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Review

Neuronal–glial interactions and behaviour

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Abstract

Both neurons *and* glia interact dynamically to enable information processing and behaviour. They have had increasingly intimate, numerous and differentiated associations during brain evolution. Radial glia form a scaffold for neuronal developmental migration and astrocytes enable later synapse elimination. Functionally syncytial glial cells are depolarised by elevated potassium to generate slow potential shifts that are quantitatively related to arousal, levels of motivation and accompany learning. Potassium stimulates astrocytic glycogenolysis and neuronal oxidative metabolism, the former of which is necessary for passive avoidance learning in chicks. Neurons oxidatively metabolise lactate/pyruvate derived from astrocytic glycolysis as their major energy source, stimulated by elevated glutamate. In astrocytes, noradrenaline activates both glycogenolysis and oxidative metabolism. Neuronal glutamate depends crucially on the supply of astrocytically derived glutamine. Released glutamate depolarises astrocytes and their handling of potassium and induces waves of elevated intracellular calcium. Serotonin causes astrocytic hyperpolarisation. Astrocytes alter their physical relationships with neurons to regulate neuronal communication in the hypothalamus during lactation, parturition and dehydration and in response to steroid hormones. There is also structural plasticity of astrocytes during learning in cortex and cerebellum.

Keywords: Central nervous system; Glia; Behaviour

Contents

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1. Introduction

1.1. Historical perspective

It was evident by the beginning of this century, that peripheral and central nervous system (CNS) tissue contained cells that were electrically excitable. These neurons, were believed to form the sole basis for integration of information within the brain by communicating at synapses [429], giving rise to the 'neuron doctrine', supported by histological studies [366,367]. This doctrine stressed the individuality of neurons, their excitability and the belief that they were the functional units of the brain, the integrative powers of which lead to behaviour and ultimately consciousness.

During the first half of this century, research on the mechanisms that underlie the excitability of nerve cells continued apace, reaching a peak in the explicit model of for the generation of the action potential [202]. This reinforced the 'neuron doctrine' in that it was now redefined to imply that information was only carried by action potentials in individual neurons, transmitted and integrated with that from other neurons by synapses. Although graded potentials were described [114] they received little credence as an important conduction mechanism, even over short distances.

The nervous system, however, is also comprised of neuroglial or 'glue' cells that have only recently attracted much attention. To many, this lack of interest in cells that in primates may comprise some 50% of the mass of the brain

may seem surprising, especially as they were first identified in 1856 [506]. Clearer descriptions however, had to wait until the beginning of this century with improved techniques in neuroanatomy (see Ref. [442] for a review). Glia were described primarily by their lack of an axon and many speculated on their possible functions. Among these was a nutritive function for neurons and as neuron insulators. Both were proved correct, but in the same era another discovery was reported that seemed at the time to be totally unrelated. This was the description of a sustained cortical 'current voltage' [50] which we now describe as a slow potential shift, probably reflecting ionic currents through glia and extracellular space (ECS).

It was not until the middle of this century that interest was reawakened in both the cell biology and the physiology of glia. The advances, especially in the last 10–20 years have been spectacular, but more recently, the previously heretical suggestion that glia play a role in nervous activity and therefore behaviour, has begun to gain respectability [20,28,135,190,256,257,382,383,436]. It is the aim of this review to describe the current knowledge of how neurons and glia interact to influence behaviour.

1.2. Types of macroglial cell

There are three main functional classes of macroglial cell in the advanced vertebrate nervous system, the ependymoglia, astrocytes and myelinating glia. Their number and degree of differentiation increase both with phylogeny and during development.

Ependymoglia include radial glia that span the neural tube during development, tanycytes that form the ventricular cerebrospinal fluid (CSF) interface, ependymal cells of the pia limitans and radial (Müller) cells of the retina. Astrocytes have diverse morphologies and probably functional capabilities. They may be regionally radially oriented [70], though as their name suggests, they have an abundance of cellular processes. Myelin forming cells include oligodendroglia in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). They have the characteristic of ensheathing axonal processes, the sheaths being interrupted by nodes of Ranvier. All these types of glial cells have long been considered to be the passive supportive cells for neurons. Until recently the only accepted useful function attributed to glia was the insulative function of myelin that reduces current loss at the inter-node and also increases the speed of conduction. The original view was that this was a passive phenomenon, like the plastic ensheathment of an electrical cable.

Electron microscope studies of the brain tissue have revealed much of the morphological relationships between glial cells and other structures and cells in the CNS. Crucially, both ependymal glia and astrocytes have processes which with those of other glial cells, often form, via gap junctions, a continuum or functional syncitium for transport of ions and small organic molecules [164,283]. Other processes invest capillaries (peri-capillary), synapses (peri-synaptic), nodes of Ranvier (peri-nodal), or abut the CSF interfaces of the ventricles and sub-arachnoid space.

Thus, the most active regions of neurons (synapses and nodes) are largely surrounded by astrocytic or ependymal processes, whilst the relatively inactive inter-nodal regions are enveloped in sheaths of oligodendroglial or Schwann cell membranes. Furthermore, the other glial processes envelop the non-neural interfaces of the brain, the blood vessels and the CSF reservoirs.

Much research on individual properties of glia has been performed on cultured cells so its applicability to the in vivo brain remains to be validated. Work on intact brains and slices of brain tissue, however, has revealed neural–glial interactions with correlations with, or direct involvement in, the expression if behaviour by the organism. In this review, the relevant evidence is partitioned into five main areas:

1. The phylogeny and ontogeny of glial–neuronal interactions.
2. Ionic signalling between neurons and glia.
3. Energetic interactions between neurons and glia.
4. Neurotransmitter interactions between neurons and glia.
5. Morphological plasticity of neuron–glia interactions.

These categories are not exclusive, but they form a framework for discussion of mechanisms by which the cells of the brain *together* lead to the evocation of behaviour.

2. The phylogeny and ontogeny of glial–neuronal interactions

Glial cells and neurons have had intimate associations since the evolutionary dawn of the CNS. Early centralisation and cephalisation of the nervous system was associated with the formation of neural circuits for the regulation of simple behavioural sequences, vital for survival. Early glial cells may have had the function of partial separation of these circuits to prevent 'cross talk'.

As brains evolved, enabling more complex behavioural activities and thus environmental exploitation, constraints on body and brain size were encountered, especially in the invertebrates with an exoskeleton. Limits on brain size in the invertebrates did not restrict the evolution of complex behaviours, though these are co-ordinated by relatively few neurons in a sophisticated circuitry, seeming to lack flexibility. Glial cells become progressively more involved in the compartmentalisation of this circuitry and maintaining a constant environment for it to operate reliably and effectively. Thus, complex *adaptive* behaviour evolved in small brains.

In the vertebrates and cephalopod molluscs, brain size was a minor constraint and so both neuronal and glial numbers could increase to allow alternative pathways for

information processing, integration and response execution. In vertebrates, myelination compartmentalised neurons in tracts and improved their performance to optimise brain mass in relation to function.

Astrocytes become more numerous and increasingly differentiated with vertebrate phylogeny, as does the intimacy of their relationship with neurons. They maintain trophic functions, though these may be modulatory rather than purely homeostatic. The peri-synaptic morphological dynamism of astrocytic processes may facilitate synaptic connectional plasticity, already enhanced by the profligacy of possible connections enabled in brains evolving without constraints on size. The flexibility of these large systems of neurons and glia has endowed the vertebrate brain with the ability to modify patterns of behaviour to be truly *adaptable* in a variety of environmental circumstances.

2.1. Invertebrate neuron–glia organisation and behaviour

There is a tremendous diversity in the nervous system of invertebrates. Relatively few species have been studied and from those that have, it has emerged that differences exist not only between phyla but also between orders and even families. The major evolutionary trends, only, are described here. In the discussion below we have followed the traditional views of phylogeny. However, it should be noted that recently, studies on the evolution of the 18S ribosomal DNA and of the Hox gene cluster have led to a new hypothesis postulating three lineages for coelomates rather than two [16]. According to this hypothesis the platyhelminths are likely to be derived secondarily simplified forms rather than a group representing an evolutionary step of increasing complexity. This alternate hypothesis could explain the great variation shown by members of this phylum. The observations with respect to the relationships between complexity of organisation and behavioural capabilities are not affected.

In Coelenterates, nerve nets or rings of bipolar and multipolar cells, the latter without differentiation of axons and dendrites characterise the hydrozoa (hydroids), the anthozoa (sea anemones) and scyphozoa (jelly fish). In medusae, local concentrations of nerve cells and fibres are associated with the nerve rings and marginal ganglia. Conduction through the multi-directional neuronal network is mainly slow and decremental. There is little evidence for glia among the coelenterates [39,84,158,159,266,433].

The behavioural repertoire is small, comprising slow locomotion and fast contraction reflexes in anthozoa (sea anemones), startle reactions in hydrozoa (siphonophores) and rhythmic swimming movements in the scyphozoa (jelly fish). Coelenterates show slow adaptive behaviour with a nervous system, that utilises through conduction rather than conventional synapses and is unaided by glia.

In the Platyhelminthes, the nerve net has condensed to form 2–5 pairs of nerve cords, connected by commissures in a ladder-like fashion. An anterior concentration of neurons, a "brain" is present in most species. Neurons are differentiated, with many bipolar and multipolar cells with definable axons and dendrites, as well as unipolar cells. In the more developed nervous systems of the polyclad flatworms, the brain is comparatively large and distinct and in these an important invertebrate feature is established, i.e. an outer crust of cells surrounding fibres in the large cords and ganglia. A few poorly differentiated spindle-shaped glial cells are found along nerve tracts, between nerve cells and in the peripheral plexuses. These may have an insulating function. In some species multiple lamellae are found around neurons or their axons and invaginations of glia into neuronal somata have been reported [151,297,374,380,381,470].

The phylum shows a range of nervous system development, though in many species there is little advance on the coelenterate nerve net, with only relatively few longitudinal fibres and little cephalisation. Behavioural activity is faster than in Coelenterates and is directional, with the head incorporating the majority of the sense organs and associated "brain" to the fore. The possession of a more sophisticated sensory apparatus allows these animals to show orientated taxes and kineses in response to light and gravity. They can be conditioned to environmental stimuli. The development of more rapid and coordinated responses in flatworms may have required some separation of neural circuits by glial cells.

In the Annelida, the CNS consists of paired ventral nerve cords that usually have segmental ganglia. The "brain" comprises the cerebral ganglion, innervating the head region with the sub-oesophageal ganglion, the largest and most anterior segmental ganglion. These ganglia are well developed with the typical invertebrate structure of a crust of cell bodies of neurons and glia surrounding an inner neuropil consisting of axons, dendrites, nerve tracts, glial processes and some glial cell bodies. All synapses are found in the neuropil. Glial cells are well developed and in the oligochaetes and polychaetes three main types may be distinguished; supportive, migratory and those forming myelin-like sheaths around giant fibres in the nerve cords. The invagination of neurons by glia (trophospongium) is well developed and may represent a trophic function of glia for neurons. Physiological studies on leech packet glial cells and their neurons established the role of glia in regulating the microenvironment of neurons especially with respect to potassium [249,319,320,414,532], which clearly has significance for behaviour, as described later (Section 2). Giant glial cells in the leech are associated with the ganglionic neuronal somata where they are involved in pH regulation that may involve glial–neuronal signalling [92]. Glia comprise 51% by volume of the leech central nervous system [218].

A new feature of nervous systems is established, the compartmentation of the CNS by glial cell processes forming partitions between the neuropil and inner fibre regions and between small masses of cells and processes. The significance of this glial compartmentation of neuropil

may be to allow discrete neuronal circuitry to produce defined patterns of behaviour.

Aquatic species of annelid, especially the polychaetes and leeches, are proficient swimmers and those with advanced mouthparts are adept at food capture and manipulation. The errant polychaetes have the largest and most partitioned brains of all the annelids and these animals are often active predators. Like platyhelminthes, annelids can be conditioned, but also show more active and complex behaviour patterns. In part this may be because of greater compartmentation of more complex neural circuitry by greater numbers of glia.

The Arthropoda (primarily crustacea and insecta) are the largest and most diverse phylum of the metazoa. They have developed a much more pronounced cephalisation and more highly developed sense organs than the annelids. The highest centres are believed to be the globuli, masses composed of small tightly packed unipolar globuli cells. Globuli are particularly concentrated in the optic neuropil and mushroom bodies of insects. Glial cells are differentiated into at least three types in the various arthropod groups and the trophospongium is well developed [46,125,169,289,330,363,408,439]. In the retina of the male bee glia constitute 57% by volume [69], where they act as metabolic substrate providers for neurons.

Separation of neuronal elements by glia is well developed. Some glial cells have thin diaphanous sheet-like processes similar to velate astrocytes, e.g. in *Limulus* brain and the optic lamina of lobster where the processes separate neurons. In the neuropil, groups of “en passant” synapses are separated as are other neuronal processes to prevent synaptic contact. Tight junctions partition the neuropil into high resistance sectors. Globuli are compartmentalised by glial processes separating groups of cells. The separation of discrete elements of neuropil, initiated in the platyhelminths, enables development of complex adaptive behaviours although these are stereotyped.

Another feature of nervous system evolution, namely stratification, is first seen in arthropods. It is especially apparent in the optic lobes of insects and crustaceans. Glial sheaths isolate the columnar synaptic region of optic cartridges of the retina above, from the other parts of the optic lobe below. Units of the compound eyes are also separated by glia. Isolation of these various components is needed for colour vision and navigation [168,407]. The evolution of an exoskeletal structure and jointed limbs paralleled the increased differentiation of the nervous system and was also associated with the development of complex patterns of behaviour. Many of these appear to be stereotyped and require relatively few neurons, necessitated by the limits on the body size imposed by the exoskeleton. Apart from habituation, classical conditioning and associative conditioning are normal components of the life of the highly evolved social insects, which learn the location of a food source and the position of the colony, and can rapidly relearn these if they are experimentally relocated. It is the complex sequences of adaptive behaviour of the social insects that gives the impression that the phylum as a whole is extremely advanced, yet the majority of arthropods do not display such complex behavioural activities.

In the Cephalopod molluscs cephalisation is advanced without skeletal constraints on size. The brain consists of a compact mass ringing the oesophagus. It has thirty distinct lobes, the most prominent of which are the optic lobes comprising half the brain tissue. Glial cells are numerous and differentiated into many types, some resembling vertebrate protoplasmic and fibrous astrocytes. There is a trophospongium but neither glomeruli of the type found in insects nor multiple layered wrappings of axons are present. Nerve cells are not confined to the outer crust but are found in clusters throughout the brain. Cephalopods have the largest brain, both in absolute terms and in relation to body size, of any invertebrate, rivalling that of the vertebrates.

2.1.1. Evolutionary trends in the invertebrates

Several trends are evident in the evolution of invertebrate nervous systems, notably condensation and cephalisation, compartmentation and stratification. Glia which differentiate into several types contribute to the latter two and probably to the well developed *adaptive* behaviours of the higher invertebrates possessing small brains. Complex behaviour, involving flexibility of response (i.e. *adaptable*) requires many separate channels that have been limited by available space in the nervous system. The eye and nervous system of cephalopods and their flexibility in terms of *adaptability* of behaviour, represent a remarkable example of convergent evolution with vertebrates.

2.2. Vertebrate neuron–glial organisation and behaviour

The vertebrates have evolved large brains with multiple and plastic cellular connectivities.

Macroglial cells outnumber neurons and account for 50% of brain volume, a figure similar to that for advanced invertebrates. There is, however, local variation and in the cerebellum, for example, there are five to six neurons per glial cell [141]. Radial glia predominate in brains with relatively thin walls, e.g. teleosts, whereas non-ependymal glia are more prevalent in thick walled brains [523]. The glia: neuron ratio increases up the phylogenetic scale of vertebrates. It also increases with axon length, brain weight and brain wall thickness [370]. These differences are related to the trophic needs of neurons, to compartmentation and to the degree of myelination. Differentiation of glia is well developed and is greatest in higher vertebrates. Multipolar neurons predominate and numerous synaptic contacts are found on the soma as well as on dendritic processes. Stratification separates grey and white matter and layers within grey matter. Glomeruli are prevalent and other forms of compartmentation are being increasingly identified. Myelin sheaths are well developed in all but the

Agnatha, with a remarkably similar morphology across the groups [390].

In modern Teleosts, ependymal-type glia predominate, though oligodendrocytes remarkably similar in structure to those in mammals occur [248]. The optic tectum, which is the main integrative centre, is highly stratified and astroglial envelopes are associated with synaptic structures, especially round dendritic spines. Differentiation of glia is not complete and there are many cells intermediate between astrocytes and oligodendrocytes [261]. Apart from the senses of olfaction, vision, audition, taste, smell, and mechanoreception, common to all vertebrates, many fish species are electro-receptive. Teleosts are capable of being classically, instrumentally and associatively conditioned [131] and exhibit a primitive form of sleep [346].

In Amphibians, there is a lesser degree of brain and behavioural complexity in comparison with modern teleosts. The brain size is similar in relation to the size of the animals, but the cerebellum is much reduced. Visual and acoustic senses are well developed and understood, but other senses may be less well developed than in fishes. As in teleosts, the optic tectum is the main integrative centre and in anurans (frogs and toads) has a similar multilayered structure [354,355]. Visual information processing pathways in the tectum have a radial organisation, paralleling that of the radial glia. In urodeles (salamanders and newts) the tectum is relatively simple and lacks multiple lamination [189]. Glia are somewhat more differentiated and there is a greater proportion of astrocytes than in teleosts. In anurans these cells combine the features of ependymal cells and astrocytes [312]. As in teleosts, sheet-like profiles envelop specific types of synapses [246]. An evolutionary advance is, however, shown by oligodendrocytes, which are fully differentiated from Schwann cells. Although many amphibian species can be classically conditioned, and they show spatial learning of territories, much of the behaviour is stereotyped in comparison to fish. Many species are inactive for long time periods, when they are unresponsive and appear to be ‘asleep’.

In aves, which may be regarded as homeothermic, feathered, flying reptiles in many respects, a relatively enormous brain has evolved. Unlike the mammals which have exploited the emergence of the advanced reptile’s isocortex, birds have massively enlarged striatum or basal ganglia. In flying species, weight constraints may have restricted brain size. Differentiation of glia is as complete as it is for mammals in contrast to the greater prevalence of intermediate forms in reptiles [132,391]. In birds, the optic tectum continues to be an important integrative centre and shows greater laminar differentiation with fifteen primary tectal layers. Birds have a large repertoire of complex behaviours, some of which are modified by parental example. These include species recognition, song learning and extensive migrations. In addition to classical, instrumental and associative learning, some species exhibit tool usage and in all territorial and migrating species there is an outstanding ability for spatial learning.

Mammals, compared to birds, have little restriction on brain size, yet instead of developing a simple mass of new brain tissue, increase in size has been achieved by expansion of the sheet of isocortex (neocortex). The development of this sheet causes a correspondingly massive increase in the number of cells and the area of the glia overlying it at the brain pial surface. Associated with this enormous increase in size is greater anatomical complexity and more elaborate architectonics.

During the course of evolution of the placental mammals, additional division into sublayers has occurred [325]. The layers themselves show an elaborate columnar organisation. During development, glia provides the scaffolding that underlies the architecture of radially arranged columns intersected by horizontal layers of neurons. These radial columns are particularly prominent in primates. Physiological and metabolic studies indicate that functional modules are associated with these columns in the adult [149].

Compartmentation of the kind seen in other animals is abundant. Glomeruli have been described in species across the orders of mammals [161,165,345,450]. The more advanced mammals exhibit, in addition to the behavioural complexity shown by birds, complex social structures and communication mechanisms, play, and in ourselves, language.

