13 The astrocyte response to neural injury: a review and reconsideration of key features

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Abbreviations

ADX – adrenalectomized; BBB – blood-brain barrier; CNS – central nervous system; CORT – corticosterone; bFGF – basic fibroblast growth factor; EAE – experimental autoimmune encephalomyelitis; EGF – epidermal growth factor; ELISA – enzyme-linked immunosorbant assay; GFAP – glial fibrillary acidic protein; GS – glutamine synthetase; HPA-axis – hypothalamic-pituitary adrenal-axis; IFN-γ – interferon-γ; IHC – immunohistochemistry; IL-1 – Interleukin; IL-1ra – IL-1 receptor antagonist; LIF – leukemia inhibitory factor; LIX – LPS-induced CXC chemokine; LPS – lipopolysaccharide; MCP-1 – monocyte chemoattractant protein-1; MHC – major histocompatibility complex; MIP-1 – macrophage inflammatory protein-1; MPTP – 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NGF – nerve growth factor; NO – nitric oxide synthase; OVX – ovariectomized; RANTES – regulated upon normal T-cell activation expressed and secreted; ROS – reactive oxygen species; SDS – sodium dodecylsulfate; T3 – triiodothyronine; TGF-β – transforming growth factor-β; TMT – trimethyl tin; TNF-α – tumor necrosis factor-α

Introduction

Over the last twenty years, considerable evidence has accumulated to suggest that gliosis represents a homotypic response of astrocytes and microglia to all types of nervous system injury, including damage resulting from exposure to chemicals or chemical mixtures. The astrocytic component of this response, often referred to as "reactive" gliosis or astrogliosis, has received the most attention and is the subject of numerous reviews (Martin and O'Callaghan, 1996; Kimelberg and Norenberg, 1994; Norenberg, 1994; Eng and Ghirnikar, 1994; Norton *et al.*, 1992; Perry and Andersson, 1992; Eng *et al.*, 1992; Kimelberg and Norenberg, 1989; Malhotra *et al.*, 1990; Eng, 1988a; Eng and DeArmond, 1981). Most of the progress in characterizing reactive gliosis over the years can be traced to the discovery of GFAP, the major protein of astrocyte intermediate filaments (Eng, 1988b; Eng, 1985; Eng *et al.*, 1985). Astrocytes accumulate intermediate filaments when they become reactive (Eng, 1988b; Eng, 1987; Brock and O'Callaghan, 1987; Aono *et al.*, 1985; Smith *et al.*, 1984; Amaducci *et al.*, 1981), therefore, by definition, reactive astrocytes show an enhanced expression of GFAP. Immunohistochemistry of GFAP has been widely utilized to monitor astrocytic responses to neural injury and has firmly established GFAP as the key biomarker of reactive gliosis. Although less prevalent in the literature, GFAP analysis by immunoblot and immunoassay also has been used to establish, quantitatively, the features of reactive gliosis (Norton *et al.*, 1992; Aono *et al.*, 1985; O'Callaghan, 1993; O'Callaghan

et al., 1995). We have used the latter approach to document regional differences in GFAP expression and the utility of GFAP as a marker of the dose-, time-, and region-dependent damage resulting from exposure to broad classes of known and suspected neurotoxic agents. Our purpose here is not to revisit these topics, but rather, it is to review and challenge some commonly held views on the nature of the astrocyte response to CNS injury. Specifically, we will discuss the potential role of GFAP in gliosis, conflicting data obtained from *in vitro* vs. *in vivo* analysis of "reactive" astrocytes, and the relative contribution of hypertrophy and hyperplasia to reactive gliosis. Because descriptions of cytokine and hormonal regulation of astroglial responses pervade the *in vitro* and *in vivo* literature, cytokine and hormonal effectors will be discussed as they relate to each feature of gliosis. Gliosis in the developing nervous system also will be examined along with the merits of analysis of gliosis by GFAP immunohistochemistry vs. GFAP immunoassay. Understanding the features of the astroglial response to injury is critical for the accurate use of GFAP as a biomarker for detecting and quantifying neurotoxicity and for designing effective strategies for neuroprotection or neurotrophic support following neurotoxic exposures.

The molecular and biochemical events that initiate gliosis and the physiological or pathological basis of this cellular reaction remain to be clarified. There are a number of reasons for a lack of progress in this area and for conflicting points of view concerning specific aspects of the glial response to injury. It is our contention that differing astrocytic responses observed between *in vitro* and *in vivo* preparations contribute to the confusion and general lack of understanding of the astroglial response to injury. As we review specific features of reactive gliosis we will focus mostly on *in vivo* data but we will intersperse our discussion with relevant data obtained from studies of astrocytes in culture. Our comments will not deal with the topic of astrocytes as targets or mediators of neurotoxicity, which have been the subject of several recent reviews (Aschner *et al.*, 1999; Aschner, 1998; Aschner, 1997; Di Monte *et al.*, 1996; Aschner, 1996; Chao *et al.*, 1996; Aschner and LoPachin, 1993). Most of our discussion of gliosis will be based on inferences made from qualitative and quantitative assessments of GFAP, as most of our knowledge of astrocytic response to injury is based on examination of this "marker." This does not exclude the significance of a host of lesser known but perhaps equally applicable markers of the reactive astrocyte (Kuhlmann and Guilarte, 1999; McKeon *et al.*, 1999; Sugaya *et al.*, 1998; Kuhlmann and Guilarte, 1997; Kawai *et al.*, 1994; May, 1993; Day *et al.*, 1990; Niquet *et al.*, 1996; LeVine *et al.*, 1986; Ghandour *et al.*, 1981; Martin and O'Callaghan, 1996).

A role for GFAP in astrogliosis?

Damage to diverse targets in the CNS by a broad array of known neurotoxic agents engenders a glial reaction at the site of injury, as assessed by qualitative or quantitative analysis of GFAP (O'Callaghan and Miller, 1993; O'Callaghan, 1993; O'Callaghan and Jensen, 1992; O'Callaghan, 1991a,c; O'Callaghan, 1988; Brock and O'Callaghan, 1987). The homotypic nature of reactive gliosis actually is quite remarkable given the extreme molecular and cellular heterogeneity of the CNS. Indeed, by virtue of the generality of this cellular reaction, enhanced expression of GFAP is taken as the "gold standard" for defining the existence of the astrocytic response to injury. Modulation of the expression of GFAP in response to neural injury often can be linked to modulation of the effect of the offending agent on its target (Miller and O'Callaghan, 1997a,b; Griffith and Sutin, 1996; Miller and O'Callaghan, 1994; O'Callaghan and Miller, 1994; Gordon *et al.*, 1991; Miller and O'Callaghan, 1993; Miller *et al.*, 1992; O'Callaghan and Seidler, 1992; O'Callaghan *et al.*, 1990b; O'Callaghan

et al., 1990a; Reinhard *et al.*, 1988). Thus, GFAP is a "biomarker," albeit an indirect one, of neurotoxicity. While there is a large body of information concerning the enhanced expression of GFAP as a hallmark of gliosis, there is little understanding of the role of this protein in relation to the molecular basis of the astrocyte response to injury.

The molecular basis of the glial response to injury, in general, has yet to be defined, and this includes the physiological or pathophysiological role of GFAP. Our lack of understanding persists despite the implementation of homologous recombination techniques to "dissect" gliosis. For example, the production of GFAP null mice yields little information with regard to its function since GFAP null mice have no obvious phenotype and still mount an astrocytic reaction to injury (Pekny *et al.*, 1995). Some subtle pathologies, however, have been reported. These include impaired myelination and abnormal white matter structure (Liedtke *et al.*, 1996), and impaired long term depression and long term potentiation (McCall *et al.*, 1996; Shibuki *et al.*, 1996).

