Immunity
Review

Reactive Astrocytes: Production, Function, and Therapeutic Potential

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Astrocytes constitute approximately 30% of the cells in the mammalian central nervous system (CNS). They are integral to brain and spinal-cord physiology and perform many functions important for normal neuronal development, synapse formation, and proper propagation of action potentials. We still know very little, however, about how these functions change in response to immune attack, chronic neurodegenerative disease, or acute trauma. In this review, we summarize recent studies that demonstrate that different initiating CNS injuries can elicit at least two types of "reactive" astrocytes with strikingly different properties, one type being helpful and the other harmful. We will also discuss new methods for purifying and investigating reactive-astrocyte functions and provide an overview of new markers for delineating these different states of reactive astrocytes. The discovery that astrocytes have different types of reactive states has important implications for the development of new therapies for CNS injury and diseases.

Introduction

Inflammatory responses are a major part of all central nervous system (CNS) insults, including acute trauma, infection, and chronic neurodegenerative diseases (see Sofroniew, 2015). In trauma and infection, the principle culprits in initiating and propagating this inflammatory response are circulating bone-marrow-derived leukocytes. In chronic neurodegenerative disease, the concept of neuroinflammation has evolved and implies an inflammatory process thought to originate primarily from CNS cell types. Chief among these CNS glial cells are microglia, the resident myeloid cells of the brain, as well as astrocytes. Both microglia and astrocytes have pro- and anti-inflammatory functions dependent on the mode of injury (Zamanian et al., 2012; Anderson et al., 2016; Crotti and Ransohoff, 2016; Liddelow et al., 2017; Herz et al., 2017; Klein and Hunter, 2017). Acute trauma, chronic infection, and other diseases in the CNS trigger a coordinated multicellular inflammatory response that involves glia as well as neurons and other CNS cells. Resident and infiltrating inflammatory cells have crucial roles in these responses, particularly in terms of neutralizing microbial pathogens. After tissue damage, they aid in clearance of debris (MacDonald et al., 2006; Tasdemir-Yilmaz and Freeman, 2014; Klein and Hunter, 2017). Recently, these resident microglia and infiltrating immune cells have been implicated in driving astrocyte reactivity (Liddelow et al., 2017). Coordinated interactions of these cells are required for the fine-tuned regulation and resolution of the inflammatory response. For instance, after acute trauma the diverse inflammatory and astrocytic cell responses balance debris clearance with preservation of healthy tissue and restriction of the spread of additional cytotoxic inflammation (Anderson et al., 2016). This review will discuss these points in the context of both the historical literature on astrocyte reactivity and CNS inflammation, as well as more modern insights into these responses.

Peripheral cells are involved in many responses to CNS injury, but because of the presence of the blood-brain barrier (BBB) and astrocytic endfeet (glia limitans), they are largely excluded from the brain and spinal cord. The BBB creates a unique "immune privileged" CNS environment, largely protecting the CNS from neurotoxic insult. This includes invasion from peripheral immune cells and the vast array of cytokines and reactive oxygen species they release—the presence of which produces an inflammatory CNS environment that is at best correlative and at worst causative of neuroinflammatory insult and neurodegeneration (see Lucin and Wyss-Coray, 2009). As techniques for astrocyte purification and visualization have improved, recent advances have shown that astrocytes are able to respond to this vast array of CNS insults. Such insults include, but are not limited to, traumatic brain injury (Bush et al., 1999; Ren et al., 2013), spinal cord injury (Bundesen et al., 2003; Bloom, 2014; Anderson et al., 2016); stroke (Gao et al., 2005; Zamanian et al., 2012; Cekanaviciute et al., 2014), brain tumor (Jacque et al., 1978), inflammation (Zamanian et al., 2012), and a wide range of neurodegenerative diseases (Liedtke et al., 1998; Lepore et al., 2008; Kraft et al., 2013; Ben Haim et al., 2015; Heppner et al., 2015; Liddelow et al., 2017). These injuries coincide with robust activation of microglia and other peripheral immune cells, and thus it has been difficult to discern the relative importance and function of individual cell-type responses. We now know the astrocyte response machinery includes phagocytosis of synapses, changes in the secretion of neurotrophins, clearance of debris and dead cells (Tasdemir-Yilmaz and Freeman, 2014; Chung et al., 2013), and repair of the BBB, as well as formation of a scar to enclose the necrotic lesion of such injuries or infection (Bush et al., 1999; Anderson et al., 2016). These effects provide benefit to the CNS, but as we will discuss, mounting evidence points to negative outcomes of reactive-astrocyte responses as well.

The large number of cell types involved in inflammatory responses in CNS injury and disease, as well as the complex cell-cell interactions among these and other neural cell types, has hampered mechanistic understanding of astrocyte reactivity. In this review, our goal is to address recent advances in understanding several key questions about the reactive-astrocyte

response to CNS disease. Are there different kinds of reactive astrocytes and, if so, how are these induced? Are reactive astrocytes helpful or harmful and, if so, how are their effects mediated? These advances have implications for the development of new therapies for CNS injury and disease.