2.2.1. Evolutionary trends in the vertebrates

The vertebrate brain was a new design, not constrained significantly in size because of the design of the skeletal system of the animal. Even in fish and amphibia, it is comparatively large and incorporates the multi-channel inputs and outputs required for flexibility of response. With the development of the isocortex and glia specialised for insulation, metabolic regulation, ionic conductance and involvement in synaptic efficacy, came the ability to make behaviour *adaptable* to the needs of the individual in the pertaining environmental circumstances.

2.3. Neuron–glial interactions in vertebrate brain development

The establishment of neuronal connections during development is governed by genetic instruction and activity-dependent changes of axonal projections. The latter mechanisms are increasingly important in the highly *adaptable* vertebrates, especially mammals.

Brain development initially involves generation of neurons and glial cells from the ventricular zone of the neural tube. This is followed by outgrowth of dendrites and axons from immature neurons and the differentiation of neuronal types and synaptic connections. The last step of development involves modification of these genetically defined connectivities as a consequence of their activity.

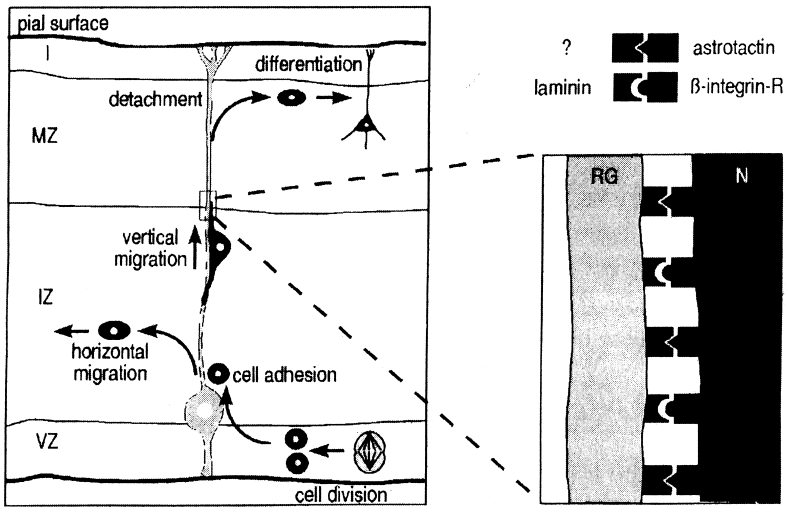

Fig. 1. Guidance of neuronal migration in neocortex by radial glial cells. Neuronal precursor cells divide in the ventricular zone and the postmitotic cells adhere to radial glial processes. Migration mainly occurs via an interaction of the neuronal surface molecule astrotactin with an unknown partner on radial glial processes. An interaction of laminin and the β-integrin receptor may also participate in radial migration. Horizontal migration is observed in about 10% of neurons and does not involve radial glial cells. Migrating neurons detach after reaching the superficial layers of the cortex and differentiate in an 'inside-out' fashion (after Ref. [300]).

This allows for *adaptability* of individuals to diverse environmental conditions.

Neuronal survival, differentiation, and maturation are considerably supported by cocultured glial cells, especially by astrocytes [18]. Brain development and function may similarily result from interactions between neurons and glial cells.

Among the first cells generated in the developing brain are radial glial cells. These cells express several characteristics of astrocytes, e.g. the expression of the cell markers vimentin, glial fibrillary acidic protein (GFAP), and S-100 protein [118]. In mammals, radial glia can later form stellate astrocytes [507]. The soma of radial glial cells first resides in the ventricular zone, while an apical process spans the entire thickness of the neural tube and contacts the pial surface to serve as a substrate for neuronal migration [364,365]. These observations have led to the hypothesis that the arrangement of radial glial fibres may be a scaffold for the vertical organization of laminated structures, like cerebellum, tectum, and cortex. Recent studies with retroviral infection of neuronal cell clones have extended this hypothesis [245,373]. This technique has shown that clonally related neural cells originate from successive cell divisions that generate one postmitotic cell per division. These then migrate along a common radial glial process and thus form a cortical column, the cells within which may have common recognition molecules to enable establishment of vertical connections (Fig. 1).

Coculturing embryonic neurons and radial glia leads to rapid adhesion of neurons to glial fibres and subsequent migration [176]. Antibodies raised against astrotactin, a surface molecule expressed by migrating neurons, reduce the interaction of neurons and radial glia suggesting that astrotactin interacts with an unidentified, partner on radial glial fibres. Distinct polypeptides present during the time of neuronal migration may serve as recognition molecules [45]. Laminin, expressed on radial glia and interacting with the neuronal β-integrin receptor also appears to participate in neuronal migration [130]. The importance of this recognition between neurons and radial glia becomes clear in mouse mutants. In weaver-mutants cerebellar granule cells fail to adhere to radial glial cells and thus, to migrate to their appropriate layer. This failure results in an ectopic location of granule cells which subsequently die [177]. However, the cell death does not seem to be causally related to the failure of migration, as weaver granule cells also die when transferred to tissue culture under conditions where wildtype cells readily survive [524]. By combining radial glial fibres and embryonic neurons from wildtype animals and weaver mutants, in vitro, it has become clear that the defective migration resides in neurons and not radial glia. Thus, neurons from mutant mice fail to adhere and migrate on wildtype radial glial cells and wildtype neurons readily migrate along radial glia from mutant animals. The defect in lamination upon failure of neuron–glia interaction clearly demonstrates that glial guidance is essential for the construction of normal cortical layers. A similar effect on neuronal migration is observed in the reeler mutation. In wildtype animals, neocortical neurons generated in later stages of development normally have to cross the laminae formed by neurons generated at earlier time periods. In mutants this process is hindered and the cortical lamination is inverted. However, despite this severe developmental malfunction and the consequent absence of cortical layering, long range axonal connections, e.g. those from thalamic nuclei, are formed between appropriate cellular populations [51]. Axonal guidance therefore appears to be a developmental process mediated by mechanisms different to and independent of neuronal migration and targeting.

Migrating neural crest cells appear to follow pathways, characterised by the expression of matrix molecules. Tenascin-C is one such molecule expressed by astroglial cells and so it appears likely that glial cells also participate in this form of non-radial migration [126].

After migration of neurons, maturation occurs. This involves the differentiation of the neuronal phenotype and the establishment of both dendritic and axonal neuronal processes. While part of the phenotype is predetermined, morphological features are malleable to environmental, especially astroglial influences. In a series of in vitro studies the influence of the astroglial environment on developing neurons has been studied [53,95,403]. Neurons cultured on a layer of astrocytes obtained from their natural environment ('homotopic co-cultures') developed a different phenotype than if cultured on astrocytes originating from a different brain region ('heterotopic co-cultures'). Similar data could be obtained by culturing the neurons in the presence of medium that had been conditioned by the different astroglial populations, indicating that soluble factors released from astrocytes influence neuronal development. Studies on cerebellar granule cells have suggested that an astroglial-derived fibroblast growth factor is essential for neurite outgrowth [178].

Axon tracts often follow pathways formed by glial cells that surround an enlarged extracellular space (ECS). A mechanical explanation suggests that glial cells may delineate the ECS for axonal growth. This concept is supported by the failure of axon elongation in mouse mutations with a significant reduction of ECS [432]. Alternatively, axons may grow along matrix or surface molecules expressed along the glial trajectories. The presence of the neural cell adhesion molecule (NCAM), and other adhesion molecules like laminin, and *N*-cadherin at locations of preferential axon growth and the synthesis of such molecules by glial cells would support such a mechanism. An additional influence of glial cells on axonal tract formation may be via molecules which repel growing axons like those expressed by myelinating oligodendrocytes [47]. Inhibition of the repulsive effect of the myelin associated growth inhibitor by a functional antibody, in vivo, leads to aberrant sprouting of growing axons [220,420].

During the developmental period when retinal axons arrive at the optic chiasm in mammals, a palisade of radial glial cell processes is present at the midline of the brain which are contacted by the growing axons [276]. Some of the axons turn their growth direction towards the ipsilateral optic tectum, most probably due to inhibitory signals encountered by growth cones [526]. The recent discovery of an axonal guidance molecule expressed by radial glial cells in the optic tectum [107] may support the notion that glial cells express such guidance signals. However, as neuronal elements are also present close to the midline where 'decision making' by retinal ganglion cell growth cones takes place, these cells cannot be excluded from participating in axonal navigation [162].

When a strip of retinal tissue that contains both nasal and temporal parts of the retina is labelled with a lipophilic fluorescent dye it allows visualisation of outgrowing axons. It can then be transferred to a membrane containing alternating strips from anterior and posterior tectum [33]. Axons from temporal retina then grow exclusively on 'anterior' strips, the in vivo target of these projections, a preference that is due to repulsive molecule(s) present in 'posterior' membrane preparations. A similar preference for 'posterior' membranes by nasal axons has also been observed in this system [508]. A role for glial cells in such effects was suggested by experiments where retinal axons were confronted with astroglial cells derived from different tectal regions and the diencephalon [152,214]. While both nasal and temporal axons extended neurites on diencephalic astrocytes, contacted by both populations during retino-tectal outgrowth, axon outgrowth from temporal retina but not nasal explants was hindered by tectal astrocytes. These data parallel the findings obtained with crude membrane preparations from tectum and suggest that glial cells may be the source of guidance activity.

The structural plasticity associated with development of neural circuits may also have glial participation, not only for promotion and suppression of axonal growth but also for elimination of synaptic contacts. In most structures of the mammalian brain, developmental, adaptive structural plasticity is confined to the so-called critical period, characterised by the presence of immature glial cells [299,459]. Experimental prolongation of the critical period in the mammalian visual cortex is paralleled by retarded maturation of the astrocytic population [184,298,457]. Interestingly, structures that maintain the capacity for structural plasticity throughout life contain astrocytes with features of immature cells, e.g. in the olfactory bulb [106].

There is direct evidence for the participation of glial cells in structural plasticity. Transplantation of immature astrocytes to the visual cortex after termination of the critical period, is capable of re-inducing the capacity for activity-dependent plasticity [302]. The exact mechanism of this effect is not yet understood but appears to be related to activity-dependent elimination of synapses rather than the induction of axonal growth. This is indicated by the finding that transplantation of immature astrocytes mainly affects regressive plasticity, like the loss of synaptic function upon unilateral sensory deprivation, but does not induce a reversal of such effects from previous deprivation when vision is restored (Müller, unpublished observation). A causal contribution of astroglial cells to synapse elimination was first deduced from electron microscopic studies in the developing spinal cord. During the time period of refinement of axonal circuitry's, astrocytic processes were often identified in synaptic clefts and shown to subsequently phagocytoze presynaptic terminals [386]. Experimental studies have supported the necessity of astroglial cells for synapse elimination. In cerebellar cultures, synapses are initially formed in exuberance and are reduced in number during subsequent

development. Elimination of astrocytes by gliotoxic manipulations prevents the process of synapse elimination [287]. When astrocytes are reintroduced to cultures previously deprived of them, the process of synapse elimination can be reinstalled. To date, it is unclear how neuron–glial signalling may guide such synapse elimination. While synapse elimination occurs quite slowly in visual cortex upon full exclusion of sensory experience, rapid changes occur in sensory deprived afferents in the presence of competing, active afferents [5]. This indicates that the mechanism of synapse elimination can be facilitated significantly by neuronal activity. As the separation of synaptic contacts by astroglial processes is linked to morphological plasticity in astrocytes, it is of interest that the neuronal activity, and the activation of astroglial transmitter receptors indeed triggers such events [75,520].

Apart from a role in synapse elimination, astrocytes have also been implicated in trophic mechanisms influencing synaptogenesis during activity-dependent plasticity. Several neurotrophic factors are synthesised and released by astrocytes [254]. Studies on the developing cat visual cortex have shown that interference with the astroglial S-100-β protein, a calcium binding protein with neurite-inducing activity on neurons, affects activity-dependent alterations in neuronal specificity [301]. Interestingly, S-100 release from astrocytes can be influenced by the neuromodulator serotonin [522], which similarly influences plasticity in kitten visual cortex [160]. It is therefore conceivable that neuron–glial signalling may also regulate synapse formation or stabilisation by causing the release of astroglial-derived growth factors.

2.4. Summary of neuron–glial interactions during evolution and development

The most complex brains of higher vertebrates have evolved to provide the ability to exhibit the most appropriate behaviour, incorporating flexibility of response. This combination of precise circuitry and local synaptic plasticity has its cost, in that the capacity of the brain to regenerate in adult reptiles, birds and mammals fails in most central nervous structures. The origins of the precise circuitry probably lie in the genetically programmed neuron–glial interactions involved in neuronal proliferation and targeting during development. The plasticity of synaptic connectivity may derive from the activity-dependent neuron–glial interactions associated with the development of the mature individual, adapted to the prevailing environmental circumstances. As we shall see, much of the plasticity of neuron–glial interactions and consequent adaptability of behaviour prevails into adulthood, though it has deep evolutionary and developmental origins.

3. Ionic signalling between neurons and glia

Apart from synaptic interactions, a common conception of the neuron is of a device divorced from the extracellular milieu, which is assumed constant. However, it is the relationship between intra and extracellular concentrations of ion species that gives rise to the neuronal resting membrane potential and will affect the magnitude of current flow along the neuron whether this be electrotonic or a propagated spike. There are three extracellular ion species, in the regulation of which, ependymal and astrocytic glia have been most commonly implicated. These are potassium $[K^+]_e$, calcium $[Ca^{2+}]_e$ and protons $[pH]_e$. Their regulation by glia may serve as a signalling mechanism between neurons and glia and/or may represent a mechanism by which glia may modulate the activity of neurons. Glia may also regulate ionic exchanges with plasma via pericapillary processes and their participation in the blood–brain barrier (BBB) at the choroid plexuses. In addition, they form via the pia limitans and the ventricular ependyma a major interface with cerebrospinal fluid (CSF). These blood and CSF relationships are involved in regulation of the ionic and fluid constituents of the brain and therefore the volume, constituents and tortuosity of the ECS. There are thus a number of ways by which glia may regulate ionic fluxes and thereby neuronal activity and behaviour. This section will briefly review the evidence for these.

3.1. Potassium

There is now considerable evidence that extracellular potassium concentration ($[K^+]_e$) may fluctuate from its 'normal' level from ca. 3.5 to up to 9+ mM in conditions of high neuronal activity [477,478,514]. If the resting potential of a neuron was entirely dependent on K^+ distribution such a change would cause a decline of ca. 20% of that potential, perhaps close to levels required for voltage-gated conductance's to be initiated. Moderate elevation of $[K^+]_e$ seems to reduce thresholds for neuronal activity and may even induce spikes, though more severe $[K^+]_e$ elevations reduce neuronal excitability [443], the latter perhaps by inactivation of Na^+ channels. Elevated $[K^+]_e$ at the synapse has been suggested to reduce transmitter release by a form of presynaptic inhibition, though many experimental studies find an increased synaptic release of transmitter with elevation of $[K^+]_e$ [477,509]. The increasing number of different K^+ channels, now being described, might account for increased excitability being associated with moderate $[K^+]_e$ increases whilst larger ones induce suppression of neuronal activity.

Glial cell membranes, like those of neurons, are polarised with a 'resting potential' (ca. 90 mV) almost entirely due to potassium distribution. They too are thus depolarised by elevated $[K^+]_e$ but in the case of glia the depolarisation extends to some extent through the syncitium. This has given rise to the 'spatial buffer concept' by which glia redistribute neuronally derived $[K^+]_e$ away from its site of release by active neurons. Thus, a region experiencing an increased extracellular K^+ current will have a more positive

K^+ equilibrium potential than the membrane potential. This leads to an inward driving force for K^+ to which the membrane is highly permeable. The K^+ will then be distributed to other parts of the syncytium via gap junctions. In regions further removed from the high K^+ region, the K^+ equilibrium potential is more negative than the membrane potential and therefore there is a driving force for K^+ out of the cell. Thus the current flows into the cells at high K^+ regions and out of the cells at regions distant from high K^+ locations and is almost completely carried intracellularly by K^+. The loop is closed by a return current in the ECS carried mainly by Na^+ and Cl^- which form the bulk of the extracellular ions. The currents generated in extracellular space by spatial buffering contribute to extracellularly recorded slow potential shifts (SPSs) with known relationships with behaviour (see later). The spread of this current must be limited by the length constant of the glial syncitium. The length constant is the distance along a process to a site where voltage amplitude has decayed to 37% of its initial value due to leakage of current across the cell membrane. The low membrane resistance of astrocytes has been considered to provide a restricted length constant and thus a limitation on a glial contribution to slow extracellular potentials [441]. This problem is compounded by the inaccessibility of the glial syncytium in situ to measurements of the length constant and by the fact that the ionic channels of astrocytes in situ seem to be unequally distributed. The length constant of an acutely isolated astrocyte of the rat optic nerve for a single process has been measured as 100 μm [21,22], whilst measurements based on K^+ transport induced by an electric field estimated a length constant of around 200 μm for the rat cerebral cortex [139].

Müller glial cells of the retina stretch through all layers starting at the photoreceptors and ending with endfeet at the inner limiting membrane adjacent to the vitreous humor. The inner and outer plexiform layer are the areas of light-induced K^+ increases, this is where K^+ ions have to enter the Muller cells, generating a current sink. The endfoot K^+ channel density is tenfold greater than in the other regions [313]. Since the endfeet border at a large liquid reservoir any K^+ released will quickly diffuse, keeping $[K^+]_e$ close to normal. If, during light stimulation the plexiform layers accumulate high K^+ and the Muller cell parts in these regions are depolarised, there is a driving force for K^+ currents into the cell in these areas and toward the hyperpolarised endfeet where K^+ is released. Again the return current will be carried by Na^+ and Cl^- ions [314]. This pattern is associated with negative extracellular potentials in the plexiform layers and a large positive potential at the endfeet–vitreous humor interface. This is the electroretinogram b-wave [314]. Since this mechanism is based on anatomically fixed loops due to the increased endfeet K^+ channel density, it is also called K^+ siphoning [314]. If such siphoning and special endfeet specialisation also applies to astrocytes in the cerebral cortex and other brain structures it might contribute to large SPSs recorded at the cortical surface adjacent to the pia limitans.

Since elevations in $[K^+]_e$ are never restricted to just one layer in a cortical structure the problem of the restricted length constant of a glial cell syncytium is less severe than earlier discussed. For example, it is quite possible that a small cluster of glial cells may be depolarised at the point of maximal potassium accumulation and this depolarisation is spread over a given distance. Potassium released from active neurons will thus be redistributed in the less active surround. There, a new cluster of spatially extended glial and/or coupled glial cells could redistribute the potassium further into the surround. The different local current gradients will then add and contribute to generation of slow potentials over the whole structure.

Laminar profiles of changes in $[K^+]_e$ in relation to the generation of slow negative field potentials have shown that slow potential shifts (SPSs) are always largest at the site of maximal rises in $[K^+]_e$ while positive field potentials occur at sites where the rises in $[K^+]_e$ are smaller. Near the source of neuronal excitation the K^+ potential reflects glial depolarisation and the SPS in mammalian cortex [384] and in the frog brain [385]. Current source density analysis of slow potential changes has supported the conclusion that at sites of maximal rises in $[K^+]_e$ there is a large enough inward current into cellular compartments to limit rises in $[K^+]_e$ [101–103,269]. Moreover, it has been found that rises in $[K^+]_e$ are associated with a shrinkage of the extracellular space at sites of maximal K^+ accumulation while at remote sites the extracellular space can get larger. This is presumably an effect of the spatial buffering.

Three factors suggest that passive spatial buffering to remove local K^+ accumulations might not be the only function of K^+ redistribution by glia. First, glia are often radially organised in cortical structures, in a manner that parallels the neuronal information processing pathway. Thus, K^+ redistribution will probably be to functionally related areas. Second, the redistribution may be more rapid than the neuronally conducted information as the gap-junctional connectivity means that there is no synaptic delay (Fig. 2).