The intermediate filament protein, vimentin, shows enhanced expression in a subpopulation of reactive astrocytes. The possibility exists that vimentin replaces the function of GFAP in GFAP null mice, but this has not been found to be the case. Vimentin "knockouts" also have been constructed and in these mice there is abnormal intermediate filament structure in astrocytes that normally co-express GFAP and vimentin, but not in astrocytes that normally express GFAP alone (Galou *et al.*, 1996). The co-expreessor astrocytes appear to undergo a hypertrophic response as indicated by β-galactosidase reporter gene expression downstream of a vimentin promoter following injury (Galou *et al.*, 1996). The predominant morphological feature of reactive gliosis, thickening of processes and enlargement of the astrocyte, surprisingly appears not to depend on GFAP or vimentin because the changes occur in both the GFAP null and the GFAP/vimentin null mouse (Pekny *et al.*, 1998; Galou *et al.*, 1996). This suggests that neither GFAP nor vimentin are necessary for, or are inducers of, the morphologic changes that astrocytes exhibit during gliosis (Galou *et al.*, 1996; Gomi *et al.*, 1995), and, therefore, their responses are secondary to gliosis. Alternatively, it is possible that there are as yet, unknown compensatory mechanisms in these knockouts, since it has been reported that transfection with an antisense GFAP construct results in defects in process extension (Weinstein *et al.*, 1991). Moreover, developmental compensation is known to occur during homologous recombination that may not occur if GFAP or vimentin were inducibly "knocked out" in the adult. In support of this notion it has been shown that in *in vitro* models of gliosis transfected with antisense GFAP do not assume a reactive phenotype when injured (Ghirnikar *et al.*, 1994; Yu *et al.*, 1993; Weinstein *et al.*, 1991; Eng, 1993). Compensation may also derive from another cytoskeletal protein, the low molecular weight microtubule associated protein, which has been shown to be important for astrocyte process extension (Pasinetti *et al.*, 1994).

One additional observation obtained with GFAP/vimentin null mice may provide insight into the function of the reactive astrocyte regardless of the specific role of GFAP (or vimentin). These double knockouts exhibit an impaired glial response to injury in that the glial "scar" was easily breached and resulted in bleeding (Pekny *et al.*, 1999). In a potentially related observation, a sophisticated transgenic approach was used to study trauma-induced gliosis that enabled investigators to target and kill astrocytes with gancyclovir treatment in the adult mouse (Bush *et al.*, 1999). The results suggest that reactive gliosis may enhance neural survival by limiting bleeding, limiting the number of invading leukocytes and/or by preventing accumulation of toxins in the extracellular milieu (e.g. excitatory amino acids) (Bush *et al.*, 1999). In summary, the role of GFAP in gliosis is unclear. While it is the best known protein expressed by reactive astrocytes, like other genes and

proteins associated with the reactive astrocyte phenotype, GFAP remains a biomarker of gliosis and not necessarily a key player in the initiation of the glial activation process.

Astrocyte response patterns: do *in vitro* models reflect the *in vivo* condition?

One of the main reasons for conflicting views concerning features of reactive gliosis stems from the fact that data obtained *in vitro* and *in vivo* often are used as though they were interchangeable in support of a given point. What is irrefutable is that the molecular and biochemical properties of astrocytes can be dramatically affected by cell culture conditions (Passaquin *et al.*, 1990). This is not an unexpected observation given the difference in the culture environment from that found *in vivo*. The artificial conditions that constitute the culture milieu represent the primary limitation associated with *in vitro* astrocyte response models. Differences between astrocyte responses observed *in vitro* and *in vivo* often are not trivial. Some of these conflicting observations are documented in Table 13.1. In addition to the different response patterns observed between *in vitro* and *in vivo* findings, often there are conflicting findings even among *in vitro* studies of astrocytes (Passaquin *et al.*, 1990). A full treatment of this subject, however, is beyond the scope of this review. Some of the conditions/features of astrocytes grown in culture that may serve as the basis for their differing response profile compared to the *in vivo* condition are discussed below and in the sections on astrocytic hyperplasia, cytokines and hormonal regulation.

Reactive astrocytes are considered to be involved with the inflammatory process because they are affected, *in vitro*, by some of the mediators of inflammation (Table 13.1; also see sections below on cytokines and hormones). For example, treatment of astroglial cultures with LPS results in up-regulation of the proinflammatory cytokine, IL-1β(Kong *et al.*, 1997; Pahan *et al.*, 1997; Letournel-Boulland *et al.*, 1994). Under the same incubation conditions, however, GFAP and glutamine synthetase (GS), another glial marker, are decreased (Letournel-Boulland *et al.*, 1994). Down-regulation of GFAP or GS is not consistent with a reactive astrocytic phenotype. Moreover, addition of IL-1 to astrocyte cultures results in cell division without affecting GFAP levels (Oh *et al.*, 1993). These findings also are inconsistent with gliosis, which is characterized by an increase in GFAP per cell.

Inflammatory mediators cause a different pattern of effects *in vivo*. For example, parenteral administration of LPS to rats does not affect GFAP or GFAP mRNA, although it does elevate cytokines in the CNS (including IL-1) (Little and O'Callaghan, 2000; Little and O'Callaghan, 1999a). When LPS is administered directly into the CNS, however, an inflammatory process results (Plata-Salaman *et al.*, 1998; Pitossi *et al.*, 1997) that involves a brain injury-induced increase in GFAP and gliosis (Lemke *et al.*, 1999; Hauss-Wegrzyniak *et al.*, 1998; Szczepanik *et al.*, 1996; Maeno *et al.*, 1991). Thus, direct and indirect avenues for manipulation of IL-1 produce conflicting findings *in vitro* and *in vivo*, results which implicate the participation of multiple cell types in the observed responses and that complicate our understanding of the relevant features of gliosis.

Astrocytes have receptors for a variety of neurotransmitters/neuromodulators and they themselves are a rich source of bioactive molecules (Oh *et al.*, 1999; Glabinski and Ransohoff, 1999; Glabinski *et al.*, 1999; Guo *et al.*, 1998; Mustafa *et al.*, 1998; Sato *et al.*, 1997; Inagaki and Wada, 1994; Sontheimer, 1994; Murphy and Pearce, 1987) (and see sections on cytokines and hormones below). Thus, it is not surprising that primary astrocytes influence and/or are influenced by the presence of other cell types. This observation also may help explain the discrepancy between some *in vivo* and *in vitro* observations concerning