Astrocyte Reactivity

Rudolf Virchow made the first description of glial cells in the mid-19th century when he described a neuroglia connective tissue (''nervenkitt'') that embedded and maintained nerve cell structure (Virchow, 1856). A decade later, Otto Dieters made the first key description and visualization of an astrocyte (Dieters, 1865). It was not until the development of more advanced microscopy and histological stains by Santiago Ramon y Cajal, Camilo Golgi, and Pio del Rio Hortega, however, that it became possible to describe with greater accuracy the intricate morphology of astrocytes and begin to unravel their astonishing diversity and varied morphology (see Somjen, 1988 for a review). The first use of the word *astrocyte* (''Astrocyten'') was used in 1895 by Michael von Lenhossék, who suggested that the star-shaped glial cells, the most common form in vertebrates, be named spider cells or astrocytes (von Lenhossék, 1895).

In addition to coining the term *neuroglia*, Rudolf Virchow made the first description of what matches with modern understanding of astrocyte reactivity and scarring (Virchow, 1855). He presented an autopsy of a 44-year-old male patient with a history of limb paralysis, worsening from age 21 in the lower extremities and ascending to affect his upper extremities at later stages. Symptoms relapsed and remitted at least once before his death. The description, though not describing glia or astrocytes specifically (these terms would not be used for the first time for several years), is remarkably specific in its account of a necrotic spinal lesion surrounded by a thick, highly fibrillary scarring. The spinal-cord white-matter lesion center contained ''ausgedehnten Schwund der Nervenfasern'' (a strong atrophy of nerve fibers; perhaps axons?), ''grosse und dicht gestreute Corpora amylacea'' (large and densely distributed corpora amylacea, or small hyaline masses often found in astrocytic endfeet), and ''Von Fett war nirgends etwas zu sehen'' (a general lack of fat—probably a loss of myelin sheathes). In addition to a necrotic core, Virchow describes that counter-staining with chromic acid revealed many ''vielfach verfilzte, äusserst feine, aber derbe Fibrillen'' (multiple matted, extremely thin but coarse fibrils—probably densely packed astrocyte processes forming a scar). Although not even using the words *glia* or *astrocyte*, Virchow gave an adept and comprehensive description of how we now describe the multifaceted reactive astrocyte response that occurs after trauma and during neurodegeneration (Eddleston and Mucke, 1993; Wanner et al., 2013; Anderson et al., 2016).

The first modern description of a broadly termed ''reactive astrocyte'' occurred during the 1970s after discovery of the intermediate filament protein GFAP (Glial Fibrillary Acidic Protein, Eng et al., 1971). Production of antisera to GFAP enabled immunostaining (Bignami et al., 1972) and rocket electrophoresis analysis of GFAP protein in human glial tumors (Jacque et al., 1978). In addition to the application of GFAP as a routine identifier of astrocytes in the healthy CNS, the molecular cloning of mouse *Gfap* (Lewis et al., 1984) led to the use of increased *Gfap* gene expression (or increased GFAP protein) in astrocytes as the standard marker of astrocyte reactivity. *Gfap*, it seems, was chosen even though it is often expressed by progenitor cells (Malatesta et al., 2003), and in disregard of other intermediate filaments, such as vimentin and nestin, that are also upregulated in reactive astrocytes.

In studies using only GFAP as a marker of astrocyte reactivity, ''reactive'' astrocytes have been reported in all mammalian species examined to date. But what of non-mammalian species? Some studies have reported minimal or no GFAP ''reactivity'' in fish (Bignami and Dahl, 1976; Murray, 1976: Hui et al., 2010; Baumgart et al., 2012), amphibians (Hung and Stelzner, 1991; Egar and Singer, 1972; Margotta et al., 1991; O'Hara et al., 1992), and the turtle (Kálmán et al., 2013). Conversely, others report some form of astrocyte ''reactivity'' in these same species (Bernstein and Bernstein, 1967; Reier 1979; Anderson et al., 1984), in addition to in lizards (Lang et al., 2008) and the chick (Székely et al., 1991; Canady and Rubel, 1992; Ajtai and Kálmán, 1998). In invertebrates, it is not yet clear to what extent reactive glia exist (MacDonald et al., 2006; Doherty et al., 2009). This lack of consensus suggests that GFAP might not be the clearest marker of astrocyte reactivity, and it provides the basis for the hypothesis that astrocyte reactivity, rather than a common singular response to injury, is highly heterogeneous.