This may be why ‘pre-emptive’ SPS’s to visual stimuli may be recorded in deep layers of the toad tectum before the visual unit responses in that region [258]. The third reason why the relocation of K^+ is not likely to be to ‘functionally irrelevant’ regions is related to the morphology of astrocytic and radial glial processes. These processes envelop nodes of Ranvier (peri-nodal) or synapses (peri-synaptic) so that, within brain tissue, much of the redistributed K^+ would be to sensitive nodal or synaptic regions which would be partially depolarised as a consequence. Indeed, because of the intimacy of astrocyte processes at these regions, it is likely that our measures of $[K^+]_e$ rise achieved with invasive introduction of large glass micro-pipettes are gross underestimates. The even more highly conductive perivascular and pial end-feet may act to siphon K^+ between nervous and non-nervous tissue.

The above arguments obtain for passive electrotonic redistribution of K^+ However, the ionic conductance for K^+ is not entirely passive as elevated K^+ causes active uptake of K^+ by glia [516]. It would thus seem that glia, especially radial glia and astrocytes, can regulate $[K^+]_e$ within bounds that still allows sufficient flexibility for neuromodulation.

3.2. Slow potential shifts and behaviour

A wide variety of behaviours are associated with a SPSs lasting for periods of 100s of milliseconds to tens of seconds whether recorded intra-cranially or epi-cranially. In those cases of intracranial recording where it was possible to record $[K^+]_{e,}$ this was highly correlated with the SPS, strongly implicating glia in its generation. Although it has not yet been possible to determine the precise relationship between intra and epi-cranially recorded SPS responses it is likely that glial 'spatial buffering' currents also contribute to the latter.

3.3. Reactive SPS responses to novel sensory stimuli

'Reactive' SPS responses of mammalian brains to sensory stimuli [406] may have a general distribution in the cortex or may be localised. In unrestrained cats, SPS responses to acoustic stimuli have been recorded in the auditory cortex [163]. Using a muscle relaxant to immobilise the animal, surface negative SPS responses have similarly been found to visual, auditory and peripheral electroshock stimuli, localised in the primary sensory region for the stimulus modality used [267]. This SPS could extend, with reduced amplitude, to other cortical regions or could appear in other regions as positivity.

The occurrence of SPS responses in the mammalian cortex led many to assume that it is a strictly cortical phenomenon, yet SPSs in response to visual stimulation have been recorded from the hyperstriatum of pigeons, a region lacking a laminated cortical structure [112,113]. This led to the proposal that a radially organised neuronal structure was not necessary for the expression of SPSs. This concept might be supported by recordings of SPSs from apparently non-radially organised or laminated regions of the fish brain, such as the telencephalon and the medulla [321].

Evidence has accumulated to emphasise that the SPS recorded intracranially is a general expression of sensory activity in vertebrates as a whole. In response to direct electrical stimulation, or sensory stimuli, the negative SPS reflects the strength of the stimulus or the activity of local neurons, respectively. Thus, in anurans, the SPS amplitude is correlated with visual unit activity at the tectal surface, where it is presumably the retinal ganglion cell input that releases K^+ and thus generates the SPS [258]. In response to the onset of illumination the tectal surface SPS also closely reflects changes in $[K^+]_e$ [385]. In mammals also,

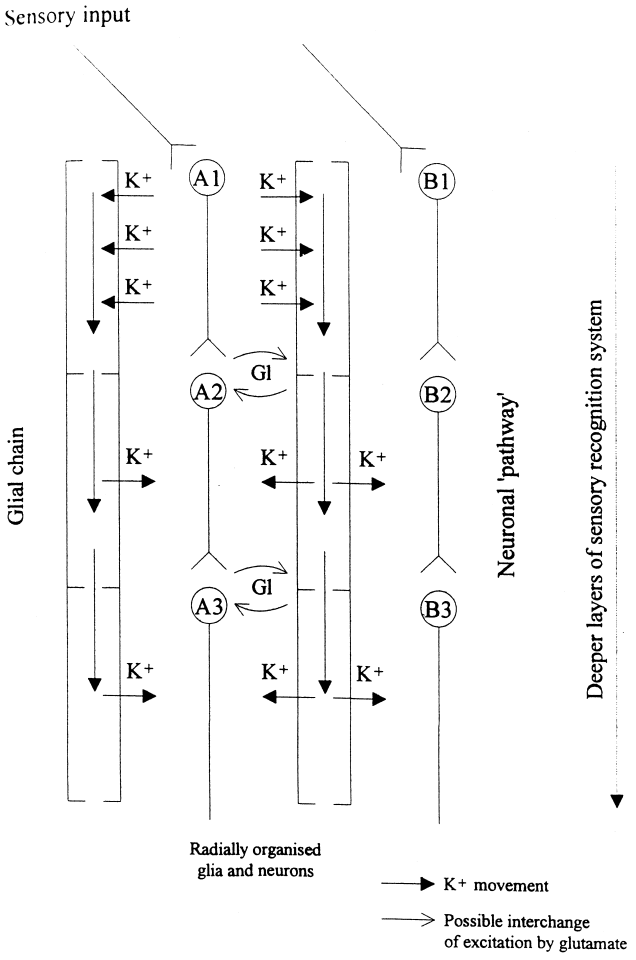

Fig. 2. A mechanism by which glia may sensitise neurons by relocation of potassium in a radially organised sensory system, like the toad tectum. On arrival of the sensory input, active neurons, e.g. A1, release K^+ which is translocated to the regions of A2 and A3 which are therefore partially depolarised prior to the specific neuronal activity being conducted through the A1/A2 and A2/A3 synapses. Spatial buffering would make this sensitisation mechanism passive, but could be more dramatic as glia have active uptake of K^+ and participate in glutamate metabolism. In sensory systems that are organised topographically to represent spatial sensory experience, like the visual system, neurons in areas close to the part of the sensory map being stimulated would also be sensitised (B1, B2, B3) and responses to moving objects would be enhanced (after Ref. [256]).

the reactive SPS to sensory stimulation is fairly closely correlated with the neuronal activity [404].

The reactive responses of animals to novel sensory input are often linked to the non-directed arousal response to novelty where the magnitude of the SPS appears to reflect the level of arousal or activation of the brain [402,404].

In fish, a bradycardia provides a quantifiable measure of the arousal response to a novel stimulus and the decline of that response with repetition of the stimulus [259]. Situations that evoke a bradycardia also induce SPSs, recorded with implanted electrodes in the midbrain, the hindbrain and the forebrain [362]. These 4–8 s SPSs were predominantly negative in the midbrain, positive in the forebrain and mildly positive in the hindbrain in response to a moving

visual stimulus, the onset of illumination or a tap to the side of the aquarium. During early presentations of the tap stimulus, when cardiac arousal responses were large, these responses were strongly related to the amplitude of the midbrain SPS response to the tap stimulus. Relationships between surface-recorded SPS responses and cardiac responses to illumination onset in fish have also been found [321].

During arousal, it is considered that all brain responses are amplified, whereas during attention there is selective response inhibition [270]. In rabbits, visual evoked potentials are enhanced in amplitude if preceded by cortical negativity [375].

3.4. SPS activity during learning

Simple repetition of an initially novel sensory experience may result in a decline in the amplitudes of SPS responses evoked by that stimulus. This was demonstrated for SPS responses to acoustic stimuli in the auditory cortex of the unrestrained cat [163], and for auditory, visual and peripheral electroshock stimuli in immobilised cats [267]. Similar observations have been made in rats [49,404].

During habituation to a variety of arousing stimuli, the decline in the cardiac arousal response of fish to all types of stimuli used has been found to be related to a decline in the positivity of SPS responses in the telencephalon [362]. This is of interest because of the considerable body of evidence linking telencephalic regions (posterior dorso-central) with habituation of arousal responses in fish [387,388]. During the period of habituation of the cardiac arousal response in goldfish to the repeated onset of illumination, SPSs recorded from the telencephalic, tectal and medullary surfaces in response to the same stimulus also declined in amplitude [321]. In a further study [322], it was found that such changes in the amplitudes of SPS responses to this stimulus are related to concurrent changes in the modulatory effects of the presentation of such stimuli on the amplitudes of AEPs to a continuous train of auditory stimuli. This was particularly apparent in the telencephalon.

In toads (*Bufo bufo*), intrinsic tectal visual unit activity frequency and burst duration habituate on repeated presentation of a prey-like object to immobilised but conscious animals. This habituation may reflect the unobtainability of the simulated prey. SPS amplitude and duration and the duration of characteristics associated with arousal in the EEG recorded with the same electrode, similarly decline [258]. However, the relationship is not a simple one. Over 20 presentations of the stimulus, unit frequency and burst duration declined to 50% of the original value; a similar decline to that of the duration of SPS and EEG responses. The decline in SPS amplitude was much larger, in the order of 70%, suggesting its habituation may be independent of the decline in unit responses. During habituation, the SPS duration was reduced to closely match that of the bursts of the local intrinsic tectal unit, suggesting that initially it was deriving some of its source $[K^+]_e$ from more superficial (retinal input) regions, perhaps by spatial buffering. It would seem that the spatial buffering currents decline in magnitude disproportionately to the decline in neuronal activity. This may be due to passive build up of $[K^+]_e$ over the repeated stimuli, or to an experiential decline in potentially sensitising, spatial buffering currents. If the former, then it would appear that 1 min is insufficient time for potassium to equilibrate (the time between successive stimulus presentations); if the latter, then it would appear that glia may be actively involved in the habituation process.

As with the habituation of responses to novelty, conditioning is a situation when there is apparent dissociation of neural activity from activity expressed in the SPS. In cats, the cortical SPS can be conditioned to a tone when it was paired with a food reward presented 5 s later [426]. Simultaneously recorded massed action potentials revealed that these were not associated with the SPS response to the tone, though they were correlated with that generated in response to the reward. SPSs have also been conditioned in the motor cortex, hypothalamus and amygdala in response to a tone in cats anticipating a food reward [209]. The importance of evocation of sub-cortical SPSs in the learning process have since been stressed [24,369].

An SPS occurring in response to the conditioned stimulus has been refered to as the proactive SPS [406]. Such SPSs occur prior to the reward and are not correlated with massed action potentials. These proactive SPSs may be similar to the CNV that occurs in the fore period of a reaction time task in humans [513]. Although the experimental paradigms are not identical to those used to elicit an epicranially recorded CNV in humans; many have tried to emulate their expression in experimental animals.

A CNV-like frontal lobe SPS in rats can be produced by pairing a tone with rewarding medial forebrain bundle (MFB) stimulation [348–350]. Simultaneously recorded frontal lobe unit activity increased at the cortical surface on pairing of the tone with the reward. The SPS was interpreted as reflecting the increased neuronal excitation. Similar experiments have been performed using a combined light/tone conditioned stimulus with MFB stimulation as the reward [406]. Again, there was a negative anticipatory potential in the visual cortex, associated with increased multiple unit activity. In this case, unlike with the food reward, the unit activity and the SPS were associated.

Several workers have described negative cortical shifts in the period between a conditioned stimulus and a motor response necessary to obtain a reward. Monkeys were trained to respond to a warning stimulus by pressing a key and holding it down until a second stimulus was given. A transcortical negative variation occurs whilst the animal waits for the second stimulus [105].

The SPS associated with movements might reflect potentials associated with motor cortex activity. Indeed, a surface negative SPS wave precedes voluntary hand movements in

premotor and motor cortices of monkeys [174]. However, a negative CNV-like wave occurs in animals not required to press the key, indicating that only part of the conditioned SPS could be so explained [105].

It has become increasingly apparent that the conditioned anticipatory epicortically recorded SPS in animals bears similarities to the human epicranially recorded CNV and is comprised of at least two components. The early component reflects the sensory response to a warning stimulus and anticipation of the imperative stimulus that requires a motor response. The second component of the SPS is the preparative phase, especially in motor cortex, associated with movement. These components have been recognised not only in monkeys [140,174], but also in rats [310].

3.5. SPS responses associated with motivation changes

In mammals, large (mV) and often sustained (min) shifts occur in response to consummatory stimuli in animals in a highly motivated state.

Shifts have been recorded in the millivolt range in hungry cats during lapping of a fish/milk homogenate [405]. A large part of the shift was lost if the oral cavity was anaesthetised, suggesting that the response was, in part, a sensory one; however, the shift magnitude was related also to the degree of motivation as greater deprivation enhanced it and feeding caused its decline [404]. On satiation, the cats rejected food and the polarity of the SPS was reversed, usually from negative to positive depending on the recording site. Further studies have shown unconditioned SPS responses to food in motor cortex, hypothalamus and amygdala that increased in magnitude with the palatability of the reward. SPS responses in the midbrain reticular formation did not show this relationship [209].

Most female mammals show regular periods of sexual activity (oestrus) that can be experimentally induced by hormone treatment. The degree of sexual responsiveness was measured behaviourally in cats [404], and found to correspond to the magnitude of negative, cortically recorded SPS responses to vaginal stimulation. The SPS to vaginal stimulation continued during the subsequent rolling and rubbing after-response of the animal, but did not occur during equivalent magnitude motor activities not related to sexual behaviour.

Most of the motivational studies of SPS responses in mammals have used the motivational state of the animal to condition SPS and behavioural responses. These have used food or rewarding electrical self-stimulation as positive reinforcement or peripheral electrical stimulation as an aversive stimulus.

In toads, the feeding motivational state of the animal can be tested and quantified by the number of times the animal will perform the behavioural components of the prey catching sequence when presented with a simulated prey object. The 'prey' is an elongate piece of black card moving in its long axis against a contrasting background; i.e. a 'worm'. The behaviours are categorised as 'orient', 'approach', 'fixate' and 'snap' [121]. After testing for the motivational state, animals prepared for recording tectal surface SPSs were tested with the simulated prey and when orienting towards the prey exhibited a negative shift on the anterior tectal surface, the retinal projection region of the frontal visual field. This negative shift was followed by a positive wave after ca. 4 s. On the surface of the posterior tectum, the reverse occurred, i.e. a positive shift was followed by negativity [260]. The amplitude of these shifts was related to the prey catching motivation prior to the operation. Animals were then re-anaesthetised and immobilised and retested to the 'worm' stimulus. The SPS response now was a monophasic negative wave in the anterior tectum and a monophasic positive wave in the posterior tectum. Again their amplitudes were related to the previously recorded motivational state. In order to determine if the motivational state had been affected by the operation and subsequent testing, animals were again anaesthetised to allow them to recover from the muscle relaxant and they were then tested again. They demonstrated no change in prey catching activity from that exhibited prior to the experiment [260].

3.6. DC changes during sleep and wakefulness

Although sleep is perhaps the most profound behavioural change exhibited by animals, it has been little explored with respect to a possible glial contribution to its onset and maintenance. There is a 'tonic' (duration's of minutes to hours) DC potential of the rat cortical surface which shifts towards negativity on awakening from sleep and towards positivity on sleep onset [48]. Several studies have shown a shift towards negativity during paradoxical sleep [224,531], in rabbits and rats, respectively, which in mammals is often equated to dreaming sleep in humans, where cortical activation occurs without behavioural activity. The shift from negativity to positivity in correlation with high to lowered brain activity, respectively, would accord with negativity in association with activation in the waking state, though whether this involves a glial mechanism has not yet been directly explored. The gross changes in extracellularly recorded 'tonic' DC levels in the brain presumably indicate changes in the ionic balance of ECS, either shunting through the blood–brain or brain–CSF glial interfaces. This possibility deserves future examination.

3.7. Protons

The oxidation of carbohydrates by CNS cells, to provide energy, results in the formation of bicarbonate, under the influence of carbonic anhydrase located within the cytoplasm or cell membrane of glial cells, and H^+ [89]. In order to maintain the slightly alkaline intracellular environment necessary for survival, all cells, including glia, actively regulate pH by ion exchangers and transporters. The protons associated with metabolic acidification [201] are crucially buffered by bicarbonate, but this involves ionic

movement resulting in membrane potential shifts that may contribute to SPS's and affect the spatial buffering of K^+.

pH changes in nerve and glial cells and in the extracellular space in nervous systems can be evoked by neuronal activity, by neurotransmitters, by active cellular pH regulation and by metabolic processes. Analogous to the signalling pattern of other ions, such as, e.g. calcium and potassium, transient shifts of protons and bicarbonate, together with the gas carbon dioxide, may influence or initiate functional processes in the nervous system, including pH-induced changes of neuronal excitability, the modulation of gap junctions and thus of electrical synapses and the glial syncytium, and the control of enzyme activities. Proton signalling in cells and in local extracellular domains, especially in the vicinity of synapses, could thus contribute to information processing in nervous systems [88–91].

pH regulation in nervous systems is an essential homeostatic function of the cellular elements, glial cells and neurons [389,489]. This is mainly achieved by transport of acid–base equivalents across the cell membranes.

Extra and intracellular pH, however, are not kept as tightly constant as often thought. In response to neuronal activity, intra and extracellular pH can rapidly and transiently change just like the activity of Ca^{2+} and K^+.

Studies on central nervous systems of both vertebrates and invertebrates have shown that neuronal activity leads to defined extra and intracellular pH changes [60,61,91,247]. These consist of mono or multiphasic pH shifts indicating that they may originate from multiple sources and/or via multiple pathways.

In the cortex, stimulus-evoked glial depolarisation is accompanied by an intracellular alkalinization of astrocytes, the amplitude of which is dependent on the amplitude of the glial depolarisation [62]. A stimulus-induced depolarisation of 15 mV was associated with a mean alkaline shift of 0.19 pH units; indicating a slope of approximately 80 mV/pH unit. Qualitatively similar pH_i shifts have been reported from glial cells in the medulla of cats [17] and from leech neuropile glial cells [393].

Several lines of evidence suggest that the depolarisation-induced alkalinization of glial cells in both vertebrate and invertebrate preparations is due to inward transport of bicarbonate via an electrogenic Na/HCO_3^--cotransport activated by the K^+-induced membrane depolarisation [93,156]. In the cortex, the glial alkaline shift was partly inhibited in Na-free saline and turned into a small acidification during the application of Ba^+ [62,156,157]. The stimulus-induced alkalinization of glial cells in the leech was turned into an acidification by all experimental protocols preventing the activation of Na/HCO_3^--cotransport.

Observations on the developing rat optic nerve and spinal cord suggest that the glial alkalinization might acidify the ECS, thereby leading to a dampening of the activity-induced extracellular alkalinization [212,368], also demonstrated for the adult leech CNS [392]. A recent report has suggested that a further function of alkalination may be to facilitate astrocytic glucose utilisation and glutamine formation [38].

Glial cells thus appear to contribute to extracellular pH transients, and play a prominent role in overall H^+ homeostasis in nervous systems.

3.8. Calcium

Calcium levels in the extracellular environment are lower than those of K^+ at about 1–2 mM. The effect of changes in extracellular calcium levels is more complex than for potassium. Low $[Ca^{2+}]_e$ would obviously affect synaptic release of transmitter as its uptake is required for vesicle fusion with the presynaptic membrane. Nevertheless, on occasion, low $[Ca^{2+}]_e$ is associated with increased neuronal excitability. In pathological cases $[Ca^{2+}]_e$ declines prior to the development of seizures [360] which may, in part, be a cause of seizure initiation. The increase in neural excitation with low $[Ca^{2+}]_e$ is probably due to shifting of the activation of Na^+ channels to less depolarised values [55], allowing greater ion influx on depolarisation or even spontaneous depolarisation in pathological conditions.

Calcium has been described as important for transmitter release at synapses, but it also offers a mechanism for neuron to glial signalling, as use of calcium-sensitive dyes have illustrated waves of increased $[Ca^{2+}]_i$ propagating through the astrocytic syncitium, especially in response to glutamate [436]. These Ca^{2+} waves could have a wide range of effects on astrocytes that could then affect neuronal function. These include: modulation of Ca^{2+}-dependent K^+ channels, regulation of $[Ca^{2+}]_e$ and regulation of metabolism of energy substrates and neurotransmitters for neurons. For example, calcium waves in retinal Müller cells modulate ganglion cell light-evoked responses [315].