Table 13.1 Conflicting astrocyte responses *in vivo* vs. *in vitro*

Endpoint	In vivo	*Reference*	In vitro	*Reference*
GFAP	steady levels *in vivo*	Nichols *et al.*, 1990; Laping *et al.*, 1994	increased with passages	Passaquin *et al.*, 1990
	decreased by cort	Loddick and Rothwell, 1999	increased by cort	Laping *et al.*, 1994; Ghirnikar *et al.*, 1994; Rozovsky *et al.*, 1995
	increases w/central LPS	Goldmuntz *et al.*, 1986	decreased by LPS	Riol *et al.*, 1997
	increases assoc. w/TNF	Hong *et al.*, 1995; Marquette *et al.*, 1996; Martin and O'Callaghan, 1996	huge decreases after TNF-α treatment	Selmaj *et al.*, 1991; Oh *et al.*, 1993; Selmaj *et al.*, 1990
	anti bFGF has no effect	Balasingam *et al.*, 1994; Rostworowski *et al.*, 1997 Rowntree and Kolb, 1997	bFGF decreases GFAP	Reilly *et al.*, 1998
	adding bFGF enhanced expression	Eclancher *et al.*, 1996; Wen *et al.*, 1995		
	K^+ causes hypertrophy	Bonthius *et al.*, 1995	K^+ causes proliferation, decreases	Del Bigio *et al.*, 1994
	IL-1 induces gliosis	Giulian and Lachman, 1985	protein unchanged, mRNA decreased by IL-1	Canady *et al.*, 1990
	mRNA increased by TNF-a neonates	Balasingam *et al.*, 1994	mRNA decreased by TNF	Selmaj *et al.*, 1991; Oh *et al.*, 1993
	thyroid-deficient mice vimentin-GFAP delayed/ impaired/decreased GFAP	Rami and Rabie, 1988 Faivre-Sarrailh *et al.*, 1991	triiodothyronine causes decrease	Andres-Barquin *et al.*, 1994
Glutamine synthetase	steady levels *in vivo*, increased by cort, increased by cAMP	Laping *et al.*, 1994; O'Callaghan *et al.*, 1991	increased with passages, decreased by cort, decreased by cAMP	Passaquin *et al.*, 1990; Laping *et al.*, 1994; Arcuri *et al.*, 1995
35, 33, and 20 kD proteins	induced by vibratory stress	Nichols *et al.*, 1989	not induced by hydrocortisone in 1° astrocytes	Nichols *et al.*, 1989
Glial proliferation	does not occur after injury	Latov *et al.*, 1979	proliferation induced by TNF and IL-1	Selmaj *et al.*, 1991
Neuron death	enhanced by IL-1	Rothwell *et al.*, 1997	IL-1 protects against	Rothwell *et al.*, 1997
Transferrin	not present *in vivo*	Passaquin *et al.*, 1990	highly expressed induced by TNF and IL-1	Oh *et al.*, 1993

astrocytes and reactive gliosis. For example, co-culture with neurons induces them to take on a stellate morphology (Pekny *et al.*, 1998) more typical of differentiated astrocytes *in vivo*. Other observations also suggest that co-culture imparts properties to astrocytes *in vitro* that are more representative of the *in vivo* condition (Table 13.1) (Pekny *et al.*, 1998; Torres-Aleman *et al.*, 1992; Hatten, 1985). For example, co-cultures treated with glucocorticoids result in down-regulation of GFAP (Rozovsky *et al.*, 1995) which is known to be the case *in vivo* (Laping *et al.*, 1994d; Laping *et al.*, 1994b; O'Callaghan *et al.*, 1991; Nichols *et al.*, 1990b; O'Callaghan *et al.*, 1989). Yet when glucocorticoids are added to primary glial cell cultures or C6 glioma cells lines, GFAP is increased (Rozovsky *et al.*, 1995; Laping *et al.*, 1994d). Consistent with the low level of expression of NGF and MHC-II *in vivo*, co-culture with neurons also inhibits secretion of NGF and expression of MHC-II compared to with monotypic astrocyte cultures (Laping *et al.*, 1994d). Taken together, these observations suggest that astrocyte gene expression is regulated within a context of neuronal activity. Thus, despite the surprising fact that the astroglial response to brain injury is uniform (i.e. astrocytes become "reactive" and show enhanced expression of GFAP), their morphology and specific gene expression patterns may be markedly affected by the presence and activity level of the adjacent neuronal or glial population. Such differences in the local environment *in vivo* or the culture/co-culture conditions *in vitro* may go a long way toward explaining the region-specific diversity of astrocytic responses to neural injury. More to the point being made here, the complex regulation of astrocyte gene expression in the intact or injured brain makes it difficult to envision culture conditions sufficient to adequately reflect the normal or reactive astrocyte.

Reactive gliosis: hypertrophy or hyperplasia?

Does enhanced expression of GFAP and other astrocyte markers reflect astrocyte hypertrophy or proliferation? Aside from the intrinsic importance of understanding the neurobiology of CNS injury, this question is important because it suggests two different functions of the glial response. A proliferative response implies a permanent change that may or may not be detrimental to recovery of function. A hypertrophic response is suggestive of a temporary ("reactive") change in cellular metabolism that most likely would be associated with a trophic, supporting role for glia in the injured CNS. This latter scenario would further suggest that understanding the process would facilitate the design and implementation of intervention strategies to limit damage and enhance recovery. Attempts to establish the relative roles of hypertrophy and hyperplasia in gliosis have resulted in conflicting or confusing data. The consensus that emerges from a review of the literature supports a major role for hypertrophy not hyperplasia (Kimelberg and Norenberg, 1994) and yet, even in recent studies, the misconception persists that hyperplasia represents the predominant response (Deng *et al.*, 1999).

The notion that the dominant astrocyte response to injury is hyperplasia has persisted for a number of reasons, one of the main ones being historical precedent. As early as 1970, Cavanagh suggested that astrocytes divided in response to a stab wound based on ^{3}H-thymidine labeling, however, astrocytes could not be definitively identified in this pre-GFAP era (Cavanagh, 1970). In 1979 however, Latov *et al.* (1979), published a paper reporting that GFAP positive astrocytes proliferated after a stab wound of the brain. However, the actual portion of proliferating astrocytes was a small percentage (1–2%) of the total identified (Latov *et al.*, 1979).

The widely cited observation of Latov *et al.* (1979), was followed in the 1980s by several papers that showed that IL-1 was mitogenic for astrocytes *in vitro* (Giulian and Baker, 1985; Giulian and Lachman, 1985). IL-1 is known to be a growth factor for astrocytes during development and is known to be elevated during the neonatal interval when astrocytes proliferate in the brain (until 2–3 weeks post-natal, see development section) (Giulian *et al.*, 1988b). Therefore, it should not be surprising that neonatal primary cultures would respond to IL-1 by proliferating. Nevertheless, elevations in IL-1 and other cytokines were known to be associated with a number of brain injury conditions (see cytokine section). Moreover, direct injection of IL-1 into the brain resulted in gliosis (Giulian *et al.*, 1988a), and IL-1 was produced by activated microglia (Giulian *et al.*, 1986). All of these observations provided strong circumstantial evidence that microglia release IL-1 after brain injury and trigger astrocytes to proliferate. In these studies, however, a causal relationship was not established. Thus, when interleukins were examined more closely using *in vivo* models, the results did not support glial proliferation. For example, in 1985 Giulian *et al.* reported that after brain injury IL-1 immunoreactivity appeared only after 10 days, by which time gliosis was already well under way (Giulian and Lachman, 1985). Even direct injection of IL-1 (Giulian and Baker, 1985; Giulian and Lachman, 1985) or TNF-α (Kahn *et al.*, 1995) into the brain resulted in proliferation only around the needle track, not at sites away from the stab that showed pronounced increases in GFAP associated with astrocytic hypertrophy.

