brain edema formation in ALF. Increased blood levels of TNF- α , IL-1 β and IL-6 were found elevated in patients with ALF who had concurrent infections (161, 162). Recently, these inflammatory cytokines were also shown to cause cell swelling in cultured astrocytes (163). Additionally,

various factors, including rise in intracellular Ca^{2+} , activation of MAPKs, NF- κ B, as well as induction of the mPT have all been shown to contribute to ammonia-induced cell swelling in cultured astrocytes (164).

Stroke

A useful and productive approach to explore mechanisms involved in stroke pathogenesis is to examine events in culture after OGD. Among one of the earliest studies of this type was that of Vibulsreth and co-workers (165) who found that astrocytes protected neurons during anoxia. The mechanism for such protection was unclear, but the authors speculated that the uptake of K^+ , the presence of glycogen as a source of glucose (166), as well the release of neurotrophic factors was contributing to such protection. Rosenberg and colleagues (167) subsequently suggested that the ability of cultured astrocytes to take up the excitatory neurotransmitter glutamate may have been a key mechanistic factor. Accordingly, neuronal cultures are able to tolerate glutamate excitotoxicity by a factor of 100-fold in the presence of astrocytes.

Compared to neurons astrocytes are able to withstand extended periods of hypoxia (168), although they are sensitive to the acidosis which often occurs during hypoxia (169, 170). Similarly, astrocytes were found to display a delayed onset of the mPT and mitochondrial dysfunction compared to neurons (171). Critical aspects of stroke relative to minimizing infarct volume are events occurring in the penumbra. Accordingly, efforts at maintaining astrocyte viability (ultimately necessary for neuronal integrity) is likely to represent a crucial strategy in stroke (172-174).

Ischemia preconditioning (IPC) is a phenomenon describing the tolerance of brain to a lethal hypoxic/ischemic insult by a prior exposure to sublethal hypoxic/ischemic insults (for review, see (175)). Analysis of mechanisms involved in IPC may provide novel therapeutic interventions for stroke. Using co-cultures of mouse primary brain microvessel endothelial and astroglial cells, Gesuete et al. (176) reported that astrocytes are the key contributor to ischemic preconditioning involved in the protection of the blood-brain barrier.

For a comprehensive review of the role of astrocytes in stroke derived from the use of astrocyte cultures, including the role of oxidative stress, prevention of apoptotic cell death, and the potential neuroprotective role exerted by astrocytes, see Voloboueva et al. (177).

Trauma

The utility of cultured astrocytes to investigate mechanisms associated with traumatic brain injury (TBI) was initiated over 15 years ago. Four different *in vitro* models of trauma evolved: scratch injury (178), stretch-induced injury (179), fluid percussion injury (179-181), and a mechanical strain model (181). Among these models the stretch-induced injury has been most frequently employed, followed by the fluid percussion injury model.

Employing the stretch model of trauma, astrocytes were shown to display oxidative/nitrosative stress (182), Ca^{2+} oscillations (183, 184), dissipation of mitochondrial membrane potential (185) and impairments of metabotropic glutamatergic and purinergic receptors (186-188). Additionally, cultured astrocytes were shown to increase the release of S-100 β , a

commonly used marker of brain injury (189). Such increase in S-100 β release was shown to delay the onset of neuronal injury (190). Employing the mechanical strain-injury model, Chen et al. (191) reported depletion of Ca²⁺ stores and associated reduction in capacitative Ca²⁺ entry into astrocytes. A study by Floyd et al. (192), reported increased activity of the Na⁺/Ca²⁺ exchanger (NCX), an ion-transporter involved in Na⁺ and Ca²⁺ homeostasis.

Cultured astrocytes subjected to fluid percussion injury (FPI) have been well characterized (147) to model cytotoxic edema (astrocyte swelling), a major complication in the early phase following TBI (148). When subjected to trauma in culture, astrocytes display a significant increase in cell volume (swelling) as a function of time (147). Additionally, FPI to cultured astrocytes resulted in the up-regulation of the water channel protein AQP4 (150), as well as increased activity of the NKCC (149), penultimate factors that lead to astrocyte swelling. Noteworthy, the increase in NKCC activity and AQP4 up-regulation correlated well with the time course of astrocyte swelling following FPI.