3.9. Summary

Ionic fluxes of $K^+$and H^+ may act as intercellular signals between neurons and glia. The currents produced in glia and the ECS by glial uptake of K^+ and 'spatial buffering' contribute significantly to SPS activity which can be recorded in intact animals in response to sensory stimulation. SPS responses alter during learning and changes in motivation in a manner that does not seem to simply reflect neuronal activity. Ionic fluxes may also occur to produce the long-lasting DC changes associated with sleep and wakefulness. Ca^{2+} waves between astrocytic glia may act as a mechanism by which the activity of the syncitium is co-ordinated.

4. Energetic interactions between neurons and glia

The brain has an enormous requirement for energy, derived from glucose uptake from the circulation. Ependymal glia form a major component of the blood–brain and brain–CSF boundaries and peri-capillary astrocytic processes form the other interface between the supply of

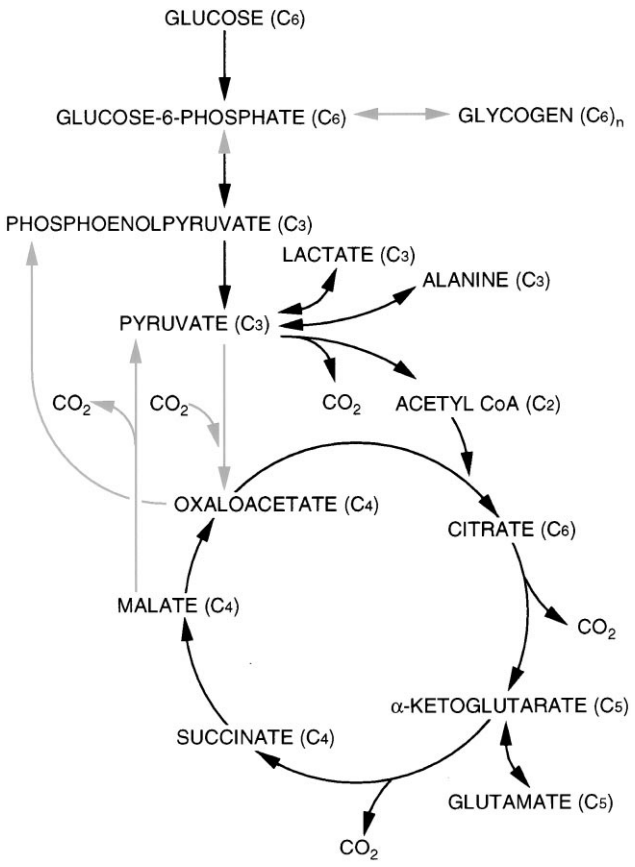

Fig. 3. Glucose metabolism: Glucose, containing six carbon atoms is completely degraded to CO_2 through glycolysis to pyruvate (three carbon atoms) and oxidative metabolism in the TCA cycle. Pyruvate is condensed with oxaloacetate (four carbon atoms) to form citrate (three carbon atoms); two CO_2 are released, and oxaloacetate is regenerated in one turn of the cycle; there is no net synthesis of any TCA cycle constituent. Pyruvate can also be condensed with CO_2 to provide net synthesis of oxaloacetate and it is interchangeable with lactate and alanine. Malate can leave the TCA cycle to form pyruvate, and phosphoenolpyruvate can be formed from oxaloacetate. α-Ketoglutarate can be used for synthesis of glutamate. The latter three reactions lead to leakage of TCA cycle constituents from the cycle, requiring synthesis of new TCA cycle intermediates. See text for neuron and glia-specific reactions.

metabolic substrates and the nervous tissue. These vital substrates are therefore taken up by the glial syncitium to be transferred to neurons. The glia therefore offer a potential control interface for the availability of metabolic substrates for neurons and the removal of the waste products of metabolism. In general though, glia operate in a far more co-operative way with neurons, not only to provide them with what they need, but also to adapt that supply to the ongoing circumstances.

A thorough knowledge of metabolic pathways in neurons and glia makes it possible to interfere with neuronal–glial interactions *via* drugs which act at very specific metabolic steps. Subsequently these drugs can be tested in behavioural paradigms. Some of the drugs that act exclusively on glia have been found to exert profound behavioural effects, thereby allowing the unequivocal conclusion that metabolic reactions occurring in glia are essential for certain behaviours.

Glucose is the only nutrient which is present *both* in the systemic circulation at high concentrations *and* readily crosses the blood–brain-barrier. Some reactions in the metabolism of glucose do not take place in both cell types. Such metabolic compartmentation necessitates the transport of metabolites from one cell type, to another where they are utilised as substrates. Such interactions have been called “metabolic trafficking” [490].

Glucose is not only needed for energy generation (in the form of ATP), but also for the production of a diverse range of metabolites, including the major excitatory neurotransmitter glutamate and inhibitory transmitter, GABA (Fig. 3).

Neurons have a considerable demand for new precursors of glutamate and GABA because only a proportion of glutamate released from glutamatergic neurons is re-accumulated by these neurons. The majority of glutamate released by neurons is taken up into adjacent glia. Part of the glutamate accumulated into astrocytes is converted to glutamine, which is subsequently released from astrocytes into the extracellular fluid (glutamine has no transmitter activity), where some is taken up by neurons, to be used as a precursor for glutamate synthesis [195,197,417,418].

The most direct evidence for metabolic differences between glia and neurons derive from immunocytochemistry of their enzyme contents. Most of the glial-specific enzymes catalyse reactions in one direction only, giving them a monopoly over the availability of critical metabolic substrates.

4.1. Mechanisms of energy metabolism

The energy supplies for brain metabolism can be derived from glycogenolysis, glycolysis or oxidative metabolism. The rates of these processes are influenced by $[K^+]_e$, or the presence of glutamate or nor-adrenaline (Table 1).

4.1.1. Glycogenolysis

Glucose can be stored as glycogen in a process that is regulated by certain transmitters, for example noradrenaline and vaso-active intestinal peptide (VIP) [274]. Glycogen can be synthesised not only from glucose but also from pyruvate, lactate, alanine or tricarboxylic acid (TCA) cycle constituents like glutamate in a gluconeogenic process [109,206,415]. *Gluconeogenesis* proceeds through the same intermediates as glycolysis but different enzymes are involved. One of these is fructose-1,6-biphosphatase, an enzyme that is restricted to glia [415].

Breakdown of glycogen (glycogenolysis) leads to the production of more ATP than glycolysis because the formation of glucose-phosphate from glucose, but not from glycogen, requires energy. In the mature central nervous system, glycogen is virtually restricted to astrocytes [170,345]. Astrocytic glycogen can be subsequently broken

Table 1
Changes in ratea of gylcolysis, glycogenolysis and oxidative metabolism in neurons and astrocytes in response to glutamate, potassium and noradrenaline. Large arrow—pronounces stimulations; small arrow—less pronounced stimulation; small arrow in parenthesis—possible small stimulation; dash—no effect. For reference, see text

	Astrocytes			Neurons		
	Glycolysis	Glycogen breakdown	Oxidative metabolism	Glycolysis	Glycogen breakdown	Oxidative metabolism
Elevated K^+	↑	↑	No change	↑	No change	↑
Elevated glutamate	(↑)	No change	No change	(↑)	No change	↑
Elevated noradrenaline	↑	↑	↑	No change	No change	No change

down (glycogenolysis) by the astrocyte-specific enzyme, glycogen phosphorylase.

CNS activity is correlated with an increased turnover of glycogen [475]. In astrocytes, glycogenolysis is stimulated by K^+-induced depolarisation. This response has been observed in a variety of preparations including retinal Müller cells [372], brain slices [203] and cultured astrocytes that have been treated with dibutyryl cyclic adenosine monophosphate (cAMP) [469]. In the latter preparation potassium-stimulated glycogenolysis proceeds twice as fast as glycolysis. At least in cultured astrocytes, the glycosyl units of glycogen are released as lactate (formed from pyruvate), not as glucose [108]. This means that the energy derived from glycolysis must be utilised by the astrocytes themselves. Energy produced during subsequent oxidative metabolism could be available for both astrocytes and neurons, provided there is metabolic trafficking of lactate from astrocytes to neurons.

The importance of glycogenolysis as an energy source must be in the short-term, since glycogen stores in the brain are depleted within minutes if glycogen serves as the only metabolic substrate. However, the breakdown of glycogen occurs so rapidly (twice as fast as glycolysis from glucose in mouse astrocytes exposed to elevated K^+ [469] that glycogen is an ideal substrate for the provision of large amounts of energy within a short time. However, astrocytic glycogen is not converted to glucose but to glucose phosphate, and then *via* pyruvate to lactate which can be released [109].

Certain neurotransmitters, including noradrenaline, VIP, adenosine and serotonin bind to astrocytic receptors and potently evoke glycogenolysis in cultured astrocytes [59,448,467]. Many of the same transmitters also stimulate formation of glycogen; this is a long-term effect, and it is dependent on protein synthesis [273,449].

4.1.2. Glycolysis

The formation of pyruvate from glucose (glycolysis) yields only a small amount of energy (ATP). However, it appears that some important processes in the central nervous system are partly dependent upon glycolytically derived energy not only in culture [223] but also in the brain in vivo [396].

Glucose utilization in cultured astrocytes is stimulated to a modest degree (20%) by an elevation of $[K^+]_e$ [80,342] and by noradrenaline [192,466,536]. However, since the absolute rate of glycolysis is high, a minor percentage increase corresponds to a large relative increase. A large increase by extracellular glutamate has been reported by some authors [340,341]. However, this occurs only when the cells are incapable of carrying out oxidative metabolism, and otherwise there is at most a very small increase [198]. The increase in glucose utilization by either glutamate or elevated K^+ is mirrored by an enhanced release of lactate

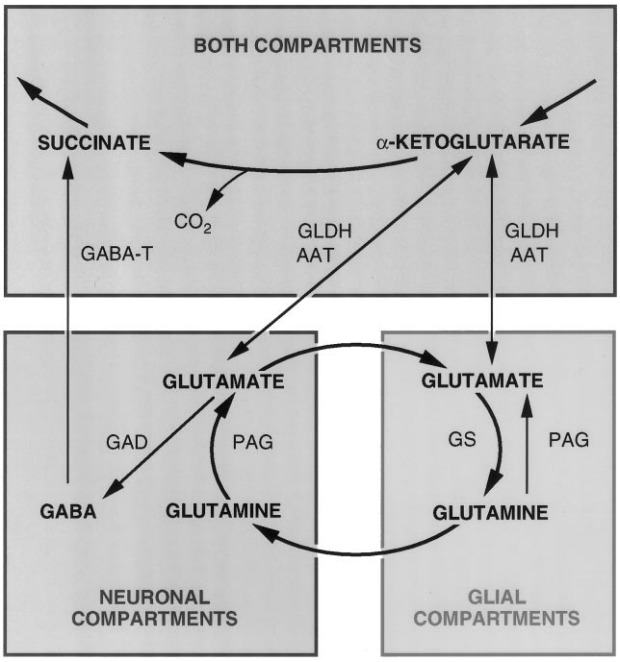

Fig. 4. Synthesis, release and metabolism of glutamate and GABA. The section of the TCA cycle from α-ketoglutarate to succinate is identical to Fig. 2. Glutamate can be formed from α-ketoglutarate in a reversible reaction catalysed by either GLDH (glutamate dehydrogenase) or AAT (aspartate amino transferase). Glutamate can be formed from glutamate in an irreversible reaction, catalysed by GS (glutamine synthetase) and reconverted to glutamate in a likewise irreversible reaction, catalysed by PAG (glutaminase). GABA can be formed from glutamate in an irreversible reaction catalysed by GAD (glutamate decarboxylase) and metabolised to succinate in a likewise irreversible reaction, catalysed by GABA transaminase (GABA-T). See text for neuron and glia-specific reactions.

[340,517]. The glutamate effect is not a result of receptor stimulation but reflects the increased energy demand for active uptake of glutamate.

The increase of glycolysis in astrocytes by glutamate and excess K^+ is secondary to a stimulation of Na^+, K^+-ATPase activity, and in both cases it is blocked by the Na^+, K^+-ATPase inhibitor, ouabain [340,341]. Elevated K^+ directly stimulates the extracellular potassium-sensitive site of this enzyme [166,286]. The stimulation by glutamate is exerted at the intracellular, sodium-sensitive site of the Na^+, K^+-ATPase and is secondary to active glutamate uptake coupled to sodium entry [340].

Glucose utilization in glutamatergic granule cells (but not in all types of neurons) can be increased by an elevation of extracellular K^+, but not appreciably by glutamate or noradrenaline ([342], Peng and Hertz, unpublished experiments). The extracellular concentrations of K^+ and glutamate are elevated during activation of a given cortical area, for example by sensory stimulation, and during seizures [273,515]. The stimulation of glycolysis by glutamate and elevated K^+ couples neuronal activity directly with glucose utilization. Since the increase in glucose utilization occurs mainly in glia, these cells are likely to be the predominant source of the 2-deoxyglucose signal detected in autoradiographic or positron emission topography (PET) studies during activation [273].

4.1.3. Oxidative metabolism

Pyruvate can be readily and reversibly reduced to lactate in a reaction that is catalysed by lactate dehydrogenase. This process enables glycolysis to continue in the absence of oxygen, with the build-up of a high concentration of lactate, but it occurs even under oxygenated conditions, when cyclic metabolic processes, such as the malate–aspartate shuttle, are capable of transferring reducing equivalents from the cytosol to the mitochondria. Pyruvate can also be transaminated to alanine, by alanine aminotransferase. The reaction velocities for these conversions are high enough, compared to the rates of generation and subsequent breakdown of pyruvate, that in most instances alanine, lactate and pyruvate can be regarded as interchangeable. It should, however, be kept in mind that the formation of alanine involves introduction of an amino group, as will be discussed in more detail later.

Subsequent metabolism of lactate or alanine requires that these compounds are initially reconverted to pyruvate. The ratio between pyruvate and lactate reflects the cytosolic redox potential, and the lactate concentration is generally about 10-fold higher than the pyruvate concentration. In the central nervous system the extracellular concentration of lactate, measured by microdialysis, is approximately 1 mM, but it increases as a result of neuronal activity [244]. In the retina, lactate is transferred from Müller cells to photoreceptors to fuel their metabolism and glutamate resynthesis [351]. The concentration of alanine in cerebrospinal fluid is 30–50 μM [129,379], but it might be higher in the extracellular fluid in brain [122].

Pyruvate generated by glycolysis can be oxidised to carbon dioxide and water in the mitochondrial TCA cycle, producing a substantial amount of ATP. This cycling could continue indefinitely (as long as glucose is available and energy is required), except for the fact that the cycle is leaky: TCA cycle intermediates are used for the synthesis of other compounds.

In the CNS, the most important diversions of TCA cycle constituents are for the synthesis of glutamate and GABA, which can be formed from α-ketoglutarate (Fig. 4).

Glutamate and the TCA cycle constituent α-ketoglutarate are rapidly interconverted in two different reactions: oxidative deamination/reductive deamination, and transamination. The former process is catalysed by glutamate dehydrogenase and ammonia is either added (for formation of glutamate) or removed (for conversion of glutamate to α-ketoglutarate). Other amino acids cannot be formed in a direct reaction between the corresponding ketoacid and ammonia. The most common transamination process is that between glutamate and oxaloacetate, yielding α-ketoglutarate and aspartate (or in the opposite direction

glutamate and oxaloacetate). This transamination is catalysed by aspartate aminotransferase.

Glutamate can be transaminated with pyruvate, forming α-ketoglutarate and alanine, or alanine can be transaminated with α-ketoglutarate, forming glutamate and pyruvate. The former of these reactions is involved in the formation of alanine during glycolysis, the latter in the re-conversion of alanine to pyruvate for further oxidative metabolism. The latter reaction may also be important in synthesis of transmitter glutamate, where alanine may provide the amino group (but not the carbon skeleton) of glutamate.

Glutamate can be amidated to glutamine by the addition of ammonia. This process requires energy (conversion of ATP to adenosine diphosphate (ADP)), and is catalysed by glutamine synthetase, which is present in glia, (both astrocytes and oligodendrocytes) [81,324,483] but absent from neurons. Hydrolysis of glutamine to glutamate requires no energy and is catalysed by a different enzyme, phosphate-activated glutaminase (PAG). The presence of this enzyme in both neurons and astrocytes was shown in primary cultures [204,252] and confirmed histochemically in intact tissue [10,530]. Therefore, neurons cannot generate glutamine from glutamate, whereas both neurons and glia can form glutamate from glutamine. GABA is produced by decarboxylation of glutamate, catalysed by glutamate decarboxylase, which only occurs in GABAergic neurons.

Due to the leakiness of the TCA cycle it is extremely important that pyruvate (or lactate or alanine) can be used for net production of new TCA cycle constituents. In this process, one molecule of pyruvate is condensed with one molecule of CO_2 (pyruvate fixation) to form one molecule of oxaloacetate, in a reaction catalysed by pyruvate carboxylase. This reaction creates one molecule of oxaloacetate (but no energy) and it is the major route for synthesis of new TCA cycle constituents. The oxaloacetate molecule that has been formed can be converted in the TCA cycle to any other TCA cycle intermediate or metabolite, including glutamate (formed from α-ketoglutarate) and GABA (formed from glutamate).

Immunohistochemical staining for pyruvate carboxylase in intact brain has shown that this enzyme is absent from neurons but is abundant in glia [427], and a similar conclusion has been made from biochemical determination of enzyme activities in neuronal and glial cultures [52,535]. This localisation means that neurons are unable to replenish their supply of TCA cycle intermediates on their own. Since neurons are being continuously depleted of TCA cycle intermediates and their derivatives, there needs to be a transfer of oxaloacetate, citrate, α-ketoglutarate or glutamine to neurons from glia. The demand for these substrates will increase after the neuronal release of glutamate or GABA.

TCA cycle intermediates can also leave the TCA cycle and be oxidatively degraded or used for gluconeogenesis [444]. Thus, malate can exit the mitochondrial membrane and be converted to pyruvate in the cytosol. This reaction is catalysed by cytosolic malic enzyme, which is restricted to glia [251,284]. Studies using radioactively labelled acetate and glucose have shown that in the mouse brain, the formation of pyruvate from TCA cycle intermediates takes place predominantly in glia [175]. Pyruvate can be reintroduced into the TCA cycle via acetyl coenzyme A for complete degradation to CO_2 and water.

Both neurons and astrocytes carry out oxidative metabolism [99,199,342] but it is likely that the rate of oxidative metabolism in astrocytes is normally lower than the corresponding rate of glycolysis. Glutamate causes a large increase in oxidative metabolism in astrocytes [120], but accumulated glutamate is used as a metabolic fuel, not only to energise its own uptake but also to partly replace glucose in general [199]. Therefore, in spite of the increased oxidative metabolism, less pyruvate is oxidised. Elevated potassium concentrations cause only a very short lasting and relatively small increase in glucose utilisation, which is not inhibited by ouabain and accordingly does not reflect stimulation of active potassium uptake [199].

Noradrenaline stimulates virtually all steps of oxidative metabolism of glucose in astrocytes [205,468], but glutamate does not. Pyruvate carboxylation, which occurs only in astrocytes, increases during exposure to an elevated K^+ concentration [222]. The extent of this increase is almost as large as the K^+-induced increase in glycolysis, but is far smaller than the stimulation of glycogenolysis by elevated K^+. Neither noradrenaline [58] nor glutamate [222] has an effect on pyruvate carboxylation.

Neuronal function, including electrophysiological activity in brain slices is readily maintained with either glucose or pyruvate/lactate as the substrate [210,262,419]. Cultured cerebellar granule cells have a rate of oxidative metabolism that is comparable to that of astrocytes. Their rate of glycolysis is somewhat higher than their rate of oxidative metabolism, but lower than the rate of glycolysis in astrocytes [342]. Cultured granule cells resemble astrocytes in the fact that they release pyruvate/lactate into the incubation medium, but they do so at about half of the rate. Furthermore, during exposure to radioactive glucose there is no release of labelled CO_2 from cultured granule cells during the first 15 min [342]. This delayed appearance of labelled CO_2 has been confirmed [482], and is in contrast to the immediate appearance of labelled CO_2 from cultured astrocytes (see above).