Astrocytic hyperplasia at the site of a penetrating wound is not unexpected. Under these conditions the blood brain barrier would be compromised allowing the influx of blood-borne astrocytic mitogens. However even with disruption of the BBB after mechanical injury other astrocyte markers such as glutamine synthetase (GS) did not increase (Condorelli *et al.*, 1990) as one would expect if astrocytes were proliferating. Clearly, the induction of reactive gliosis is influenced by, but does not require, disruption of the blood brain barrier. Large increases in GFAP levels and immunoreactivity can be observed in the absence of a disrupted blood brain barrier and, perhaps as a consequence, the absence of astrocyte mitogens (O'Callaghan and Jensen, 1992; O'Callaghan *et al.*, 1990a,b). To limit the influence of blood-borne factors in the study of reactive gliosis, it is preferable to preserve the integrity of the blood brain barrier through the use of toxic insult models (O'Callaghan and Miller, 1993; O'Callaghan, 1993) or models of brain damage that result from surgical procedures performed in the periphery (Raivich *et al.*, 1999; Raivich *et al.*, 1996; Tetzlaff *et al.*, 1988; Graeber and Kreutzberg, 1986).

One of the strongest arguments against injury-induced astrocytic hyperplasia is the fact that gliosis is not permanent because it resolves over time (Torre *et al.*, 1993). For example trimethyltin-induced neuronal death results in an increase in GFAP in the CA3 and CA4 regions of the hippocampus that is thousands of percent above control but that subsides over time to control levels (Brock and O'Callaghan, 1987). A massive die-off of astrocytes must occur to explain the return to control levels of GFAP if glial proliferation is solely or even largely responsible for the initial increase. There is no evidence to support an event. Indeed, 3H-thymidine incorporation studies combined with GFAP immunohistochemistry have shown that only a small percentage (~1%) of astrocytes proliferate after trimethyltin-induced hippocampal damage, therefore, hypertrophy is the predominant response (Brock and O'Callaghan, 1987; Latov *et al.*, 1979). Evidence obtained from the dopaminergic neurotoxicants, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), methamphetamine (METH), methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) also support a major role for astrocytic hypertrophy. In these cases, the glial reaction is extremely dynamic, with GFAP levels reaching 400 % of control within as little as 48 hours

post dosing (O'Callaghan *et al.*, 1990b; O'Callaghan *et al.*, 1990a; O'Callaghan and Miller, 1994) followed by a return to control in 2 weeks. While corresponding GFAP immunohistochemistry is suggestive of an explosive expansion of the astrocyte population (O'Callaghan and Jensen, 1992; Deng *et al.*, 1999), the appearance of several hundred-fold increases in mature astrocytes within such a short period of time would be highly unlikely. Moreover, the loss of these astrocytes over the ensuing 2 weeks would again have to be the result of massive cell death if the initial increase was due to proliferation. These apparently inconsistent results can be explained on the basis of the inherently low levels of GFAP present in the target region (striatum), such that they fail to be detected by immunohistochemistry (see further discussion under immunohistochemistry section below). Thus, any injury-induced increase in GFAP raises this protein to the level of detectability and gives the appearance of astrocytic hyperplasia. Studies with ^{3}H-thymidine labeling after MPTP also do not support the existence of astrocytic proliferation (W. G. McAuliffe, personal communication). Examination of GFAP levels following exposure to a variety of other neurotoxic agents also demonstrates the transient nature of gliosis (O'Callaghan and Miller, 1993; O'Callaghan, 1993; Sparber *et al.*, 1992; O'Callaghan and Jensen, 1992; O'Callaghan, 1991a; O'Callaghan, 1988; Brock and O'Callaghan, 1987). The variation in the onset and decline in gliosis from toxicant to toxicant appears to reflect the onset and duration of the insult (O'Callaghan, 1993). Thus, continued gliosis occurs under conditions of continued damage as is seen with neurological disease states such as Alzheimer's disease (Le Prince *et al.*, 1993; Delacourte, 1990), EAE (the rodent model of multiple sclerosis) (Kothavale *et al.*, 1995; Smith and Eng, 1987) etc. Advanced age may be considered one form of "brain injury" and it is associated with an enhanced expression of GFAP immunoreactivity (Kohama *et al.*, 1995; Goss *et al.*, 1991; Goss *et al.*, 1990) and content (O'Callaghan and Miller, 1991). Again, however, evaluation of the hyperplasia vs. hypertrophy issue supports the latter (Bjorklund *et al.*, 1985). Many studies now have demonstrated that senescence-related increases in GFAP and reactive gliosis were due to astrocyte hypertrophy and not glial proliferation (Gordon *et al.*, 1997; Bjorklund *et al.*, 1985).

Precedent for a dynamic role of astrocytes in brain injury stems from data observed in the intact animal. For example, astrocytes extend GFAP-positive processes (hypertrophy) in an extremely fluid fashion, withdrawing processes from between axons and engulfing axons to prevent/regulate transmitter release (Hawrylak *et al.*, 1999; Laping *et al.*, 1994d; Hatton *et al.*, 1984; Tweedle and Hatton, 1980). This physiological increase and decrease in GFAP and astrocyte size suggests that a similar process may underlie reactive gliosis and its resolution. Together, these observations strongly argue for a trophic rather than a detrimental role for astrocytes in injury. In summary, the evidence supports the conclusion that gliosis, as reflected by increased expression of GFAP, is predominantly the result of astrocytic hypertrophy; little evidence exists for injury-driven astrocytic proliferation *in vivo*.

Do IL-1, TNF or other cytokines induce gliosis?

As mentioned earlier, it has been widely suggested that reactive gliosis may be a component of an inflammatory process within the CNS (Scripter *et al.*, 1997; Hong *et al.*, 1995; Balasingam *et al.*, 1994; da Cunha *et al.*, 1993). In particular, the expression of the key proinflammatory cytokines, IL-1 and/or TNF-α, have been implicated as early signals that lead to gliosis. As discussed below, we feel that the role for IL-1, TNF-α or other cytokines in gliosis has yet to be established. A resolution of this issue is important, however, because an understanding of the role of these (or other) cytokines in gliosis would dictate the appropriate strategy for treating CNS injury. For example, the use of anti-inflammatory agents

following CNS injury would not be indicated if such treatment diminished the potential trophic effects of gliosis by interfering with cytokine-activated pathways.