Investigations into mechanisms of FPI-mediated astrocyte swelling showed increased production of free radicals, activation of MAPKs, and inhibition of these events significantly mitigated the FPI-mediated astrocyte swelling (147). Further, FPI to cultured astrocytes resulted in an induction of the mPT, a Ca²⁺-dependent opening of the permeability transition pore in the inner mitochondrial membrane. The mPT results in a collapse of the mitochondrial inner membrane potential, ultimately leading to decreased oxidative phosphorylation and bioenergetic failure (193). The induction of the mPT can also result in secondary oxidative stress. All of these factors were shown to contribute to trauma-induced astrocyte swelling.

Epilepsy

It has been known for decades that aberrations in GABAergic neurotransmission are associated with seizure activity and epilepsy (91, 194, 195) and the two clinically active antiepileptic drugs, tiagabine and vigabatrin have a mechanism of action involving GABA metabolism and high affinity transport (91). With regard to vigabatrin, which acts as a catalytic site-directed suicide inhibitor of GABA-transaminase (GABA-T), the enzyme catalyzing GABA degradation (91), experiments involving primary cultures of neurons and astrocytes were used to gain information about the relationship between drug action and its ability to increase the availability of synaptic GABA. Thus, it was shown that exposure of GABAergic neurons in culture to vigabatrin facilitated synaptic release of GABA (196). Additionally, cultured neurons and astrocytes documented that vigabatrin had a more potent inhibitory action on GABA-T in neurons as compared to that in astrocytes (197), which could at least be partly explained by the lack of a high affinity transport system for vigabatrin in astrocytes and its presence in neurons (198). It is highly unlikely that this information could have been obtained without the use of cultured neural cells as a model system.

Based on numerous studies employing cultured neurons and astrocytes and pharmacological agents mimicking the structure of the GABA molecule it was demonstrated that high affinity GABA uptake into GABAergic neurons and astrocytes, respectively, exhibits differences with regard to potency of inhibitors (199, 200). Using this knowledge it was demonstrated

that for GABA transport inhibitors, a correlation exists between anticonvulsant efficiency and inhibitory potency on astrocytic GABA transport. Surprisingly, such a correlation could not be established regarding inhibition of neuronal GABA transport (115). This observation underscored the previous suggestion that selective inhibition of astrocytic GABA transport would likely be related to seizure protection (201), a hypothesis based on an analysis of neuronal and astroglial GABA transport performed using primary cultures. It should be emphasized that the basic experimental work leading to the development of tiagabine as a clinically active antiepileptic drug involved the use of primary cultures of neurons and astrocytes in addition to animal models (202, 203).

Seizure activity additionally involves aberrations in ion homeostasis due to increased excitation and depolarization of both neurons and astrocytes (204, 205). Such aberrations in ion homeostasis, including changes in intracellular Ca^{2+} concentration, have been studied in cell cultures of human epileptic tissue and abnormalities in both neurons and astrocytes, as compared to cells from non-epileptic tissue, were reported (206, 207). It thus appears that the use of these types of primary cultures will facilitate studies aimed at better elucidating mechanisms involved in epileptogenesis.

Alzheimer's disease

Alzheimer's disease (AD), the most prevalent neurodegenerative disease, is histopathologically characterized by extracellular deposits of β -amyloid ($\text{A}\beta$) peptides resulting in the formation of neuritic (senile) plaques. $\text{A}\beta$ is believed to be neurotoxic and such accumulation has been strongly implicated in the neurodegeneration associated with AD. It is generally believed that $\text{A}\beta$ accumulates in the extracellular space and triggers reactive astrogliosis. While the presence of reactive astrogliosis is often viewed as simply a "reactive" event, such reactive astrocytes likely play an important pathogenic role in AD. Cultured astrocytes have been shown to phagocytize and degrade $\text{A}\beta$ (208, 209). While such uptake of $\text{A}\beta$ may have a protective effect, Abramov et al. (210) established that the accumulation of $\text{A}\beta$ resulted in oxidative stress by the activation of NADPH oxidase. Such activation of astrocytic NADPH oxidase and associated oxidative stress may have been due to $\text{A}\beta$ -induced Ca^{2+} transients (211). Similar Ca^{2+} transients were not observed in cultured neurons exposed to $\text{A}\beta$ (212). It is likely that such changes in calcium homeostasis may have impaired astroglial functions which may have impacted on neuronal integrity.