4.2. Metabolic involvement in memory formation in the chick

Glia are involved in the supply of energy to neurons during one-trial passive avoidance learning in 1–2 day old chicks where they learn in a single 10 s trial to avoid a coloured bead treated with a noxious chemical (methylanthranilate (MeA)), [4,142,316,338,394]. Memory for the passive avoidance learning experience in the chick appears to develop in three sequentially dependent stages, inferred both from purely behavioural [143] and from

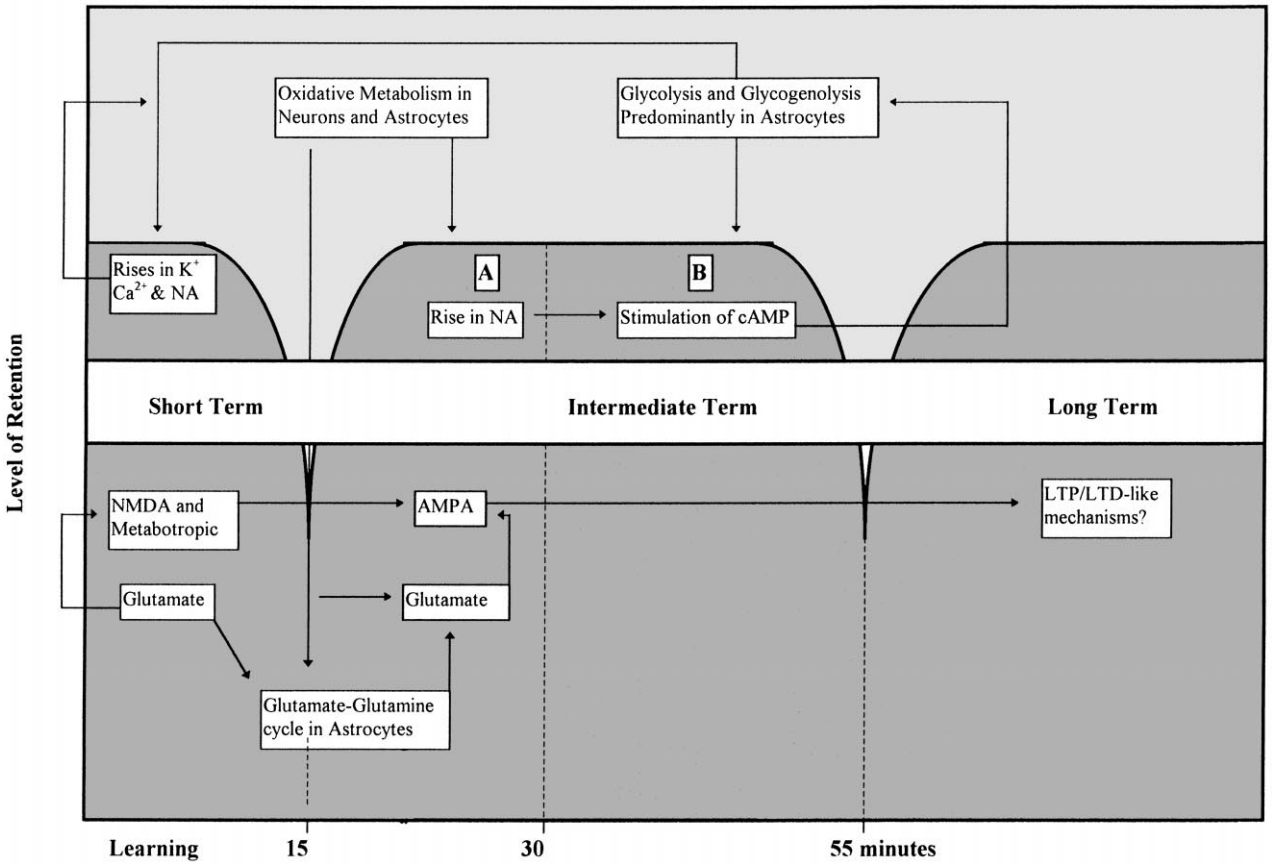

Fig. 5. Schematic diagram of the Gibbs–Ng three-stage model of passive avoidance memory formation in the day-old chick showing astrocyte–neuron interactions in glutamate turnover and in energy metabolism. Glutamate is released from neurons immediately after training (stimulating NMDA and metabotropic receptors), and during phase A of the intermediate memory stage (stimulating AMPA receptors). This glutamate may activate LTP/LTD-like mechanisms related to long-term memory, and some other glutamate-sensitive process operating during phase A of intermediate memory. The glutamate–glutamine cycle in astrocytes is involved in the restocking of neuronal glutamate supplies following release. The rise in forebrain noradrenaline (NA) during phase A of intermediate memory may stimulate glycolysis and the astrocyte-specific process of glycogenolysis via the cAMP second messenger system, providing energy or substrates necessary to drive the memory-related mechanisms active during phase B of this stage. The release of K^+, Ca^{2+} or NA following training may also stimulate glycolysis and glycogenolysis in astrocytes. Finally, neuronal and astrocytic oxidative metabolism energizes memory-related processes necessary for phase A of intermediate memory.

pharmacological intervention studies [142,144,316,338]. A short-term (STM) stage, formed by 5 min post-training and decaying after 10 min post-training, is followed by an intermediate-term (ITM) stage, expressed between 20 and 50 min post-training, and a long-term (LTM) stage formed 60 min after training. Behaviourally, the stages are shown to be separated by transient retention deficits at 15 and 55 min post-training. Pharmacologically, the stages are distinguished by their differential susceptibility to abolishment by different classes of pharmacological agents. Inhibition of any given stage results in the loss of all stages thereafter, suggesting a sequential dependence across stages (Fig. 5). Although pharmacological agents targeting learning have only shown effects for hours after the training trial, experiments to explore mechanisms of recall, suggest that consolidation may occur for days after training [471,472].

4.2.1. A short-term stage

In untrained anaesthesized chicks, stimulation of the beak with methylanthranilate results in a prolonged increase in $[K^+]_e$ in the neostriatal/hyperstriatal region of the forebrain [479], a region shown to be metabolically active during memory formation [395,421]. Local application of 4mM monosodium glutamate to this forebrain region also results in significant increases in $[K^+]_e$ [317]. Clearance of accumulated extracellular K^+ involves astrocytic uptake of K^+, glial spatial buffering, and activity of the electrogenic Na^+/K^+ pump and an electroneutral $Na^+/K^+/Cl^-$-cotransport system [478].

Formation of the STM stage in the chick is prevented by depolarizing treatments with isotonic (154 mM) KCl or a low concentration (4 mM) of monosodium glutamate [142]. Formation of STM may depend upon an initial phase of neuronal hyperpolarization mediated by K^+ conductance changes following neural input and dependent on $[Ca^{2+}]_e$ [211], since this stage is also abolished by the Ca^{2+} channel blocker lanthanum chloride and its duration is extended by increased $[Ca^{2+}]_e$ [145].

4.2.2. The intermediate-term stage

The ITM stage itself consists of two phases: ITMA (approximately 20–35 min) and ITMB (approximately 35–50 min). These phases are uniquely distinguished by the ability of the uncoupler of oxidative phosphorylation 2,4-dinitrophenol (DNP) to abolish ITMA but not ITMB. Furthermore, while all three stages are formed when the chicks are trained with concentrated MeA, reducing the intensity of this reinforcer by dilution to 20% in alcohol results in the formation of STM and ITMA, but not ITMB or LTM [77], with normal retention levels observed only until about 35–40 min post-training. However, both ITMB and LTM are reinstated in the presence of adreno corticotrophic hormone $(ACTH)_{1\text{-}24}$ or noradrenaline (NA) [78,79].

Formation of the ITM stage is prevented by inhibition of Na^+/K^+-ATPase activity with ouabain and the astrocytic $Na^+/K^+/Cl^-$ cotransport system with ethacrynic acid [142]. ITM formation is thought to involve a second phase of neuronal hyperpolarization brought about by the coupled action of the electrogenic sodium pump and the astrocytic cotransport system [211].

Na^+/K^+-ATPase activity is present in both neurons and astrocytes [205,476]. In both cell types the enzyme activity increases with an increase in $[K^+]_e$ up to the normal extracellular concentration. However, in astrocytes, but not in neurons, there is a further stimulation of the enzyme when $[K^+]_e$ rises above normal levels [205]. In addition, an ionic cotransport system in astrocytes, but probably not in neurons, accumulates one K^+ ion, one Na^+ ion, and two Cl^- ions. Once inside the cell, the accumulated Na^+ ions will stimulate Na^+/K^+-ATPase activity at sodium sensitive sites, resulting in an active energy-dependent extrusion of Na^+ ions and a concomitant uptake of K^+ ions. The joint operation of the Na^+/K^+ pump and the cotransport system in astrocytes will result, therefore, in an uptake of K^+ and Cl^- ions from the interstitial space without any net movement of Na^+ ions. The intensity of the K^+ transport mechanisms operating across the astrocytic membrane and the absence of any net movement of Na^+ ions during the operation of these mechanisms may result in the $[K^+]_e$ returning to normal before the $[Na^+]_e$ is normalized. Continued stimulation of Na^+/K^+-ATPase beyond this point may lead not only to Na^+ extrusion from neurons and glia but also a coupled active uptake of K^+ ions, resulting in an $[K^+]_e$ "undershoot". This could lead to the neuronal hyperpolarization hypothesized to be associated with the formation of ITM and may account for the amnestic effects of ouabain and ethacrynic acid.

The inhibitor of oxidative phosphorylation dinitrophenol (DNP) abolishes the ITMA phase of the intermediate memory stage in chicks trained on the passive avoidance task, but has no direct effect on the succeeding ITMB phase [144]. Since glycolysis and oxidative metabolism occur in both neurons and astrocytes [194], it is unclear as to whether the amnestic effects of DNP are due to inhibition of neuronal or of glial metabolism. Neuronal metabolism must be involved since astrocytes survive [438] and accumulate glutamate on glycolysis alone. The possibility that astrocytic metabolism also may be involved cannot be ruled out since fluoroacetate has the same effect as DNP. However, this effect of FA is likely to be due to interference with glutamate/glutamine synthesis rather than astrocytic energy production. It is reasonable to conclude that the energy demands of at least some of the cellular processes underlying ITMA are met by neuronal and astrocytic oxidative metabolism. However, memory processing during ITMB appears to be able to proceed in the absence of oxidative metabolism, such as would occur in the presence of DNP. Glycogenolysis in astrocytes may be a sufficient alternative source of energy. This is supported by the finding that iodoacetate (IO), which inhibits glycolysis in both neurons and astrocytes, has no effect on ITMA but abolishes ITMB, with significant retention losses appearing after 30 min post-training [328,329]. The use of IO does not distinguish between neuronal glycolysis, astrocytic glycolysis, astrocyte-specific glycogenolysis or any combination of these. However, a significant increase in uptake of 2-deoxyglucose has been observed during ITMB [421], a process described earlier as probably being exclusive to glia. Moreover, the mean forebrain level of glycogen (restricted to astrocytes) in the chick shows a rapid reduction beginning 30 min post-training, reaching a nadir by 55 min and recovering quickly to untrained levels by 65 min post-training [328].

The appearance of ITMB is preceded by a significant increase in whole forebrain NA levels by 30 min post-training [79]. β-Adrenergic blockers, such as propranolol [78] and sotalol [456], abolish ITMB but not ITMA. These findings suggest that consolidation of memory traces beyond ITMA may depend upon adequate reinforcement promoting sufficient arousal mediated by increased noradrenergic activity. Both NA and the β-adrenergic agonist isoproterenol have been shown to amplify glycogen turnover and glycolysis in mouse astrocytes [44,468], and propranolol inhibits this stimulation [466]. These findings are consistent with increasing evidence for a β-receptor-mediated metabolic response to NA in mammalian glia, but not in neurons [440,448,460].

Mammalian astrocytes express large numbers of β-adrenergic receptors [460] and compared to neurons show particularly marked rises in cAMP if these receptors are stimulated [461–463]. Glycogen levels in the chick brain peak at the time of hatching [115]. In 10–12 day-old chick primary astrocyte cultures (corresponding approximately to hatching age), glycogenolysis is stimulated by NA, as is cAMP formation [326,327]. It seems reasonable to suggest that NA released by neurons, in response to adequate reinforcement before the transition from ITMA to ITMB, may act as a metabolic signal that stimulates astrocytic glycogenolysis, and possibly neuronal and astrocytic glycolysis, to provide energy or substrates (alanine, pyruvate or lactate) required to drive the cellular processes underlying the expression and maintenance of ITMB. The finding that

inhibition of the activity of the cAMP-dependent protein kinase A results in memory loss only after 50 min post-training [541] is consistent with this view.

4.2.3. Long-term stage

For many decades, investigations into how the brain forms long-term memories have been shaped by the hypothesis that memorial representation of an experience involves increases in synaptic efficacy [188] brought about by relatively permanent changes to neuronal connectivity in a network activated by the experience, possibly in the number and properties of synapses [277]. Such a network would yield a preferred pathway for subsequent stimulation by both internal and external stimuli associated in the course of the learning experience.

Formation of LTM in chicks is prevented by protein synthesis inhibitors such as cycloheximide, anisomysin and emetine [142,338] suggesting that consolidation of memory requires structural or enzymatic processes that increase synaptic efficiency. That the initiation of these changes depends on activity in glial cells has already been evidenced above, but whether the long term modifications are restricted to neurons or affect both neurons and glial cells is still open to question (but see Sections 5 and 6).

There is, however, increasing evidence from chick studies linking specific aspects of phosphorylative activity to specific stages in memory formation [542]. Inhibition of the cAMP-dependent protein kinase A (PKA) results in memory loss in the chick from 60 min post-training when administered into either hemisphere [541].

Direct evidence implicating phosphatase activity in memory formation per se is much less extensive [26,540]. In passive avoidance training in the chick, okadaic acid, which preferentially inhibits protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), abolishes ITMB and the subsequent LTM stage [540]. It is unclear as to which protein phosphatase is involved. The immunosuppressant cyclosporin A (CyA), in contrast to okadaic acid, induces retention deficits only after 85 min post-training [26], well after formation of the antibiotic-sensitive LTM stage This phosphate appears to exert its effect via second messenger activation of calcineurin [241,539].

In chicks trained on the passive avoidance task, blockade of NMDA receptors by AP5, AMPA receptors by DNQX, and mGluRs by MCPG all yielded retention deficits around 80–85 min post-training [376,377]. Significantly, however, the NMDA and mGluR antagonists have to be administered within 5 min following training to produce their amnestic effects. In contrast, the effective time-of-administration window for the AMPA antagonist is between 15 and 25 min post-training, during the ITMA phase of the intermediate memory stage. These results would seem to suggest two periods of glutamate release critical to long term memory consolidation, one immediately after learning, binding to NMDA and mGluR receptors, and one during ITMA, binding to AMPA receptors. The memory consequences of these processes are not expressed until after formation of a distinct protein synthesis-dependent LTM stage.

4.3. Astrocytic regulation of glutamate, and its role in memory formation

The glial specific inhibitor of the citric acid cycle, fluoroacetate (FA), inhibits de novo astrocytic formation of glutamine from glutamate and α-ketoglutarate with little, if any effect, on ATP content [474]. In contrast, neurons do not readily oxidize glutamate or glutamine as metabolic fuels. Astrocytic conversion of glutamate to glutamine can also be blocked by methionine sulfoximine (MSO), an inhibitor of glutamine synthetase activity. MSO abolishes memory in day-old chicks trained on the passive avoidance task by 20 min post-training, provided it is administered shortly before the training trial [146]. MSO would appear to abolish memory from the ITMA phase of the intermediate memory stage onwards. A similar effect is observed with FA [329]. Unlike MSO, however, FA can be administered slightly later. This may not be too surprising since MSO affects synthesis of glutamine directly from glutamate accumulated into astrocytes immediately upon release while FA affects de novo glutamine synthesis from glucose. It is interesting too that blockade of glutamate uptake (into both glia and neurons) by ℓ-aspartic acid β-hydroxamate induces amnesia only if the drug is given within the first few seconds following training [318]. If the amnestic effects of MSO are indeed due to restricted replenishment of neuronal glutamate, they should be challenged by alternative supplies of precursors and perhaps by glutamate itself. Extracellular glutamine, glutamate or a combination of α-ketoglutarate and alanine effectively overcomes MSO-induced amnesia [146,318]. α-Ketoglutarate or alanine alone had little or no effect. The amnestic effects of FA are also successfully challenged by extracellular glutamine. Since FA also inhibits energy production in astrocytes, it is possible that this counteractive effect of glutamine may be attributed to its acting as a metabolic fuel. However, FA toxicity appears to derive mainly from its inhibition of synthesis of glutamate/glutamine precursors rather than from interference with astrocytic energy production [474]. Finally, the fact that interference with glutamate production results in loss of memory from the ITMA phase (20 min post-training) onwards strongly suggests that glutamate may play a role in intermediate memory processing [318], in addition to its role in inducing and sustaining LTP- and LTD-related memory processes since blockade of glutamate receptors associated with LTP and LTD did not yield amnesia until some 85 min following training.

4.4. Summary

We have seen that glycolytic flux is greater in astrocytes than in neurons and that at least half of glucose carbon is converted to lactate or alanine before complete degradation

of glucose. Neuronal, in contrast to glial, function is readily maintained with lactate as the substrate. Thus, astrocytically produced pyruvate/lactate or alanine is almost certainly accumulated into neurons and utilised as a metabolic substrate in the mammalian retina and central nervous system. This may especially be the case when neuronal activity is suddenly increased.

An increase in extracellular glutamate stimulates glycolysis in astrocytes slightly, but decreases oxidation of glucose because glutamate is used as an alternative fuel for oxidative metabolism, and has no effect on glycogenolysis in these cells, while an increase in extracellular K^+ massively stimulates glycogenolysis, and mildly stimulates astrocytic glycolysis, but has no effect on their oxidative metabolism. In other words, elevated extracellular K^+ or glutamate can increase pyruvate production by astrocytes substantially, but this is not accompanied by a comparable increase in their utilization of pyruvate. In at least some types of neurons, by contrast, an increase in extracellular glutamate has little effect on glycolysis but it dramatically increases their oxidative metabolism. An increase in extracellular K^+ concentration also increases glycolysis in some neurons, but the effect on oxidative metabolism is much more pronounced. Taken together, these differences indicate that when neuronal activity is increased, there *must* be a net loss of pyruvate, lactate and/or alanine from astrocytes.

Noradrenaline must act in a different way, since it stimulates both glycogenolysis and oxidative metabolism in astrocytes, yet leaves neuronal metabolism unchanged. This is consistent with conclusions from immunohistochemical studies and lesion experiments that many monoaminergic transmitter systems, especially noradrenaline, have glia as a major target [7,460,462,463]. Therefore, when noradrenaline stimulates glycogenolysis, excess pyruvate, lactate and/or alanine must be oxidised by astrocytes instead of by neurons.

In one-trial passive avoidance learning in the chick, at least the ITMB stage seems to be dependent on energy derived from astrocytic glycogenolysis and adrenergically stimulated oxidative metabolism. The ITMA stage of memory formation is dependent on astrocytically derived glutamine synthesis.

5. Transmitter interactions between neurons and glia

Behavioural changes ranging from sensory–motor through mood and cognitive behaviour depend on and are affected by changes in transmitter–receptor interactions. These interactions are generally thought to be purely attributable to interactions of transmitter with receptors located on neurons, particularly at synapses. With the identification of receptors and uptake systems for transmitters on astrocytes in primary cultures it became self-evident that behaviourally related transmitter effects could also involve astrocytes via transmitter–astrocyte receptor interactions [193].