Proinflammatory cytokines and associated chemokines have been implicated in a variety of brain injury conditions including LPS-induced inflammation in the brain (Dinarello, 1988; Fontana *et al.*, 1984), scrapie (Kim *et al.*, 1999; Williams *et al.*, 1994), multiple sclerosis/EAE (Tanuma *et al.*, 1999; Glabinski *et al.*, 1999; Eng *et al.*, 1996; Tani *et al.*, 1996b; Ransohoff *et al.*, 1993; Merrill, 1992; Hauser *et al.*, 1990), Alzheimer's disease (Griffin *et al.*, 1998; Mattson *et al.*, 1997; Giulian *et al.*, 1995; Stanley *et al.*, 1994; Wood *et al.*, 1993; Griffin *et al.*, 1989), AIDS dementia (Persidsky *et al.*, 1997; Stanley *et al.*, 1994; Merrill, 1992), viral infection (Sauder and de la Torre, 1999; Thomas *et al.*, 1998; Wege *et al.*, 1998; Marquette *et al.*, 1996; Campbell *et al.*, 1994b; Lieberman *et al.*, 1989), trauma (Lemke *et al.*, 1999; Bell *et al.*, 1997; Shohami *et al.*, 1994; Balasingam *et al.*, 1994; Ransohoff and Tani, 1998; Ghirnikar *et al.*, 1998a; Ghirnikar *et al.*, 1996; Glabinski *et al.*, 1995), siezure (Probert *et al.*, 1995), irradiation (Hong *et al.*, 1995), ischemia, and stroke (Rothwell, 1999; Stroemer and Rothwell, 1998; Zhai *et al.*, 1997; Rothwell *et al.*, 1997a; Loddick *et al.*, 1997; Rothwell *et al.*, 1997b; Feuerstein *et al.*, 1997; Martin and O'Callaghan, 1996). The cyto- or chemokines associated with these various injury and disease scenarios include TNF (Fiala *et al.*, 1997; Martino *et al.*, 1997; Renno *et al.*, 1995), IL-1 (Griffin *et al.*, 1998; Griffin *et al.*, 1995), IL-6 (Lemke *et al.*, 1999; Kim *et al.*, 1999; Streit *et al.*, 1998; Marquette *et al.*, 1996; Williams *et al.*, 1994; Campbell *et al.*, 1994a; Hunter *et al.*, 1992; Hauser *et al.*, 1990), IL-1ra (Panegyres and Hughes, 1998), MCP-1 (Glabinski *et al.*, 1999; McManus *et al.*, 1998; McTigue *et al.*, 1998; Mustafa *et al.*, 1998; Ghirnikar *et al.*, 1998b; Carroll and Frohnert, 1998; Glabinski *et al.*, 1996; Godiska *et al.*, 1995), MCP-5 (McTigue *et al.*, 1998), MIP-1α (Hausmann *et al.*, 1998; Godiska *et al.*, 1995), MIP-1β(Mustafa *et al.*, 1998; Ghirnikar *et al.*, 1996; Godiska *et al.*, 1995), MIP-2 (Glabinski *et al.*, 1998), MIP-3 α(McTigue *et al.*, 1998), RANTES (Hausmann *et al.*, 1998; Mustafa *et al.*, 1998) GRO (Glabinski *et al.*, 1998), IP-10 (Glabinski *et al.*, 1999; McTigue *et al.*, 1998; Hausmann *et al.*, 1998), and TGF-β (da Cunha *et al.*, 1993; Glabinski and Ransohoff, 1999; Hill *et al.*, 1999; Hausmann *et al.*, 1998; Mitrovic *et al.*, 1994; Owens *et al.*, 1994; Balasingam *et al.*, 1994; Merrill, 1992; Merrill *et al.*, 1992). Abundant evidence exists to support a role for microglia and astrocytes as a source for synthesis and release of these mediators following brain injury. What has not been shown *in vivo*, however, is a direct association of inflammatory mediators with the induction of gliosis.

A few fairly straightforward strategies have been used in an attempt to obtain direct evidence for a role of pro-inflammatory cytokines and related trophic factors in gliosis. These include direct administration (usually i.c.v.) into the brain, the use of transgenic mice over-expressing specific cytokines or growth factors (usually down-stream from the GFAP promoter) and, finally, the use of mice lacking a variety of cytokines implicated in gliosis. In large measure, these approaches have been uninformative because almost all of the manipulations resulted in the induction of gliosis or did not affect the glial response to injury. Thus, no specific mediator or combination of related factors has been directly linked to the initiation of gliosis.

Direct (i.c.v.) administration of a number of candidate mediators results in gliosis. This includes administration of IFN-γ (Balasingam *et al.*, 1994), IL-1 (Guilian and Baker, 1985), IL-2, IL-6 (Balasingam *et al.*, 1994), CNTF and TNF-α (singly or together) (Kahn *et al.*, 1995), bFGF (Eclancher *et al.*, 1996), and TGF-β1 (Laping *et al.*, 1994a). In these experiments, however, one needs to be mindful of the fact that the control condition is a stab wound. Penetrating lesions of the brain constitute the classic stimulus for induction of

gliosis, potentially through pathways involving combinations of the very factors (or their antagonists) being introduced into the brain as the "experimental" condition. Thus, local injection may be a confounder in these experiments.

The results of experiments using transgenic mice also fail to illuminate specific aspects of inflammation involved in the induction of gliosis. For example, neuronal degeneration and gliosis are part of the syndromes that occur in transgenic mice overexpressing TNF-α (Probert *et al.*, 1995; Douni *et al.*, 1995; Stalder *et al.*, 1993), TGF-β (Rabchevsky *et al.*, 1998; Wyss-Coray *et al.*, 1995), GFAP-IL-3 (Asensio *et al.*, 1999; Campbell and Powell, 1996; Chiang *et al.*, 1996; Powell *et al.*, 1993), GFAP-IFN-α (Akwa *et al.*, 1998), KC (in oligodendroglia) (Tani *et al.*, 1996a; Ransohoff *et al.*, 1996), and GFAP (Messing *et al.*, 1998). IL-6 transgenic mice develop gliosis and increases in TNF-α, IL-1α and β throughout their life, with neurological deterioration (Hernandez *et al.*, 1997; Castelnau *et al.*, 1997; Di Santo *et al.*, 1996; Tani *et al.*, 1996b; Fattori *et al.*, 1995; Chiang *et al.*, 1994; Campbell *et al.*, 1993; Raber *et al.*, 1998; Campbell *et al.*, 1998; Campbell, 1998; Gruol and Nelson, 1997; Campbell and Powell, 1996; Gold *et al.*, 1996; Chiang *et al.*, 1994). IL-6 transgenic mice have chronic up-regulation of complement C3 expression (Barnum *et al.*, 1996), a heightened stress response (Raber *et al.*, 1997), abnormal iron deposition (Castelnau *et al.*, 1998), and disrupted BBB (Brett *et al.*, 1995). In all of these examples, the generality of the gliotic condition observed is suggestive of the generality of an effect caused by the transgene: neural injury, the most widely accepted "stimulus" for gliosis.

Precedent exists in the literature for secondary effects of relatively specific genetic "lesions" to result in gliosis. For example, gliosis occurs secondary to neurodegeneration in the naturally occurring mutants *staggerer* (Aono *et al.*, 1985; Monnier *et al.*, 1999), weaver (Blum and Weickert, 1995), *reeler* (Bignami and Dahl, 1986; Benjelloun-Touimi *et al.*, 1985; Ghandour *et al.*, 1981), *twitcher* (Pedchenko and LeVine, 1999; LeVine *et al.*, 1994), *pcd* (Purkinje cell death) (Zhang *et al.*, 1997), *jimpy* (Cammer and Tansey, 1988; Imamoto, 1985; Jacque *et al.*, 1980), *quaking* (Sapirstein, 1992; Jacque *et al.*, 1980), *shiverer* (Nagaike, 1985), Gunn rat (O'Callaghan and Miller, 1985), and many others. What is known for many of these mutants is that widespread neural degeneration is secondary to relatively specific events occurring during development (e.g. loss of cerebellar granule cells (weaver); failure to myelinate (jimpy) and inversion of developmental layers (reeler) as appears to be the case for transgenics overexpressing various genes associated with the inflammatory cascade.

Relatively few studies have employed mice lacking specific cytokines/chemokines or trophic factors to determine their role in gliosis. Mice null for (IFN-γ) did not have an altered glial response (Rostworowski *et al.*, 1997). TNF-α p75 receptor and IL-6 knockouts showed elevations in GFAP levels that did not differ from wild type mice following administration of the dopaminergic neurotoxicant, MPTP (J. P. O'Callaghan) (unpublished data). In contrast to our findings with IL-6, however, Kreutzberg and coworkers (Klein *et al.*, 1997) found IL-6 knockouts to exhibit both an attenuated microglial and astroglial response to facial nucleus injury from peripheral nerve cut. Given the wide variety of knockout mice available, however, the general lack of publications in this area is suggestive of the failure to implicate an involvement of specific inflammatory mediators in gliosis. A definitive demonstration that a "knock down" of any of the putative glial activating factors results in blocked, delayed, or attenuated gliosis would provide the most convincing demonstration of the causative role of cytokines in gliosis.