An additional component of AD is the intracellular accumulation of hyperphosphorylated tau filaments (neurofibrillary tangles). Garwood et al. (213) showed that astrocytes are essential for the $\text{A}\beta$ -induced tau phosphorylation observed in primary neurons. A soluble inflammatory factor released from $\text{A}\beta$ -treated astrocytes appears to be involved in the tau phosphorylation as these changes were inhibited by minocycline, an anti-inflammatory agent. Some of the factors released by $\text{A}\beta$ -treated astrocytes include cytokines that have been implicated in the pathogenesis of AD (214, 215).

Parkinson's disease

Parkinson's disease (PD) results from a degeneration of dopaminergic neurons of the midbrain (substantia nigra and noradrenergic neurons in the locus coeruleus in the pontine

tegmentum). Mitochondrial dysfunction, oxidative stress and inflammation have long been implicated in its pathogenesis (216). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin which causes PD-like symptoms and is arguably the most commonly used agent to investigate PD pathogenesis. Astrocytes take up MPTP and convert it to MPP⁺ through the action of monoamine oxidase B (MAO-B) (217, 218). Such discoveries made by using cultured astrocytes have ultimately led to the development of MAO-B inhibitors (selegiline, rasagiline) for the treatment of PD (219). The released MPP⁺ is taken up by dopaminergic neurons via the dopamine transporter. In neurons MPP⁺ inhibits complex I of the mitochondrial electron transport chain resulting in the formation of free radicals and cell death. Using primary astrocyte cultures a defect in glutamate uptake was observed, which could contribute to excitotoxic injury (220). More recently the potential involvement of the Wnt1/Fzd-1 β -catenin pathway in MPTP toxicity was proposed which could lead to novel therapeutic therapies for PD (221). For an excellent review of the use of MPTP in teasing out mechanisms of MPTP toxicity, see (222).

Huntington's disease

Huntington's disease (HD) is a hereditary neurodegenerative disease resulting in choreaform movements and dementia by affecting medium spiny neurons in the striatum (caudate/putamen). The disorder is due to an expansion of polyglutamine repeats on chromosome 4. HD is also associated with an inhibition of complex II of respiratory chain resulting in an increase in free radical production (223). The complex II inhibitor, 3-nitropropionic acid (3NP), reproduces many of the features of HD in rats (224). 3NP was shown to induce an increase in intracellular Ca²⁺ in cultured astrocytes which was due to an influx of Ca²⁺ by the reverse operation of the Na⁺-Ca²⁺ exchanger, possibly explaining the gliotoxic action of 3NP (225). HD has also been examined in cultured astrocytes infected with an adenovirus carrying the codon gene of N-terminal 552 glutamine repeats (226). In these cells the GLT-1 glutamate transporter and glutamate uptake were found to be significantly reduced. This comports with the well-known excitotoxicity associated with HD (227). These studies also showed that rapamycin, an autophagy stimulator, prevented the suppression of GLT-1 expression and glutamate uptake by mutant Htt-552 in cultured astrocytes.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disorder affecting upper and lower motor neurons. Defects in glutamate transport and associated excitotoxicity have been strongly implicated in its pathogenesis. Considerable evidence exists indicating that a loss or dysfunction of astrocytic glutamate transporters represents a major component of ALS pathogenesis (228-231).

Approximately 20% of cases with familial ALS and 5% of cases of sporadic ALS exhibit mutations of the superoxide dismutase (SOD) gene (232). Expression of mutant SOD in astrocytes leads to a toxic "gain of function" resulting in the production of molecules that are toxic to motor neurons (233, 234); the nature of these toxins is unclear.