Transmitter uptake systems also play a critical role in the control of neural activity by terminating transmitter activity. These systems were also earlier considered to be restricted to neuronal presynaptic sites, but work on cultures and in situ has shown transporters, especially for excitatory amino-acids and GABA, on astrocytes.

5.1. Receptors in cultured vs. acutely isolated astrocytes

Receptors for transmitters on glial cells were first shown in predominantly non-neuronal primary cultures prepared from neonatal rats [147,281,311,501]. When the astrocyte-specific marker glial fibrillary acidic protein (GFAP) [117] became available and widely used it was found that these same cultures were 95% or more astrocytic as determined by immunocytochemical staining for GFAP [31]. It was subsequently shown that these astrocyte cultures could also exhibit a number of "neuron-like" properties, such as voltage-gated Na^+, K^+, and Ca^{2+} channels, as well as receptors and high-affinity uptake systems for neurotransmitters [110,128,231,416,446,451]. The receptors found on cultured and other astrocytes have been summarised in several reviews [14,110,128,191,196,228–230,232,275, 283,295,305,306,500]. Therefore the details of receptor expression and action seen in primary astrocyte cultures will not be recapitulated here. Suffice to say that around ten adrenergic and related aminergic receptors, including their subtypes, nine amino acid and two purinergic, and at least eight peptide receptors have so far been found and studied in cultured astrocytes. This surprised many neuroscientists [100,232,445,493]. Although primary astrocyte cultures were very important in sensitising the neuroscience community to the complexity of astrocyte properties and their potential involvement in many aspects of CNS function it is apparent that the fidelity of such cultures in reflecting the properties of astrocytes in vivo, could not be assumed.

A number of the properties shown by the cultures have been shown to be a property of astrocytes in situ, such as glutamate uptake [265,333,492], and adrenergic β-receptor expression [6]. However, examples of differences between in vitro and in vivo have emerged as more in situ studies have been done [282]. For example, in the case of receptors it has been recently reported that the 5-hydroxy tryptamine (serotonin, 5-HT_{2A}) receptor, routinely found in primary astrocyte cultures [87,323], is rarely found on GFAP(+) astrocytes in rat brain sections when probed with cyclic deoxy ribose nucleic acids (cDNAs) for the receptor [200], although it has been reported in rat brain sections using an antibody [521] and messenger ribose nucleic acid (mRNA) for 5-HT receptor has been detected in corpus callosum [279]. Glial cells, either oligodendrocytes or astrocytes, identified electrophysiologically in spinal cord slices from P3 to P18 rats, were found to have glycine receptors,

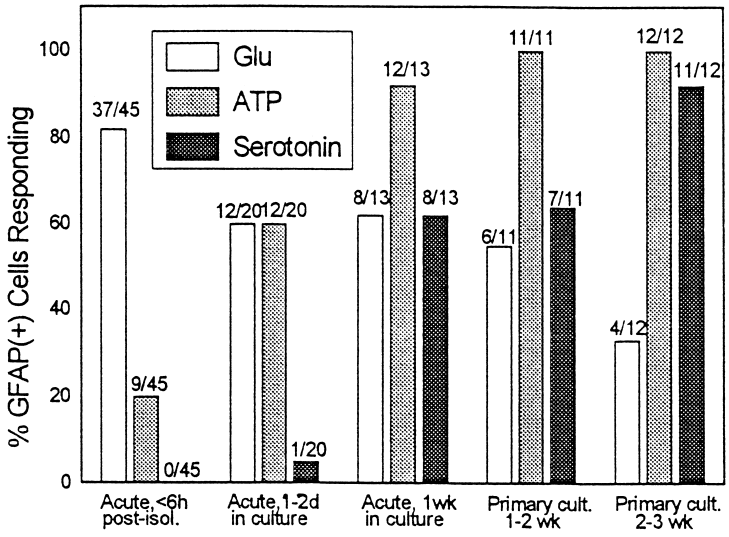

Fig. 6. Shows the percent frequency of all the GFAP(+) cells isolated from hippocampi of P1-10 rats that showed a Ca^{2+} response to 50 μM Glu, 10 μM ATP and 10 μM 5-HT. The data for the acutely isolated cells were from cells studied <6 h after isolation, the same cells after culturing for 1–2 days or one week in serum-containing medium. The primary cultures were from P1 rats and were cultured for 7–14 or 15–21 days in serum-containing medium, as indicated. All cells were tested for all three transmitters.

whereas they have not been found in primary cultures of these cells [239].

Using freshly isolated cells from the hippocampus, the Ca^{2+} responses of acutely isolated GFAP(+) astrocytes have been studied < 6 h after isolation, to glutamate, ATP, adenosine and 5-HT [233,234]. These cells frequently showed increases in intracellular Ca^{2+} in response to 50 μM ℓ-glutamate, less to ATP and no response to 5-HT (Fig. 6). This figure also shows that responses of GFAP(+) astrocytes in primary culture was almost a mirror image, whilst culturing the acutely isolated cells caused the response to resemble that of primary cultures.

5.2. Studies on astrocytes in non-cultured systems

Successful cloning of the gene for the mammalian adrenergic receptor [104] led immediately to elucidation of the receptor molecule's functional domains, including understanding of its topography in relation to the plasma membrane and identification of sites that are phosphorylated or involved in determining ligand-binding specificity, desensitisation and sequestration [34,104,464,538]. Another consequence of the successful cloning was the generation of antibodies directed specifically to the N-terminus, C-terminus and the third intracellular loop portion of the receptor molecule [465]. These antibodies have provided data that complement those gained previously by using antisera directed against whole receptor molecules [9,464]. With the use of these antibodies it became possible to localise receptors within intact tissue of various brain regions at varying developmental stages. Moreover, by combining the immunocytochemical localisation of β-adrenergic receptors with the immunocytochemical localisation of catecholaminergic terminals, it is possible to determine whether the two elements are likely to form synaptic and/or non-synaptic relationships.

The antibody directed against the third intracellular loop portion of β_2-adrenergic receptors recognises neurons. Within neurons, perikaryal organelles involved in protein synthesis, i.e. the Golgi apparatus and endoplasmic reticulum, as well as plasma membranes, dendritic shafts and spines are immunoreactive [7,8,13]. What was surprising, however, was the frequent recognition of plasma membranes that are apparently not part of or continuous with pre- or postsynaptic regions. This observation supported the proposal that norepinephrine may operate as a neurotransmitter via "volume transmission" in addition to conventional point-to-point synaptic transmission. This was an idea put forth first by investigators noting that noradrenergic terminals rarely form morphologically identifiable synaptic junctions [96]. This view is compatible with the notion that the locus coeruleus–norepinephrine system is involved in setting the behavioural states of vigilance and orientation toward sensory stimuli, while the GABAergic and glutamatergic systems mediate fast point-to-point transmission, such as sensory perception via circuits to and within the visual cortex [431,494].

More surprising are the results obtained when using the C-terminus antiserum to the β-receptor. It was observed that the predominant sites of immunoreactivity were the fine processes of astrocytes [6] (Fig. 7). Within layer 1 of adult rat visual cortices, greater than 90% of the immunoreactive profiles encountered were astrocytic. It is apparent that, in spite of the fact that these astrocytic sites cannot be directly involved in synaptic transmission, they are in the immediate proximity of or within a few micrometers of catecholaminergic (presumably noradrenergic) axons, and can therefore presumably be receptive to neuronally

released norepinephrine. The remaining 10% of the immunoreactive profiles are mostly dendritic, although axonal labelling also is evident. These immunoreactive neuronal profiles are not necessarily adjacent to immunoreactive astrocytic processes. Instead, the immunoreactive astrocytic profiles typically are adjacent to asymmetric (presumably excitatory [153]) axo-spinous junctions. Based on these observations, it was [6] hypothesised that β-adrenergic receptors on astrocytes may be involved in boosting neural processing of sensory information by stimulating glycogenolysis (reviewed in Refs. [272,460], see also Refs. [3,11] and Section 4). Stimulation of β-adrenergic receptors has also been reported to reduce the uptake of ℓ-glutamate in primary astrocyte cultures which, if it occurs in vivo, would also lead to increasing glutamatergic activity prior to desensitisation [172].

The reason for the rather selective immunolabelling of astrocytic processes by the C-terminus antiserum remains unknown. One possibility is that there is prevalence of one conformational state of the receptor within astrocytes, relating to the degree of phosphorylation of the C-terminus by β-adrenergic receptor kinase and of the agonist-promoted desensitisation [34].

Other receptors have been localised to glia in situ using the approach described above. Use of antibodies to dopamine D2 receptors [425], NR1 subunits of the NMDA-type glutamate receptors [12] on astrocyte plasma membranes, and more recently NR2A/B also have been found, predominantly on the membranes of the processes of cortical astrocytes [71]. Alpha amino, 3-hydroxyl, 5-methyl, 4-isoxazole proprionic acid (AMPA) subunits of glutamate receptors [72,127] and α_2-adrenergic receptors [12,399] also have shown antigenic sites on astrocytic plasma membranes.

5.3. Transmitter-induced responses in astrocytes in slice preparations

The hippocampus has long been recognised as an excellent model for examining synaptic interactions between specific neuronal groups, largely due to its laminar organisation which permits the preparation of brain slices where the major neuronal pathways remain intact. Within the hippocampus CA3 region, pyramidal neurons send glutamatergic input to CA1 pyramidal neurons via the Schaeffer collateral pathway, forming excitatory synapses in several regions of the CA1 including the stratum radiatum. When hippocampal slices from young animals ranging in age from 7 to 16 days are loaded with calcium-sensitive indicator dyes, astrocytes are the principal cells that take up and trap the dye [352,353]. Confocal microscopy, which has the ability to collect light from optically thin sections tens of microns deep into tissue slices was used to study the neuroligand Ca^{2+} responsiveness of astrocytes in situ using calcium-sensitive indicator dyes. All cells studied were subsequently identified as astrocytes by their staining for GFAP. Perfusion with glutamatergic ligands showed that astrocytes within hippocampal slices respond to glutamate, kainate, AMPA, *trans* 1-amino, cyclopentane-1,3-dicarboxylic acid (*t*-ACPD) and NMDA with increases in

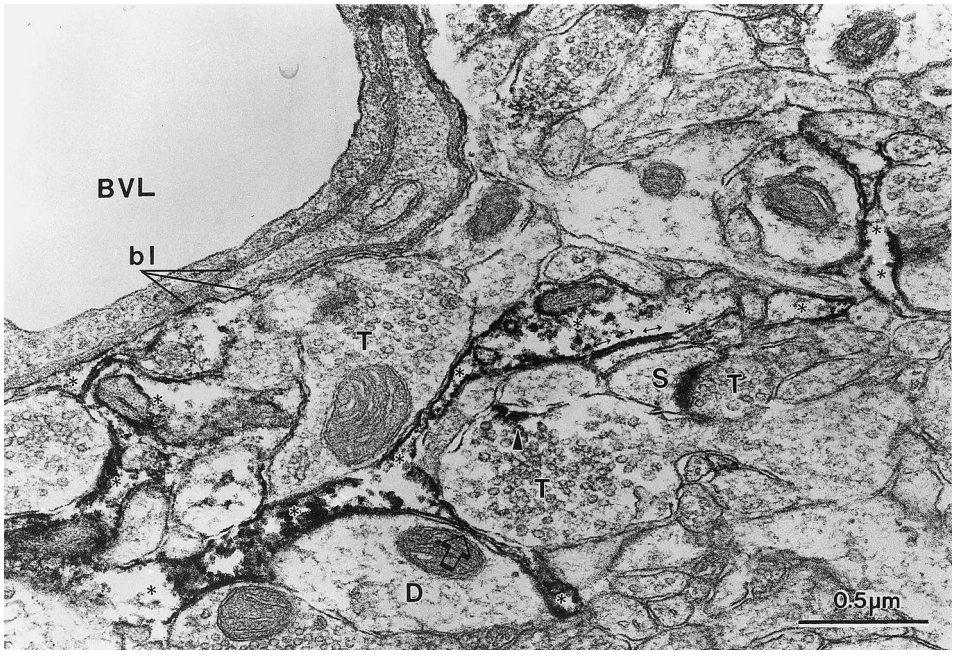

Fig. 7. Electron micrograph showing astrocytic profiles immunopositive to antiserum for the C-terminus end of the β-adrenergic receptor (see text), near a blood vessel. Asterisks are within the cytoplasm of immunoreactive astrocytic processes. Note the immunoreactivity along a gap junction formed by an astrocytic profile (three double-sided arrows). Frequently, astrocytic processes containing β-adrenergic receptor-immunoreactivity are so fine that they are the thickness of two plasma membranes (large open arrow within a dendritic profile, D). T = terminal; S = dendritic spine; bl = basal laminae; BVL = blood vessel.

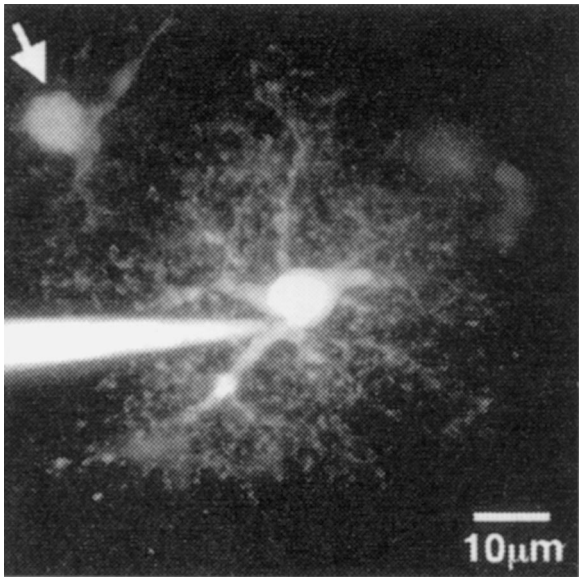

Fig. 8. Fluorescent image of an astrocyte located in a hippocampal slice from 7–16 days old rat. Cell was filled with a dye (Cy5-EDA) from a patch pipette visible on the left of the image. The arrow shows another astrocyte coupled to it as indicated by dye transfer (from Ref. [27], reproduced with permission).

$[Ca^{2+}]_i$ [353]. Preincubation with tetradotoxin (TTX) to block Na-dependent neuronal action potentials failed to block calcium increases mediated by either glutamate, kainate, AMPA or *t*-ACPD, but did block approximately half the responses to NMDA. Preincubation with receptor antagonists or removal of extracellular calcium suggested that hippocampal astrocytes exhibit both ionotropic and metabotropic glutamatergic receptors.

Both ATP (P2-receptor agonist) and adenosine (P1-receptor agonists) are known to be important neurotransmitters in the CNS. In vitro, astroglia exhibit calcium responses primarily to ATP and not adenosine [221]. Perfusion with purinergic ligands caused the majority of astrocytes to respond to both adenosine and ATP; however, most of the responses to ATP (but not all) were blocked by the adenosine receptor antagonist, theophylline, in situ [353]. Furthermore, a selective P2-receptor antagonist that is less susceptible to breakdown than ATP failed to increase Ca^{2+} in all but a few astrocytes. The above findings suggest that the release of glutamate or adenosine/ATP (converted in vivo to adenosine via ecto-ATPases) from neurons leads to calcium increases in astrocytes in vivo. This behaviour corresponds well with recent findings on GFAP(+) astrocytes acutely isolated from rat cerebral cortex [233,234,347]. An α_1-mediated $[Ca^{2+}]_i$ increase in astrocytes has been identified electrophysiologically and by dye-coupling, in the CA1-3 region of hippocampal slices from P21-42 rats to perfusion with specific agonists [111].

Electrophysiological studies on astrocytes in situ in mammalian brain slices or tissue using patch–clamp methodology [40,64,271,453,454], have shown conductance changes in voltage-clamped astrocytes or presumed glial cell bodies in hippocampal slices when glutamate and GABA were perfused (see Fig. 8 for a dye-filled astrocyte from the hippocampus with an attached patch pipette).

In a recent study using whole-cell voltage clamping, Steinhauser et al. [453] perfused GABA and glutamate onto P9-12 mouse hippocampal slices. Besides $GABA_A$ receptor-mediated currents, it was found that in GFAP(+) astrocytes, glutamate decreased the resting K^+ conductance and that most glutamate-mediated responses were through the kainate receptor. A small group of these cells also had NMDA-activated currents. High concentrations of glutamate of 1mM were presumably needed because of glutamate uptake. However, in a study on P21-42 rat hippocampal slices, there was a failure to show any astrocytic responses to glutamate or the metabotropic glutamate receptor agonist *t*-ACPD, and kainate caused brief Ca^{2+} changes in only 25% of the cells [111]. In contrast, norepinephrine and the α-1-agonist phenylephrine evoked complex $[Ca^{2+}]_i$ signals in 100% of the astrocytes tested. The differences in the glutamate responses in these two studies could be because of species differences, age-difference, or because too low glutamate concentrations were used in the second set of studies. However, clear indication of age-dependency of the glutamate-mediated events has been shown in a study on rat hippocampal slices, where metabotropic glutamate receptors mediated the phosphorylation of GFAP in immature but not in adult rats [527]. GABA- and glycine-induced chloride currents and block of K^+ conductance mediated by $GABA_A$ receptor activation have also been shown in astrocytes in rat spinal cord slices [336].

Various complexities and difficulties do arise in the interpretation of whole-cell recordings from slice preparations. A decrease in detected net current could indicate either an inhibition of one type of K^+ current or increase in another K^+ current in the opposite direction; e.g. decrease in delayed rectifier K^+ outward current or increase in an inward rectifying K^+ current with the outward current being unaffected [21]. This increased inward current could, besides being a direct result of transmitter exposure, also result from elevated $[K^+]_e$ due to K^+ release from contiguous, glutamate-activated neurons in the tissue preparation. However, it is clearly a response of astrocytes in situ, not subject to possible culture changes.

5.4. Transmitter-induced electrical changes in astrocytes

Work in primary astrocyte cultures showed marked depolarizations to applied glutamate and kainate [35,225]. Since these effects could not be due to accumulation of extracellular K^+ in these neuron-free, perfused astrocyte monolayers, the depolarizations were attributed to glutamate receptors or electrogenic glutamate uptake. Later work clearly showed that in these cortical astrocyte cultures the depolarisation was via a Kainic acid (KA)/AMPA type receptor [447]. However, electrogenic glutamate uptake

was found to be the basis of the glutamate-induced depolarisation in acutely isolated astrocytic Müller cells from the amphibian retina [19,37], and primary cultures of cerebellar astrocytes [534]. Later, in situ hybridisation studies detected transcripts for the AMPA receptor in Bergmann glial cells in tissue sections. It was found that this receptor lacked the GluR2 subunit, making it Ca^{2+} permeable [41,303,422]. This type of receptor was also expressed by primary cultures of the Bergmann glia [215], and is thus an example of how primary cultures can reproduce the in vivo state. In contrast, acutely isolated glial cells from P9-12 hippocampus showed a high level of GluR2 by single-cell reverse transcriptase polymerase chain reaction (RT-PCR) [452]. It is also beginning to appear that NMDA receptors, absent from cultured astrocytes, are present in some glial cells in situ [452].

Serotonin (5-HT) has been shown to hyperpolarise glial cells [518], using, as did earlier workers [250], the neuropile glial cells of the leech CNS, which could also be conveniently impaled with sharp microelectrodes. The hyperpolarisation was likely due to activation of a K^+ conductance. Recently these studies in the neuropile glial cells have been extended [304], measuring $[Ca^{2+}]_i$ with fura-2 in the exposed cell. It was found that 5-HT increased $[Ca^{2+}]_i$, and that this was largely dependent on external Ca^{2+}. Using current injection with 2 electrodes, a decrease in membrane resistance was also found which was ascribed to an increased Ca^{2+}-dependent K^+ conductance.