Although the avenues of future investigation suggested above might shed light on the potential inflammation-gliosis link, an examination of the recent literature reveals several

lines of evidence that do not support a role of cytokines as direct activators of reactive gliosis. First, gliosis can occur in the absence of up-regulation of the proinflammatory cytokines, IL-1β or TNF-α. Thus, a cholinergic-specific toxin destroyed neurons without increases in either of these cytokines but with reactive gliosis (Lemke *et al.*, 1999). The dopaminergic neurotoxicant, MPTP, destroys dopaminergic nerve terminals and causes a marked gliosis in the damage region without an elevation of IL-1 (O'Callaghan *et al.*, 1990b). Our studies also indicate that loss of hippocampal neurons caused by the organometallic neurotoxicant, TMT, results in several thousand-fold increases in GFAP commensurate with enhanced immunostaining of the protein in the target region, but there is no preceding or accompaning change in pro-inflammatory cytokines (Little and O'Callaghan, 1999b,c). In contrast to our findings, Maier and Coworkers have reported early sustained (up to 8 days) increases in IL-1α, IL-6 and TNF-α after trimethyl tin treatment (Maier *et al.*, 1995). It is doubtful that these effects were related to gliosis, however, because the time course observed did not relate to the time-course of microglial (McCann *et al.*, 1996) or astroglial activation (Brock and O'Callaghan, 1987), nor were the effects observed in both the known targets of the compound (frontal cortex and hippocampus). Thus, these discrepancies indicate that cytokine expression patterns are not related to the time course or region-specific patterns of toxicant-induced damage and the ensuing gliosis.

A second line of evidence also does not favor a role of cytokines in gliosis, which is that cytokines may be elevated without an accompanying gliosis. For example, peripheral administration of LPS results in the increase in IL-1β, IL-2, TNF-α, and IL-6 mRNA levels in the CNS in the absence of gliosis and neuronal damage (Little and O'Callaghan, 1999a; Rothwell *et al.*, 1997b; Buttini *et al.*, 1997; Buttini *et al.*, 1996; Goujon *et al.*, 1996; Little and O'Callaghan, 2000, submitted). In addition, peripheral cytokines are known to be transported across the blood-brain barrier (McLay *et al.*, 1997; Banks and Kastin, 1997; Pan *et al.*, 1997; Banks *et al.*, 1995b; Gutierrez *et al.*, 1994; Banks *et al.*, 1994; Banks *et al.*, 1993; Gutierrez *et al.*, 1993; Banks *et al.*, 1991; Banks and Kastin, 1991; Banks *et al.*, 1989; Banks *et al.*, 1995a), but conditions that result in inflammation in the periphery, such as infection or sepsis, do not cause gliosis. Furthermore, after peripheral injection of Freunds adjuvant the BBB opens allowing peptides (including presumably cytokines) into the brain parenchyma without evidence for induction of gliosis (Rabchevsky *et al.*, 1999).

A third line of evidence that does not favor a role for cytokines in gliosis is the lack of association of the temporal, regional or cellular pattern of cytokine expression with the temporal and regional pattern of astrogliosis. For example, different expression profiles were observed after cholinergic cell loss compared to those that received direct injection of LPS and interferon. Only IL-1α was expressed in astrocytes (exclusively) after cholinergic cell destruction (Lemke *et al.*, 1998; Lemke *et al.*, 1999) but after LPS/interferon IL-1α was expressed only in microglia and TNF-α and IL-6 were also expressed (Lemke *et al.*, 1999). The temporal expression of IL-1α also differed between the two types of damage (Lemke *et al.*, 1999). Production of IL-1, IL-1ra, and TNF-α (and others) in response to i.c.v. LPS differs in hypothalamus, cerebellum, and hippocampus (Ilyin *et al.*, 1998). After ischemia (permanent or with reperfusion) the expression of TGF-β2, IL-1α, IL-1α, and IL-1ra differed in their temporal expression pattern (Hill *et al.*, 1999). Expression of TNF-α was differentially regulated in different brain regions after traumatic brain injury (Fan *et al.*, 1996). Differences in type of cytokine and temporal expression also were reported for trauma vs. burn injury in the rat cornea (Planck *et al.*, 1997) and between trimethyl tin and LPS (Little and O'Callaghan, 1999a; Little and O'Callaghan, 2000). Thus, astrogliosis, a homotypic response at sites of damage throughout the CNS, is not related to specific types of

cytokine signals in terms of their order of expression, the amounts expressed, and the region of the brain in which they are expressed. It is difficult to reconcile how such differing patterns of cytokine responses could lead to the same outcome, gliosis.

A final line of evidence that does not support a role of inflammation in gliosis is that suppression of cytokine expression does not prevent gliosis from occurring. Glucocorticoids (Buttini *et al.*, 1997; Buttini *et al.*, 1996) suppress IL-1β–(Grosset *et al.*, 1999; Nguyen *et al.*, 1998; Goujon *et al.*, 1996; Goujon *et al.*, 1995; Lee *et al.*, 1988; Besedovsky *et al.*, 1986) and TNF-α -(Goujon *et al.*, 1996; Brenner *et al.*, 1993) and their induction of NF-κB (Kleinert *et al.*, 1996) and IL-6 (Miyazawa *et al.*, 1998), while adrenalectomy results in increased levels of these cytokines centrally and peripherally in response to LPS (Goujon *et al.*, 1996). Glucocorticoids also suppress the induction of many other cytokines and chemokines IFN-γ (van der Velden *et al.*, 1998), IL-8 (Krishnaswamy et al., 1998; van der Velden *et al.*, 1998), and other related factors (e.g. RANTES (Meyer *et al.*, 1998), LIF (Miyazawa *et al.*, 1998), NOS (Brenner *et al.*, 1994), JE (Kawahara *et al.*, 1991)). Conversely, glucocorticoids have been shown to up-regulate the expression of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (Barnes, 1998). Despite the anti-inflammatory action of glucocorticoids, their administration in very high dosages fails to attenuate trimethyltin-induced gliosis (O'Callaghan *et al.*, 1991). In the facial nucleus injury model, dexamethasone pretreatment down-regulates MHC-2 expression but does not block gliosis (Kiefer and Kreutzberg, 1991). Consistent with the effects of glucocorticoids, the use of an anti-sense construct to the chemokine MCP-1 reduced the number of infiltrating macrophages to a stab wound by 30% but does not alter gliosis (Ghirnikar *et al.*, 1998b). Additionally, in immune system deficient scid mice, gliosis was actually exacerbated in the facial nerve axotomy injury model (Serpe *et al.*, 1999). Furthermore, in mice in which both TNF receptors are knocked out there is a delayed injury response and an exacerbation of damage (Sullivan *et al.*, 1999), suggesting that TNF is neuroprotective after traumatic brain injury (Sullivan *et al.*, 1999). Together, these observations suggest that gliosis is activated by factors that are not regulated by glucocorticoids and that pro-inflammatory cytokines (IL-1, TNF and IL-6) are not essential factors for astroglial activation.