Using mitochondria derived from cultured astrocytes from SOD1(G93A) rats, investigators found defective respiratory function, including decreased oxygen consumption, lack of ADP-dependent respiratory control, and decreased membrane potential. These mitochondrial defects were associated with nitrative-oxidative damage. The authors concluded that mitochondrial dysfunction in astrocytes critically influences motor neuron survival. The finding that β -lactam antibiotics can stimulate expression of glutamate transporters in astrocytes, and thereby enhance glutamate uptake providing neuroprotection in animal models of ALS and stroke (235), provides hope for a treatment for this tragic condition.

Miscellaneous conditions

Space limitations preclude an exhaustive treatment of all neurological conditions in which astrocytes play a key role. In view of the critical involvement of these cells in the workings of the nervous system it would be difficult to identify any neurological conditions in which astrocytes do not play an important role. We here provide citations of articles that the interested reader may find helpful. These include articles on AIDS (astrocytes are believed to represent the major neuroepithelial “reservoir” for HIV-1 in the CNS) (236-240); metal toxicity (241, 242); Wernicke's encephalopathy (vitamin B1 deficiency) (243); experimental autoimmune encephalomyelitis (EAE, a model of multiple sclerosis) (244); neuromyelitis optica (245), and Alexander's disease (246, 247).

CONCLUSIONS

This review has provided information about the major progress achieved regarding normal functions of astrocytes by the use of primary astrocyte cultures derived from rodents. These include the key roles that astrocytes play in the glutamate-glutamine cycle, neurotransmitter uptake, detoxification of noxious agents, and the crucial role of calcium in astrocyte activation and stimulation of gliotransmitter synthesis and release – the latter representing the principal means for regulation of neuronal activity and cerebral blood flow. Not discussed in this review are several other well-known functions gleaned from the use of primary cultured astrocytes such as the generation of trophic factors, free radical scavenging, brain water and ion regulation and the provision of nutrients and metabolites for neurons. Additionally, these cultures have been invaluable in deciphering mechanisms of neurologic disease, including stroke, trauma, brain edema, hepatic encephalopathy, epilepsy, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, among many others. In many instances, findings identified in culture were subsequently found to occur *in vivo*.

Despite these remarkable achievements, some investigators have questioned the validity of this culture system. Using “freshly isolated” astrocytes, some differences were described when compared to primary cultured astrocytes. Yet, the freshly isolated astrocytes were subsequently “cultured” (i.e. “traumatized” during removal from the skull, treated with enzymes to disperse them). Accordingly, it is impossible to assign which system is superior or more accurate. Moreover, all cell culture systems have inherent problems in that the cells are out of their normal environment and no culture system can fully replicate the *in vivo* condition. All investigators are fully aware of that confound.

Regardless, whatever critical novel data is generated *in vitro*, these findings will ultimately have to be verified *in vivo* before such findings can gain general acceptance. The clear advantage of the culture system is that mechanistic data can be generated that would be impossible to achieve *in vivo*. More importantly, the *in vitro* approach often provides the crucial questions to ask from the *in vivo* system. We submit, no “paradigm shift” is needed.

In summary, many vital advances have been made with the use of primary astrocyte cultures, and indeed, much of our knowledge about the roles of astrocytes in health and disease could not have been attained without the use of a simple model, the primary cultured astrocyte.

It gives the authors enormous pleasure to submit this article in honor of Leif Hertz. Leif has been an inspiration and role model to all of the authors. His pioneering studies with cultured astrocytes literally opened up an entire new field of neuroscience, as much of our current understanding of the functions of astrocytes has been derived from his work. We also would like to acknowledge Leif, as well as his charming wife Elna, for their refinement of the astrocyte culture technique.

Acknowledgments

This work was supported by a Merit Review from the Department of Veterans Affairs and by National Institutes of Health grants DK063311. The authors express their appreciation for the helpful assistance of Drs. K.V. Rama Rao and A.R. Jayakumar.

SCL was partly supported by The Danish Medical Research Council grant 09-066319.

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