5.5. Ionotropic transmitter receptor channels in astrocytes

Ionotropic glutamate receptor channels activated by ℓ-glutamate or its specific analogues NMDA, AMPA or kainate and variably permeable to K^+, Na^+ and Ca^{2+}, are some of the most intensively studied receptor ion channels. The ionotropic glutamate receptor channels, especially the NMDA receptor channel, have been suggested to play important roles in brain maturation [123], some pathological conditions and in behavioural disorders such as schizophrenia [150]. The NMDA receptor has not been found in cultured astrocytes, but is sometimes seen in astrocytes in situ [361,453]. The AMPA or kainate-activated receptors are found in most astrocyte preparations [213,236,339]. In cultured trout astrocytes glutamate, kainate and quisqualate-induced inward currents are carried mainly by Na^+ [65]. Comparison of the neuronal (granule cells) and astrocytic (type-2 astrocytes) receptor channels in cerebellar cultures showed striking similarities in the whole-cell currents, single-channel function and pharmacology of their AMPA/kainate receptor channels [533]. In cultured cortical type-1 astrocytes, glutamate activated inward currents are mainly carried by the electrogenic glutamate uptake system and kainate-activated Na^+-dependent currents [455]. In type-2 cortical astrocytes, kainate seems to activate currents carried by both Na^+ and Ca^{2+} [288,455].

Other ionotropic receptor channels found in astrocytes are the $GABA_A$ receptor chloride channels, which have been found in rat optic nerve and acutely isolated hippocampal astrocytes [42,133]. In astrocytes cultured from spinal cord two different types of $GABA_A$ receptors have been characterised; in both fibrous and protoplasmic astrocytes the local anaesthetic pentobarbital, as well as the benzodiazepine diazepam, increased GABA-induced currents, but the inverse benzodiazepine agonist DMCM reduced GABA-induced currents in "fibrous" astrocytes and increased them in "protoplasmic" astrocytes [398]. Similar results have been obtained from acutely isolated rat hippocampal astrocytes [133]. Ion channel activities related to adrenergic receptor activation have surprisingly, in view of the evidence that these receptors exist on astrocytes in situ, has not been studied much in astrocytes [428,460]. However, conductance changes have been found in type 1 astrocytes in primary culture which were mediated by α_1 receptors and seemed to involve a change in Cl conductance [36].

5.6. Metabotropic receptors and calcium-mediated events in astrocytes

Astrocytes are known to show great plasticity in their receptor expression and function depending on their developmental stage, region or experimental conditions. For example the metabotropic glutamate receptor agonist *t*-ACPD induces phosphoinositol hydrolysis over 10 times more in astrocytes cultured in serum-free compared to serum-containing culture media. This difference was shown to be specific for glutamate receptors as no such difference was detected for Acetyl choline or norepinephrine [291]. Metabotropic ($mGluR_5$) receptor expression was found to be enhanced when astrocytes were cultured in chemically defined media, and it was suggested that this resembles reactive gliosis in vivo [292]. The amount of $mGluR_5$ present in astrocytes has been found to decrease drastically in brain with development [505]. Metabotropic RNAs encoding glutamate, acetylcholine and serotonin receptors have been found in corpus callosum tissue, and by in situ hybridisation studies [279].

One consequence of the activation of metabotropic receptors can be a rise in intracellular Ca^{2+}. This usually involves a first, fast peak of calcium release from intracellular stores via the IP_3 pathway, and often a second long-lasting elevation and/or oscillation of Ca^{2+} mediated by Ca^{2+} influx at least partially through Ca^{2+} channels, such as the voltage-sensitive ℓ-type Ca^{2+} channel [57,110,227,504]. Among a plethora of possible effects, the increased $[Ca^{2+}]_i$ can lead to increased and transient activation of Ca^{2+}-sensitive K^+, Cl^- and Ca^{2+} channels, which may then cause alteration of the astrocyte membrane potential, followed by further voltage-dependent channel activation. Other metabotropic receptors linked to increases in $[Ca^{2+}]_i$ in primary astrocyte cultures are bradykinin B_2 receptors inducing $[Ca^{2+}]_i$ elevation together with mainly inward currents [148,455]. Many of the metabotropic receptors also activate cyclic adenosine

monophosphate (cAMP)-mediated intracellular pathways, which in contrast may lead to decreased Ca^{2+} currents, but increased K^+ currents (see Ref. [296] for a review).

In conditions where long-lasting excessive transmitter release from neurons, or reduced uptake by astrocytes, occurs, $[Ca^{2+}]_i$ may stay permanently elevated, which will lead to overactivation of Ca^{2+}-sensitive ion channels, triggering Ca^{2+}-mediated cell metabolism, and, if prolonged, eventually to cell death.

5.7. Transmitter transporter systems in astrocytes

Uptake systems are very important in determining the concentrations of transmitters remaining in the synaptic cleft and therefore the magnitude and duration of transmitter effects. Three separate cyclic deoxy ribose nucleic acids (cDNAs) encoding three different glutamate transporters in brain have now been described. In rat brain two of these localise to astrocytes (GLT-1 and ℓ-glutamate/ℓ-aspartate transporter (GLAST)) while a third (EAAC1) is found in neurons [219,265,400].

Altering the activities of the astrocytic EAA transporters has been shown to alter neuronal function. Lesioning astrocytes in guinea pig hippocampal slices with fluoroacetate resulted in inhibition of post-synaptic potentials, which could have been due to loss of a variety of metabolic or other support functions of astrocytes [226]. It was also found that the response to added glutamate was prolonged. More recently, specifically reduced levels and activities of the three EAA transporters individually has been acheived by chronic intraventricular administration of the specific antisense mRNAs in mice [401]. It was found that only reduction of the astrocytic GLT-1 or GLAST increased steady state $[glu]_e$ levels while similar reductions in the neuronal EAAC1 had no effect. Thus such lesion studies can address the question of functional relevance. Raising medium $[K^+]$, as seen in cerebral ischemia, leads to reversal of the glial transporter in cultured astrocytes and this may be one source of the raised $[EAAs]_e$ seen in these conditions which are considered to lead to neuronal toxicity (excitoxicity) [234,480]. All these data suggest that alterations in the functioning of the astrocyte EAA transporter will have profound effects on extracellular glutamate levels thereby affecting normal as well as pathological brain function.

The presence of other transporters on astrocytes have only been established in primary cultures and are at much lower activities than those for the EAA transmitters. These include the Na^+ -dependent and fluoxetine-sensitive high affinity uptake system for 5-HT. In cultures the 5-HT system is dependent on serum, in contrast to the glutamate uptake system in culture which is unaffected by growth in serum-free medium [235]. High affinity uptake has also been shown by autoradiography in astrocytes freshly isolated from rat cerebral cortex [83]. It may be that many transporters are present in very localised regions, so that they would only be infrequently seen in isolated cells or in situ. Inhibition of uptake systems for transmitters such as serotonin have profound effects on mood, appetite and other affective and non-affective behaviours [76,134].

5.8. Neuron–glia interactions in turnover of glutamate and glutamine

The strongest evidence for the operation of a glutamate–glutamine cycle in the intact CNS comes from exposure of the isolated rabbit retina to methionine sulfoximine, a relatively selective inhibitor of the glial enzyme, glutamine synthetase. This drug completely depletes some retinal neurons (bipolar cells and ganglion cells) of glutamate, even when the incubation medium contains large amounts of glutamate and glutamine [358]. This finding unequivocally shows that these neurons depend upon glutamine synthesis in their glial Müller cell neighbours for their entire supply of glutamate, and that they have no access to extracellular glutamate or glutamine, probably because the neurons are completely invested by glial processes [371]. These findings have been confirmed in organotypic cultures of hippocampal slices [253] with the exception that extracellular glutamine could be utilised directly by the neurons, perhaps because the glial ensheathment is less extensive in hippocampal slices.

In an even more convincing experiment, rabbit retinae were incubated in medium containing only the non-physiological ɗ-isomer of glutamate, which was absent from the tissue prior to incubation [356]. This isomer is transported by a glutamate uptake carrier and converted to ɗ-glutamine by glutamine synthetase, which is not stereospecific. However, the subsequent hydrolysis of glutamine to glutamate by glutaminase can only take place with ℓ-glutamine, not ɗ-glutamine, as the substrate. Therefore, the neurons which depend upon astrocytic glutamine for their supply of glutamate stain for ɗ-glutamine, supplied from adjacent Müller cells, but not for ɗ-glutamate. The adjacent Müller cells must rapidly release the synthesised ɗ-glutamine, since they stain intensely for accumulated ɗ-glutamate, but only little for ɗ-glutamine.

Immunocytochemical studies of retina have not yet examined whether α-ketoglutarate is an additional precursor for neuronal glutamate. In any case, the sheath of Müller cell processes that encloses each retinal neuron may act as a barrier to exogenous substrates, thereby ensuring that the neurons can only access α-ketoglutarate that has been released from neighbouring Müller cells.

5.9. Summary

A surprisingly large number of transmitters have been shown to have receptors on astroglial cells in primary culture. Several of these have been shown on astroglia in situ but, to date, the relevance of only a few of these to neural–glial interactions has been revealed. Of these, β-adrenergic receptors and those for excitatory amino acids, especially glutamate, are most prominently involved.

These receptors mediate profound energetic interactions between neurons and glia, as described in the last section. However, glutamate may also activate ion channels and therefore affect spatial buffering of K^+. Glutamate receptors and transporters are also responsible for Ca^{2+} currents in glia and the removal of glutamate from the ECS, respectively. Crucially glia are responsible for the provision of the glutamate substrate, glutamine for neurons.

6. Morphological plasticity of neuron–glial interactions

Current evidence indicates that astrocytes alter their physical relationships with neurons, termed structural plasticity, to regulate neuronal communication. This is thought to be accomplished by astrocytes rearranging their processes to modulate synaptic contacts [136,181,287,332] and through the establishment and maintenance of neuronal subgroupings [73,491]. In each region where astrocytic structural plasticity has been documented in the normal adult CNS, the commonality is that a fairly discrete group of neurons is challenged to adapt to a changing internal or external milieu.

6.1. Structural plasticity of hypothalamic astrocytes

Stimulation of the hypothalamic supraoptic nucleus (SON) and nucleus circularis of rats by water deprivation results in a profound remodelling of these nuclei, seemingly brought about by a deensheathment of magnocellular neuroendocrine cells (MNC's) by their neighbouring astrocytes [496,497]. Similar observations in the SON of lactating animals have been described [487]. This work was soon extended with the finding that astrocytes of the posterior pituitary, [412] pituicytes, also change their ensheathment of neurosecretory axons with stimulation [498].

The hypothalamic neurohypophysial neurosecretory system (HNS), consisting primarily of the SON, the paraventricular nucleus, nucleus circularis and the posterior pituitary, responds to a variety of stimuli by activating MNC's to synthesise and/or release their neuropeptides oxytocin, vasopressin, or both. These stimuli include parturition, lactation, and dehydration brought about by 2% saline substitution for drinking water, intraperitoneal injection of hypertonic saline, or water deprivation. Structural changes of the SON have also been observed in virgin female rats induced to behave maternally by exposure to rat pups [409], behaviours known to be associated with oxytocin [124] and in response to restraint stress [293].

From electron microscopic studies showing an absence of astrocyte processes where they were normally seen, it was hypothesised [496,497] that HNS astrocytes and pituicytes [498] *actively retract* their processes from between MNC's and posterior pituitary axons, respectively, concurrent with stimulation. Further observations also indicated that retraction of astrocytic processes leads to an "uncovering" of postsynaptic sites and the establishment of additional synaptic contacts [181,487] in the form of novel "multiple" synapses (one presynaptic profile in synaptic contact with 2 or 3 postsynaptic elements) in both the somatic and dendritic zones [342,485]. The absence of glial processes was thereafter implicated in the formation of dendritic bundles [343,344,409] and gap junctions [3,66]. With the cessation of stimulation, most of these changes are entirely reversed [182], with astrocytes apparently reinserting their processes around neighbouring neuronal structures. Analogously, in the posterior pituitary, the resident pituicytes re-envelope neurosecretory axon terminals, again segregating them from the fenestrated capillaries there and hampering peptide release.

The evidence thus indicates that HNS astrocytes are involved with events associated with neurosecretion. The original observations and their functional import have been recent subjects of extensive reviews [179,180,486].

To further assess the possible active rearrangement of SON astrocytic processes, immunoreactivity for GFAP [499] was investigated [413], reasoning that changes in GFAP might accompany gross structural changes of these cells. GFAP immunoreactivity in the SON of lactating rats was reduced, relative to control rats in the oestrous phase of their cycle. These results are consistent with the notion that astrocyte processes retract. Dehydration also has the same reversible effect on GFAP immunoreactivity [186]. The GFAP immunoreactivity of the SON significantly decreased in the dehydrated group compared to controls and subsequently returned to control levels with rehydration.

While changes in GFAP immunoreactivity are consistent with the idea of morphological remodelling, they are not entirely irrefutable evidence for active structural plasticity [413].

*6.2. Direct light and electron microscopic evidence of structural plasticity of SON-*VGL *astrocytes*

Astrocytes in the ventral glial limitans subjacent to the SON (SON-*VGL*) send exceptionally long GFAP- and vimentin-immunoreactive processes dorsally into the SON where they surround and ensheathe magnocellular neuronal somata and dendrites [32,537]. The thickness of the SON-*VGL* was significantly decreased in 9-day dehydrated animals when compared with controls [30]. This reversed toward control levels with rehydration. An electron microscopic evaluation of individual astrocytes in the same material revealed that a reversible overall reorientation of astrocyte cytoplasm, from a direction perpendicular to the pial surface to one parallel to the pial surface also occurred with stimulation. The orientation of individual astrocytes was much more likely to be vertical in control animals. Conversely, astrocytes with a horizontal orientation were significantly greater in number in 9-day dehydrated animals. It appears that astrocytes in the SON-*VGL* have a preferred orientation, depending on hydration state.

The possible functional significance of astrocyte

reorientation may be to contribute to the clearance of extracellular potassium from the SON to the cerebrospinal fluid (CSF) of the subarachnoid space (SAS). An increase in K^+ in the SAS occurs at the inferior limit of the SON during antidromic stimulation of the pituitary stalk [68] suggesting that K^+ clearance occurs through spatial buffering by glial cells to the SAS during stimulation of the SON. Astrocyte reorientation may enhance K^+ clearance by increasing the astrocytic surface area interfaced between the SON and SAS, or by otherwise making an existing astrocytic syncytium more efficient. One possible functional consequences of decreased SON-*VGL* thickness is that it may facilitate increased CSF-SON communication. It is known that diffusible molecules may pass into the parenchyma from the CSF [85,510] or vice versa. Given that peptides are released by exocytosis from SON dendrites [357], a thinner SON-*VGL* might enable peptide release directly into the CSF. This may provide a means of communication between the two supraoptic nuclei, as well as other circumventricular structures [473] and explain, at least in part, how neurohypophysial peptides get into the CSF [378]. A second possibility is that a thinner VGL enables an osmotic signal contributed by a hyperosmotic CSF [334,359] to reach osmosensitive magnocellular neurons in the SON [331].

6.3. A possible role for pituicyte proliferation in structural plasticity of the neurohypophysis

It has been known for a long time that there is significant proliferation of astrocytes in the SON and posterior pituitary of very young rats when the HNS is stimulated by dehydration [307,337]. Observations of astroglia in vitro, which often lose their processes prior to cell division, suggested that re-entry into the cell cycle may account, in part, for the changes seen in pituicyte ensheathment of axons in response to stimulation. Pituicytes in fully adult rats are also capable of robust proliferation with dehydration [308] when they lack the well-developed processes seen in non-proliferating pituicytes. Hence, reentering the cell cycle may account for at least some of the reduction of pituicyte coverage of HNS axon terminals seen with dehydration.

6.4. A possible role of glycoconjugate molecules in SON plasticity

The plasticity observed in the stimulated SON may also involve tenascin [119,434], a molecule thought to influence the direction of outgrowing axons and dendrites and, ultimately, determine specific cell–cell interactions. In the normal adult CNS, tenascin expression generally has been reported to be minimal except that in tissue sections from control adult rats the tenascin antibody stained the SON-*VGL* and dendritic zone subjacent to the SON [434]. This staining was absent in tissues obtained from dehydrated rats. Likewise, Coomassie stained mini-gels of SON-*VGL* tissues revealed a decrease in the 210–220 kD tenascin band after prolonged dehydration. An increase in tenascin expression back to or beyond control levels was also repeatedly observed with rehydration.

Since immunocytochemistry of tissue sections revealed tenascin to be predominantly present in the SON-*VGL* itself and in the dendritic zone of the SON, through which son-*VGL* astrocytes project, its effects are likely to be limited to dendrites, their associated synapses, and local astrocytes. One major impact of its decrease in the SON-*VGL* with dehydration might be to enable dendritic bundling [344] and gap junction formation [66]. Both are thought to enhance the neurosecretory activity of magnocellular neurons. In this regard, it has been shown that, with stimulation, gap junctions form only between dendrites of like-peptide containing neurons [67,183]. Similarly, at least for oxytocinergic somata, it has also been reported that direct membrane appositions usually occur only with other oxytocinergic somata or dendrites [56].

A role for cell-surface-associated molecules in the HSN has been suggested by the presence of the polysialic acid-linked form of the neural cell adhesion molecule, PSA-NCAM [488]. The deposition of PSA-NCAM is very similar to that of tenascin, being distributed most heavily in the SON-*VGL* and dendritic zone. Although these authors did not report variations in PSA-NCAM expression with changes in stimulus conditions, it is possible that PSA-NCAM, tenascin, and perhaps other extracellular matrix molecules work together to regulate plasticity in the SON. PSA-NCAM has also been reported on both neurosecretory axons and pituicytes of the posterior pituitary [240]. PSA-NCAM expression by pituicytes was dependent on intact neurosecretory axons: stalk transection led to its disappearance from the neural lobe, and suggests that extracellular matrix molecules can be regulated by neuronal signals from adjacent axons.

6.5. Astrocytic regulation of magnocellular neuron activity

In recent years additional interactions of SON and posterior pituitary astrocytes with magnocellular neurons have been discovered. Astrocytes show intense immunoreactivity in the SON-*VGL*, as well as lesser staining in astrocytes in the nucleus proper [86]. This is of interest since it has been known for some time that cultured astroglia release taurine in response to hypo-osmotic conditions [335] and that, acting at glycine receptors, taurine has an inhibitory effect on neuronal activity [208]. The cause of the low basal firing rates of MNCs had previously been unclear and, these new observations, suggested that it might be due to a tonic release of taurine from SON astrocytes [86]. Studies on dissociated SON MNCs determined that taurine, indeed, acts as an inhibitory agonist at MNC glycine receptors and as a partial inhibitory agonist at $GABA_A$ receptors [207]. By recording extracellularly in vivo in normally hydrated rats there was also seen a decrease in firing rates of phasic, vasopressinergic, neurons with application of taurine. A tonic release of taurine was measured from

dissected pieces of the SON. This could account for at least a portion of the basal inhibition of MNCs. Subsequent switching to a hypo-osmotic medium resulted in a much enhanced release of taurine, but not glycine or β-alanine. This indicates that astrocyte derived taurine may be the principle agonist at glycine receptors during osmotic regulation. The release of taurine from SON dissections are sensitive to fluorocitrate, an inhibitor of glial metabolism, further confirming its origin in SON astrocytes [94]. It was concluded that the hypo-osmotic dependent release of taurine from SON astrocytes is similar to that reported for cultured astroglia [335] and is due to a passive diffusion of taurine through volume sensitive chloride channels. It is of interest to note that under hypo-osmotic conditions, cultured astrocytes also swell [335]. Although it has yet to be established whether SON astrocytes also undergo swelling in response to hypo-osmotic conditions, such changes would represent another form of structural plasticity. Functionally, it is clear that the reduction in astrocytic elements seen with dehydration could increase neuronal excitability by removing the source of tonically released taurine.