As the aforementioned examples attest, inflammatory mediators can be associated with conditions that lead to gliosis, but they are not implicated as initiators of the activation process. Although a wide variety of stimuli can result in gliosis (e.g., trauma, disease, chemicals, etc.), the common feature underlying these insults is damage to neurons and/or glia (Herrera *et al.*, 1998; Del Cerro *et al.*, 1996; Bonthius *et al.*, 1995; Del Bigio *et al.*, 1994; Yokel *et al.*, 1991; Canady *et al.*, 1990; O'Callaghan, 1993; O'Callaghan *et al.*, 1995). Thus, we favor the view that the association of inflammation with gliosis is due to the direct or indirect role of inflammatory mediators in neural injury. These include such diverse effects as abnormal iron deposition, disruption of the hypothalamic-pituitary-adrenal axis, disruption of the BBB, overwhelming metabolic compensatory or stress responses, or influx of lymphocytes elaborating toxic factors (e.g. C3, reactive oxygen species etc.) (Hall *et al.*, 1998). Viewed in this context, inflammation serves as a modifier of gliosis, by directly or indirectly affecting the primary stimulus for glial activation, neural damage.

The central immune system: same as the peripheral immune system?

One reason for the conflicting views of the role of inflammation in CNS injury responses, including gliosis, is the assumption that the immune process is the same in the CNS as it is in the periphery. There are many reasons to believe that it is not. For example, the

LPS-induced chemokine (LIX) is negatively regulated by CORT in the periphery but is induced by CORT in the CNS (Rovai *et al.*, 1998). Further, each tissue has its own resident phagocytic cells that may or may not be biochemically similar in their response to antigen. In the case of braintissue, the resident phagocytes are the microglia and it is known that they are dissimilar to peripheral macrophages in some important ways. They express much less MHC surface protein than is the case for other tissue macrophages (Gehrmann *et al.*, 1993) and their response to LPS is delayed and more prolonged compared to peripheral macrophages (Andersson *et al.*, 1992). Astrocytes also express MHC-1 and 2 antigens implicating them as active participants in inflammatory/immune processes (Powell *et al.*, 1993). Astrocytes are active phagocytes and are also capable of pinocytosis (Powell *et al.*, 1993). Interestingly, glucocorticoids and adrenalectomy have brain-region-specific effects on cytokine production both in saline and LPS-treated mice (Goujon *et al.*, 1996), suggesting that there are not only differences in the inflammatory process between brain and the periphery, but also among different brain regions. Thus, given the possibility that immune function may be different in the brain, and given that IL-1 (Scripter *et al.*, 1997; da Cunha *et al.*, 1993; Giulian and Baker, 1985; Giulian and Lachman, 1985), TGF-β (Rozovsky *et al.*, 1998; da Cunha *et al.*, 1993), and other cytokines (Gomes and Paulin, 1999; Rozovsky *et al.*, 1998; Selmaj *et al.*, 1990) have been shown to be glial growth factors, it is possible that cytokines have a signaling role in the adult brain not associated with inflammation.

Hormonal regulation of GFAP

Because a variety of hormones are known to regulate the expression of GFAP in the intact animal, there is a misconception that hormones negatively regulate GFAP expression both physiologically and after gliosis (Gomes and Paulin, 1999). The data indicate that not only is hormonal regulation itself complex but so too are the effects of hormones on GFAP. There is little evidence, however, to support a large role for hormones in gliosis.

Exogenous 17-β-estradiol or progesterone was found to decrease the glial response after traumatic brain injury (Garcia-Estrada *et al.*, 1993). Testosterone regulates GFAP in a brain region-specific manner (Day *et al.*, 1993; Day *et al.*, 1990) and in a developmentally-dependent manner (Garcia-Segura *et al.*, 1988). There was an additive increase in GFAP to injury and castration (Day *et al.*, 1990), while exogenous testosterone reportedly moderated the glial response to facial nerve axotomy (Jones *et al.*, 1999). Exogenous testosterone also decreased the glial response to traumatic brain injury (Garcia-Estrada *et al.*, 1993; Garcia-Estrada *et al.*, 1999) as did dihydroepiandrosterone (DHEA) and pregnenalone (Garcia-Estrada *et al.*, 1999). However, in castrated mice, estrogen but not testosterone was neuroprotective from MPTP damage (Dluzen, 1996), but neuroprotection after MPTP has been shown to be strain-dependent in male mice (Dluzen *et al.*, 1994). Estrogen treatment results in a small (20%) decrease in GFAP after MPTP induced dopaminergic nerve terminal damage (Miller *et al.*, 1998) and was neuroprotective as measured by dopamine levels in males and females (Dluzen *et al.*, 1996; Miller, 1998).

Adrenal steroids such as corticosterone and its analogs are powerful negative regulators of GFAP at the transcriptional level *in vivo* but do not alter the reactive glial (GFAP) response to injury (Nichols *et al.*, 1990a,b; Laping *et al.*, 1994b; O'Callaghan *et al.*, 1989; O'Callaghan *et al.*, 1991). Thyroid hormone may partially regulate the transition from vimentin to GFAP as the primary intermediate filament since this transition was delayed and impaired in thyroid deficient mice (Rami and Rabie, 1988). There are developmental

and regional differences in astrocyte response to thyroid hormones (Andres-Barquin *et al.*, 1994). Hypothyroid or thyroidectomized rats had greatly reduced GFAP immunoreactivity and mRNA in some brain regions (Faivre-Sarrailh *et al.*, 1991; Kalman *et al.*, 1991; Rami and Rabie, 1988) and hypothyroid mice had delayed astrocyte response to injury (Miyake *et al.*, 1989). Thus, most data support a possible role for sex steroids and thyroid hormone, but not glucocorticoids, in *modulating* GFAP levels during gliosis. The hormonally induced changes in astrocyte process extension occur rapidly and result from changes in GFAP levels, suggesting a physiological precedent for how gliosis might be regulated.

While a number of factors and conditions have been shown to modulate the glial reaction to injury and the associated expression of GFAP, it is premature to implicate any particular signaling entity or pathway. Figure 13.1 summarizes some of the salient features of gliosis based on *in vivo* observations: (1) cytokines need not be elaborated; (2) the BBB need not be compromised; (3) modifying factors such as hormone status (prepubescent, pregnancy, estrus, post-menopause), gender and age may influence the onset, intensity, and duration of the glial response to injury; (4) glial hyperplasia is a relatively minor aspect of reactive gliosis.

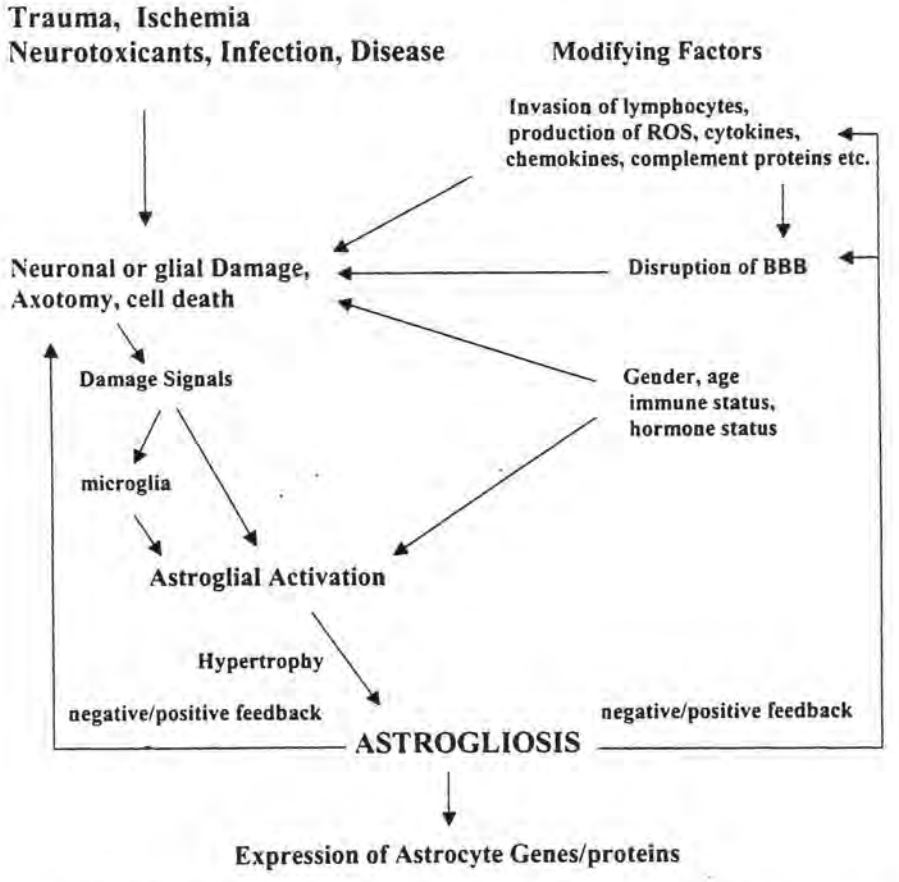

Figure 13.1 Schematic of pathways that lead to gliosis and modifying factors.