In the posterior pituitary, the role of taurine in the HNS was expanded when it was demonstrated that pituicytes contain high amounts of immunoreactive taurine [294]. Incubation of neural lobes in hypo-osmotic medium also led to a release of taurine, but not other amino acids, into the bathing solution. Since pituicytes engulf the neurosecretory terminals of the posterior pituitary under basal conditions, taurine may be having an inhibitory effect on neurosecretory axons as well as their somata in the SON.

It is of note that despite similar observations made under dissimilar stimulus conditions in various parts of the HNS, not all astrocytes are the same, even within the individual nuclei of the HNS. In the SON alone, data indicate that SON-*VGL* astrocytes are a different population to those in the nucleus proper. First, SON astrocytes proliferate readily when the nucleus is activated [307,337], whereas SON-*VGL* astrocytes do not (Salm and Moats, unpublished observations). In addition, SON-*VGL* astrocytes express relatively high levels of vimentin [32] and tenascin [434] and taurine [86] whereas SON astrocytes do not. This suggests that there are a least two distinct populations of astrocytes serving the SON.

6.6. Structural plasticity of hypothalamic astrocytes in regions involved with reproduction

A number of investigators have examined the arcuate nucleus and median eminence of rodents and the preoptic area, mediobasal hypothalamus, and infundibular areas of primates as models to study gonadal steroid influences on brain plasticity [136,137], and have found evidence for structural plasticity of astrocytes. Synaptic remodelling has been studied in the arcuate nuclei of the rodent over the oestrous cycle [332]. By electron microscopy, it was determined that the percentage of neuronal membrane covered by synapses, as well as the number of synapses per 1000 μm of membrane decreased at oestrus, a time of high plasma oestrogen. This change occurred quickly, within the 24 h period between pro-oestrus and oestrus. Concurrently, they also observed an increase in the percentage of neuronal membranes, ensheathed by glial processes.

These observations have been extended to determine whether gonadal steroids affect synapses and glial appositions onto immunocytochemically identified gonadotrophin releasing hormone (GnRH) neurons in the mediobasal hypothalamus and preoptic area of Rhesus monkeys [525]. These areas may be analogous to the rodent arcuate, although the latter does not contain GnRH neurons. Ovariectomised animals demonstrated an increase in glial ensheathment in both the preoptic area and mediobasal hypothalamus, relative to animals receiving silastic implants with steroids or intact animals. This was concomitant with a decrease in synaptic density in both regions. While these results are consistent with the idea that glial ensheathment and synaptic coverage are inversely related [332], the impact of oestrogen appears to be opposite in the two experimental systems.

Astrocyte ensheathment/synapse relationships in the African green monkey found that ovariectomised animals had significantly more synapses and less glial ensheathment of hypothalamic infundibular neurons (equivalent to arcuate neurons in the rat) than did ovariectomised animals that had received intramuscular injections of 1 mg/kg estradiol valerate three days before sacrifice [309]. These results were the same as in the rat arcuate: oestrogen replacement led to an increased ensheathment of infundibular neurons and a concomitant decrease in synaptic density.

Astrocyte structural plasticity and its relationship to synaptic ensheathment has been re-examined in the rat arcuate nucleus [138]. Using modern stereological methods, this work again reported an increase in the neuronal membrane coverage by astrocytic profiles on the afternoon of pro-oestrus/morning of oestrus. As seen by glial fibrillary acid protein (GFAP) immunoreactivity, it was found that there was a simultaneous, reversible, increase in the surface density (amount of astrocyte surface in the neuropil) of astrocytic processes during the afternoon of pro-oestrus and the morning of oestrus. These then declined to baseline for the rest of the cycle. With electron microscopy, the incidence and size of filament bundles was measured to find that the size of filament bundles decreased on the afternoon of pro-oestrus and morning of oestrus, whereas the number of astrocyte profiles containing filament bundles increased at the same time. Since filament bundles decreased, but the number of glial profiles containing filament bundles increases, we can surmise that new GFAP is being assembled into nascent, smaller, filament bundles on the afternoon of pro-oestrus. GFAP messenger ribose nucleic acid (mRNA) increases on the afternoon of pro-oestrus and in response to injections of estradiol into ovariectomised female rats, confirming that changes of

GFAP immunoreactivity are related to alterations in GFAP mRNA synthesis [63,243].

Recently, it has been found that as plasma LHRH increased the distance of LHRH axon terminals from the median eminence portal system decreased [238]. That this may be due to changes in coverage by tanycytes is suggested by preliminary reports of dynamic interaction between these two elements. Confocal microscopic imaging has been used to demonstrate a dynamic relationship between luteinising hormone releasing hormone (LHRH) immunoreactive axon terminals and vimentin immunoreactive tanycytes (astrocytes) in the median eminence during the preovulatory surge of LHRH [237]. This change in tanycyte ensheathment appears similar to the structural plasticity of pituicytes observed in the posterior pituitary.

6.7. Studies of astrocyte plasticity in the suprachiasmatic nucleus

Another area of the hypothalamus where the activity of the resident neurons must vary in a matter of hours for the nucleus to perform its function is the suprachiasmatic nucleus (SCN). Here it has been shown that neurons in this region are responsible for encoding photic information and entraining circadian rhythms in rodents [290]. Rhythms in glucose consumption, electrical activity and peptide synthesis have been documented for this nucleus. Hence, the diurnal "clock" is contained in the SCN and determining its mechanism has been the subject of much interest [290]. Although, relative numbers of astrocytic cell bodies per neuron in the SCN-proper are a great deal lower than in surrounding areas of the anterior hypothalamus [502], the SCN is heavily invested with extensive astrocytic processes. Recently, evidence has been presented that GFAP immunoreactivity in the SCN is cyclic in Syrian hamsters, reaching a nadir approximately 2 h after activity onset, [263]. At this time, immunoreactivity was seen only in isolated stellate astrocytes, rather than spread in a complex meshwork throughout the SCN. Interestingly, this pattern persisted in animals that had been exposed to eight days of constant darkness. GFAP immunocytochemistry has also revealed that SCN astrocytes are larger during oestrus, relative to pro-oestrus in two strains of hamsters [268]. It was hypothesised that this "glial swelling" acted to block electrotonic coupling between SCN neurons because a light pulse given to animals after two weeks of constant darkness induced a much larger phase-shift in their activity rhythms when administered during oestrus versus pro-oestrus.

These data offer the tantalising possibility that SCN astrocytes might be involved with very profound aspects of behaviour: regulation of diurnal activities. However, it should be noted that electron microscopic investigations in the rat failed to detect any changes in the amount of astrocytic coverage of SCN neurons over the circadian cycle [116]. Hence, the significance and interspecies prevalence of these observations have yet to be determined.

6.8. Structural plasticity of hypothalamic astrocytes and behaviour

To summarise, regions of the hypothalamus where structural plasticity of astrocytes may ultimately prove to influence behaviour are the HNS, the arcuate nucleus and median eminence (rodents), preoptic area, infundibular nucleus, and mediobasal hypothalamus (primates) and the suprachiasmatic nucleus. The overall function of the hypothalamus, that of integrating sensory, endocrine, visceral, and internal metabolic signals to maintain homeostasis, is accomplished in part by adjusting motivational states and resultant overt behaviours. This includes ingestive behaviours necessary to maintain the internal milieu, i.e. feeding and drinking. It also applies to reproductive and parenting behaviours including parturition and lactation, lordosis, crouching, nest building, pup retrieval and nursing, and overall activity levels.

6.9. Correlation of astrocytic structural plasticity with long term potentiation and kindling in the hippocampus

Hippocampal long-term potentiation (LTP) has become a model for a cellular explanation of biological information storage, i.e. memory [23]. Kindling, a technique whereby hippocampal tissue is rendered seizure-prone, shares many similarities with LTP [43,511]. A dynamic correlation of astrocytic structural changes with synaptic plasticity has been demonstrated with both LTP and kindling [43,511]. An increase in the number of synapses in the hippocampus is a feature of both LTP [54,264] and kindling [185]. In rat, hippocampal dentate gyrus potentiations resulted in increased astrocytic coverage of dendritic buttons and spines compared to controls [520]. Similar ultrastructural changes of astrocytic processes occurred in the hippocampal CA1 region when rats were kindled to five generalised seizures [185]. In addition, the kindled rats demonstrated an increase in the density of synapses on dendritic shafts simultaneously with an increase in the volume fraction of astrocytic processes (a measure of how much of the neuropil is composed of astrocytes). Shorter intervals were not examined, therefore it is not known how rapidly these changes occur or if they involve an initial retraction of processes followed by an overall increase of astrocytic membrane. The most parsimonious interpretation of the astrocytic change with kindling is that it subserves the metabolic needs associated with the increased number of synapses and the greater activity of neurons with kindled synapses.

The expression of GFAP may be linked to mechanisms associated with neuronal synaptic efficiency, as mutant mice, created to be deficient in GFAP, demonstrate enhanced LTP in the hippocampus, compared to wild types [280]. Corroborative reports indicate GFAP up-regulation in kindling [82,171], beginning as soon as after the first afterdischarge [458].

The link between LTP and astrocytes is further

established in GFAP-null mice [280]. These mice lack GFAP, do not compensate by upregulating vimentin or nestin and have astrocytic prosesses at the pial surface that were smaller and lacking in intermediate filaments in comparison to wild type. Most interesting, these mice also displayed LTP in response to submaximal stimulus intensity compared to wild type, strongly suggesting a role for GFAP in regulating LTP.

The effects of oestrogen on hypothalamic tissues are also seen in the hippocampus. Electrophysiological studies of the hippocampus have demonstrated that neuronal excitability increases with the rising levels of oestrogen that occur during the afternoon of pro-oestrus. Seizure threshold is lowered during pro-oestrus [484] and female rats examined during the afternoon of pro-oestrus demonstrated the greatest degree of LTP compared to male rats and female examined at each phase of the oestrous cycle either in the morning or afternoon [519]. Anatomically, the number of dendritic spines and axo-spinous synapses in the hippocampus fluctuates across the rat oestrous cycle, being high on pro-oestrus and low on oestrus [528,529]. Interestingly, the volume fraction of astrocytic processes, as estimated in electron micrographs, also fluctuates across the oestrous cycle, being significantly lower on the afternoon of pro-oestrus than on the afternoon of oestrus [242].

6.10. Experience induces structural plasticity of astrocytes in the visual cortex

Unlike LTP and kindling, the use of the "enriched" or "complex" environment procedure, originated by Hebb [187] has since been extensively used [154,397]. It permits the assessment of structural plasticity of all cortical neural elements in relation to an animal's interaction with the environment.

Neurons in the visual cortex of rats raised in enriched conditions (EC) have increased dendritic arborization, greater numbers of synapses per neuron and greater lengths of synaptic contact zone, compared to individually caged (IC) rats [155]. These neuronal changes are paralleled by an increased volume fraction of glial nuclei [495] and a time-dependent increase in the surface density (the amount of astrocyte surface in the neuropil) and number of astrocytes positive for GFAP [435]. Recent findings also demonstrate that astrocytic processes directly apposed to synapses are altered by experience. EC rats have a greater surface area of astrocytic processes surrounding synapses than the IC rats [216]. These data suggest that astrocytes are not just filling up the extra volume like glue or packing material, but appear to be selectively participating in synaptic formation and plasticity.

Time-course work suggests that the response of astrocytes in EC rats appears to be two-phased: a period of astrocyte growth, followed by one of astrocyte proliferation. GFAP immunoreactive astrocytes grew larger in the EC rats over the first month of exposure. With an additional month of EC exposure the relative size of the astrocytes decreased while the number of GFAP-positive cells increased [435]. Similar to astrocytes in the activated HNS, the postnatal proliferation of visual cortical astrocytes appears to be greatly influenced by environmental complexity: a consistent result from a number of laboratories is an increase in the number of glial cells in EC rats compared to those raised in standard conditions [1,97,481]. Because the visual cortex of young–adult rats exposed to complex environments shows dendritic growth and synaptogenesis, events that parallel those of development, it is possible that the proliferation of astrocytes provides a transient population of "young" cells that recapitulate the conditions necessary for early developmental levels of neuronal plasticity.

It is of interest that the two phase response of astrocytes measured in layer IV of the visual cortex was not seen in layers II/III. In contrast to the slow change of astrocytes within layer IV, measurements of GFAP-IR in cortical lamina II/III revealed significant increases within 4–10 days after the onset of complex housing [217]. These measurements are consistent with the detectable increase in dendritic length in layer III that occurs within four days after the onset of differential rearing [512]. The sluggish astrocytic response in layer IV in the presence of rapid neuronal and astrocytic changes in layer III is a subtle example of the remarkable heterogeneity in GFAP-IR found across brain regions. Indeed, studies of regional differences in the morphology and biochemistry of astrocytes indicate a heterogeneity of astrocytes which may rival the variations in form and function of neurons [15,167].

6.11. Motor learning induces synaptogenesis and astrocytic structural plasticity in the cerebellum

As we have seen in the visual cortex and hippocampus, astrocytic volume fraction and process surface density increases in association with the addition of synapses. The increased astrocytic parameters could reflect responses to either increased synaptic activity, synapse number, or to both. A paradigm that allows these two influences to be separated is to subject a "learning" group of rats to daily practice on a series of difficult motor tasks. Two "activity" control groups are additionally subjected to either forced exercise on a treadmill or voluntary exercise in a running wheel attached to the home cage. In the cerebellar paramedian lobule, the exercise group has a higher density of capillaries relative to both a third, "inactive" control and the learning group, indicating that the exercise manipulation placed increased metabolic demands on the tissue [29]. In the learning group, synapse number per neuron was increased relative to all other groups, suggesting that synapse addition correspond specifically to learning. Using the same tissue, it has been found that an increased volume of astrocytic processes per Purkinje cell occurred only in the learning animals [2]. Hence, in this case, structural plasticity of astrocytes appeared to correspond with

learning-related synaptogenesis rather than to a general increase in metabolic or synaptic activity.

With respect to dynamic astrocyte–neuron interactions in the cerebellum, in vitro evidence indicates that cerebellar astrocytes act to modulate synaptic density. Purkinje cells in glia-free cultures form aberrant synapses with their own somata and dendrites. When cerebellar astrocytes were added to these cultures the number of synapses decreased and the number of non-innervated spines increased [287,424]. Astrocyte-conditioned medium, similarly, increased the number of non-innervated spines, apparently by proliferation of spines in the absence of presynaptic innervation [423].

The cerebellum is important for the control and fine tuning of movements that appear to occur through a motor learning process involving both synaptogenesis and astrocytic structural change. A critical role for astrocytes in motor learning is further suggested by the fact that mutant mice lacking GFAP display specific deficiencies in eyeblink conditioning (a form of discrete motor learning involving the cerebellum) and long term depression, a putative synaptic mechanism for eye-blink conditioning [430].

6.12. Possible mechanisms for inducing structural changes of astrocytes

Given the diversity of the regions where structural plasticity of astrocytes has been documented, there are undoubtedly multiple signalling pathways that can bring it about. It is likely to be accomplished in different brain regions by the stimulation of astrocyte receptors for locally available neurotransmitters, for example norepinephrine and/or epinephrine in the SON [25,285,410,411] and epinephrine in the posterior pituitary [25]. In the SCN, glutamate would appear to be a good candidate as it is released from the retinohypothalamic afferents that entrain the SCN clock and is known to influence the motility of astroglial filopodia [74,503]. As we have seen, in the rodent arcuate, median eminence, globus pallidus, and hippocampus as well as the primate mediobasal hypothalamus and infundibular nucleus, oestrogen is a likely stimulus for astrocytic structural plasticity. GABA may be another candidate for inducing astrocytic morphological change, as cultures of olfactory astrocytes respond to either GABAergic agonists or the presence of GABAergic neurons [278]. However, at the moment, perhaps the most complete case can be made for stimulation of β-adrenergic receptors as a means of inducing structural plasticity of astrocytes.

Several lines of evidence suggest that astrocytic β-ARs in adulthood regulate structural plasticity in selected populations of astrocytes. The SON-*VGL*, predominantly astrocytic, receives a dense catecholaminergic innervation [285] and β_2-ARs on SON-*VGL* astrocytes are upregulated in response to dehydration [255]. Further, in the posterior pituitary, structural changes of pituicytes have been induced in explanted pituitaries by incubation in β-AR agonists [437]. Also, structural changes of pituicytes and SON astrocytes after a hypertonic intra-peritoneal saline injection are blocked by ablation of the adrenal medulla [25], suggesting that these cells need an adrenergic signal to alter their shapes.

Recently, it has been observed also that monocular enucleation in adulthood causes upregulation of β-adrenergic receptor immunoreactivity in the corresponding cortical column. This change is evident more than a year after deafferentation and without apparent changes in GFAP levels indicative of gliosis [11]. It is possible that the observed change serves to help conserve glutamate released from the remaining few glutamatergic inputs arriving at the deprived columns. This could be accomplished by decreasing glutamate uptake into local astrocytes [173], particularly during alert behavioural states when glial β-ARs would be activated following firing of locus coeruleus neurons. As we have seen, this could also be brought about by changing the configuration of astrocytic processes near individual synapses.

A possible mechanism leading to structural plasticity of arcuate astrocytes arises from evidence that tanycytes and astrocytes in this region are immunopositive to antibodies to kainate glutamate receptors but not to oestrogen or androgen receptors [98]. It is suggested that oestrogen act on arcuate neurons which then release glutamate from their axon terminals to induced morphological changes in arcuate tanycytes and astrocytes. This, then, may lead to the synaptic remodelling of that nucleus. Likewise these receptors might also be involved with the release of LHRH containing axon terminals in the median eminence which occurs on the day of pro-oestrus.

6.13. Summary

Currently, it seems reasonable to speculate that astrocytic structural plasticity acts to enhance communication in the CNS. By altering coverage of neuronal elements astrocytes may diversely inhibit or facilitate the formation of synapses. By selectively associating with synaptic complexes, astrocytes may be in a position to modulate synaptic physiology through their well-known abilities to regulate the extracellular ionic environment or to take up and metabolise neurotransmitters. Nevertheless, to ultimately determine the functional significance of such changes, it will first be necessary to experimentally verify that astrocytic structural changes are indeed necessary or sufficient for neuronal changes to occur or for the smooth functioning of the nervous system to proceed.

7. Concluding remarks

There is a growing wealth of evidence that glia are intimately involved in neuronal function and therefore behaviour. Much of this evidence is inferential, like the relationship between glial differentiation and numbers

with behavioural complexity through phylogeny, the diversity of glial transmitter receptors or the glial morphological changes associated with changes in neural activity and behaviour. More definitive neural–glial interactions include the sole provision of energy and glutamate substrates by glia for neurons, necessary for learning and visual sensory function, respectively. In this category also is the regulation of extracellular K^+ by glia, a process which contributes to the evocation of slow potential shifts with functional relationships to sensory activity, learning and motivational states. Much of the direct evidence for active participation of glia in neural function derives from their involvement in guidance of neurons, the subsequent neuronal plasticity during development and the metabolic implications of glial β-adrenergic receptor activation for neural function.

These combined areas of evidence, however, make a compelling argument for increasing our research efforts to understand the mechanisms by which neurons and glia interact. At the very least, glial function can no longer be ignored.

Problems still exist in studying neuronal–glial interactions, especially in vivo. These include extrapolation of data from cell culture experiments to the in vivo situation or determining what is a glial rather than a neuronal function as e.g. with respect to ionic distributions in ECS. Also, the relative contributions of glia and neurons to synaptic plasticity and demand for metabolic and transmitter substrates need to be determined.

Slowly, however, these problems are being solved by the identification of specific cytotoxins, inhibitors of glial-specific enzymes and by the use of cell-specific markers. Development of new techniques for visualising ionic fluxes, enzyme activities and receptor activity are promising to make evidence, already available, about associations between neuronal and glial activity more causally interpretable. It has been the aim of this paper to help inspire both the development and use of available and new technologies to research the true level of intimacy of neural–glial interactions and their contributions to behaviour.

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