Gliosis in the developing nervous system

There are reports that gliosis does not occur in neonates or during fetal development (Sievers *et al.*, 1993). It has been shown, however, that gliosis indeed does occur after developmental exposures to diverse neurotoxicants (Morse *et al.*, 1996; Goldey *et al.*, 1994; Breese *et al.*, 1994; O'Callaghan and Miller, 1989; Eng, 1987; Miller and O'Callaghan, 1984). For example, administration of trimethyltin to the neonatal rat results in astroglial hypertrophy and an increase in GFAP expression that is more robust, although shorter in duration, than that observed in the adult after trimethyltin (O'Callaghan and Miller, 1989). We note, however, that studies of gliosis in the developing CNS are complicated by the normal developmental proliferation of astrocytes and the switch from vimentin to GFAP as the primary glial intermediate filament (Laping *et al.*, 1994d; Riol *et al.*, 1992; Tardy *et al.*, 1989). Furthermore, the neonatal rat brain is more resilient to injury and can regain function to a greater degree than is possible for an adult (Scripter *et al.*, 1997) and this may affect the duration of glial responses associated with injuries during development. Conversely, at the other end of the developmental spectrum, aged animals have been shown to exhibit mild to severe gliosis (Blum and Weickert, 1995; Laping *et al.*, 1994c) without any obvious injury, but are still able to mount a gliotic response after injury (Gordon *et al.*, 1997).

The limitations associated with studying gliosis during the developmental period, *in vivo*, underscore the fact that *in vitro* studies of astrocytes may be problematic for similar (age-related) reasons. For example, embryonic glial cells cultured with factors that induce GFAP expression (EFG, bFGF, LIF) result in differentiation and a cessation of cell division (Nishiyama *et al.*, 1993). Thus, it appears that the phenotype of a reactive glial cell and that of a proliferating glial cell are exclusive.

The variables highlighted above represent significant obstacles to studying gliosis in the neonate or aged animals. However gliosis clearly occurs in both groups. These observations emphasize the heterogeneity of astrocytes at different developmental stages, and suggests caution when interpreting results of *in vitro* studies using astrocytes or animals at early or late developmental stages. These data also suggest the dynamic nature of the astrocyte and how exquisitely sensitive it is to its environment, and that these cells are constantly changing over time in response to age-, nutrient-, and disease-status of the organism.

Detection and quantification of GFAP: immunocytochemistry vs. ELISA

Immunohistochemistry is the most widely applied technique for both qualitative and quantitative analysis of GFAP and, by extension, of reactive gliosis. From an immunohistochemical standpoint, quantification usually takes the form of cell counts although the amount of GFAP per cell also has been estimated (Bjorklund *et al.*, 1985). From a biochemical standpoint, GFAP has routinely been quantified by immunoblot analysis (O'Callaghan *et al.*, 1999) or by a variety of liquid or solid phase immunoassays (Martin and O'Callaghan, 1995a,b; O'Callaghan, 1991b). The relative merits of GFAP immunohistochemistry vs. GFAP assays will not be discussed in detail here as they were the subject of two recent papers (Martin and O'Callaghan, 1995a,b). Suffice it to say that both approaches have advantages and disadvantages. Immunohistochemistry of GFAP can reveal the presence of astrocytes in very discrete areas of the nervous system and estimates of the increases in GFAP associated with reactive gliosis often are reported using this technique. Biochemical assays of GFAP content in large tissue samples might miss even large

effects confined to small areas of the sample because of the dilution of signal. However, routine aldehyde fixation reduces antigen–antibody recognition (Martin and O'Callaghan, 1995b; Shehab *et al.*, 1990) and makes GFAP immunohistochemistry inherently less sensitive than biochemical assays of GFAP. In practical terms this often results in the detection of GFAP by immunohistochemistry only when the protein is elevated by injury. As noted above (hyperplasia vs. hypertrophy section), this situation gives the appearance of astrocytic proliferation in response to injury, because so few astrocytes are observed under the control condition. The inaccuracy of these results can easily be verified by subjecting the same tissue to immunoassay and the control tissue will be found to contain GFAP. While GFAP immunohistochemistry overestimates the proliferative capacity of astrocytes, we recently demonstrated that GFAP immunoblots underestimate injury-induced expression of GFAP. Immunoassays of GFAP, however, are not subject to this limitation. Furthermore, our long-term experience with such immunoassays suggests that they can be used to standardize astrocytic responses to a variety of neurotoxic insults. By standardize, we mean that the time-, dose- and region dependent-features of reactive gliosis can reliably be reproduced. Even small effects of response mediators, modifiers or antagonists of the gliotic condition can be analyzed using these sensitive immunoassays. A clearer, less ambiguous understanding of the features underlying reactive gliosis will likely emerge from the process.

Summary

We have attempted to reanalyze what we perceive to be the current dogma concerning the features associated with reactive gliosis and the induction of GFAP. Enhanced expression of GFAP remains the most accurate "biomarker" of gliosis regardless of the source of brain injury, yet little is known about the role of this protein in the astrocyte under physiological or pathophysiological conditions. We have suggested that the questions about glial hypertrophy vs. proliferation have arisen in part due to conflicting findings from *in vitro* studies compared to *in vivo* findings as well as inappropriate interpretation of immunohistochemical analysis of GFAP. The vast weight of evidence from *in vivo* studies consistently shows that increases in GFAP are the result of astrocytic hypertrophy. The rapidity and reversibility of the glial reaction to injury are suggestive of a trophic rather than a detrimental role for the activated astrocyte. We feel that another important misconception concerning reactive gliosis is the obligatory involvement of inflammatory mediatiors. IL-1, TNF (and/or other cytokines) do not appear to induce gliosis; cytokines can be induced in the absence of gliosis and gliosis can be induced in the absence of cytokines. Damage to cellular and subcellular elements of the mature and developing CNS represents the dominant "stimulus" for reactive gliosis. The "damage factors" common to this stimulus, probably in combination with cytokines, chemokines, and other growth factors may influence the course of gliosis. Given the compartmentalization and cellular heterogeneity of the CNS, multiple signal transduction pathways are likely to be involved in initiating and regulating this complex injury response. The expression of GFAP constitutes only one element of gliosis, but its multi-factorial regulation through interactions among hormones, cytokines, chemokines, and growth factors serves to illustrate the potential complexity of the gliotic condition. Despite these complexities, reactive gliosis remains remarkably generalized in its response in that it represents a microsensor (Kreutzberg, 1996) for injury to all regions and components of the CNS.

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