So why has GFAP enjoyed such a long existence as *the* marker of astrocyte reactivity? Original descriptions of immunofluorescent staining in human postmortem tissue reported that GFAP antisera stained astrocytes in pathological (''gliosed'') human Alzheimer's brain (Bignami et al., 1972). Although the authors carefully describe the presence of $GFAP^+$ astrocytes in the non-gliosed rodent brain, the overwhelming immunoreactivity in the human brain sections affected by Alzheimer's disease is impressive. The original definition of astrocyte reactivity also relied heavily on the incorrect assumption that astrocytes were always highly proliferative. Given the extreme heterogeneity in the amounts of GFAP present in adjacent astrocytes in all brain regions under normal physiological conditions (Boulay et al., 2017; John Lin et al., 2017), it is easy to see how an increase in GFAP positivity could be misinterpreted as a proliferation in response to injury. Depending on context, some $GFAP^+$ ''reactive'' astrocytes incorporate BrdU (5-bromo-2′-deoxyuridine, a synthetic nucleoside that is an analog of thymidine) or stain positive for Ki67 (a cellular marker for proliferation). However, both methods reveal astrocyte proliferation is limited, especially in the context of neuroinflammation and neurodegenerative disease. This modest proliferation is around 10% after a stab wound (Simon et al., 2011), 0%–3% in mouse models of Alzheimer's disease (Kamphuis et al., 2012; Sirko et al., 2013), and 7% in an amyotrophic lateral sclerosis (ALS) model (Lepore et al., 2008). There is no increase in the number of astrocytes after systemic injection of the bacterial cell-wall endotoxin lipopolysaccharide (LPS) in vivo or in in vitro models of this neuroinflammatory insult (Zamanian et al., 2012; Liddelow et al., 2017). It should be noted, however, that considerable proliferation of astrocytes is seen after trauma when the reactive response is to produce a protective scar around the injury (Anderson et al., 2016). The analyses therefore suggest that in some instances the higher density of $GFAP^+$ cells is due to higher protein amounts and cortical atrophy, rather than to a proliferation of cells after infection or injury (see also Serrano-Pozo et al., 2013).

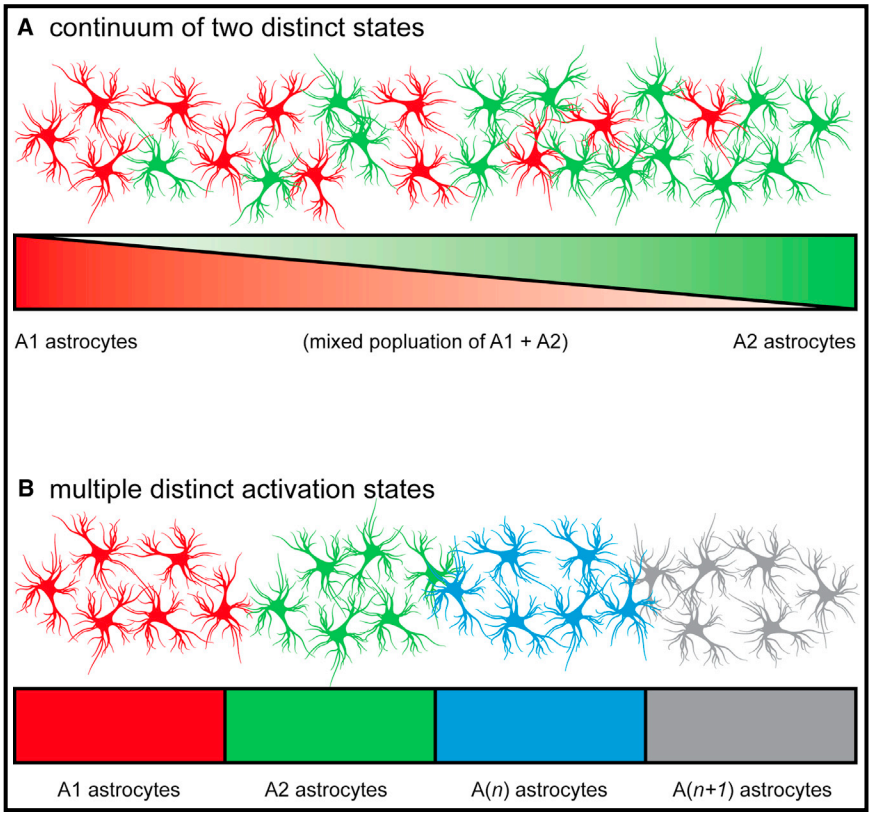

Figure 1. Possible Delineations of Reactive-Astrocyte Profiles

(A) Astrocytes so far have been shown to exist in two distinct reactive states. If these are the only two states, it is possible that they exist as a continuum, with a heterogeneous mixed population of both A1 (inflammatory) and A2 (ischemic) states. (B) In a model involving alternative reactive-astrocyte polarization states, there are multiple reactive profiles (similar to microglia and macrophages), with n number of possible activation states. Currently, it is unknown which model best describes astrocyte reactivity (although most data suggests two distinct populations, as in [A]). Single-cell genomic analysis should effectively answer this question in the future.

Thus, although changes in GFAP protein amounts and the level of *Gfap* gene expression have been a useful marker for assessing astrocyte reactivity in animal models of injury and disease and in human pathological specimens to date, better markers of astrocyte reactivity are required. Fortunately, as will be discussed below, recent transcriptomic studies have revealed other reactive-astrocyte genes that are more specific to certain types of reactive astrocytes and that are more highly expressed than *Gfap*; these genes might prove to be useful in future pathological studies.

Transcriptome Analysis of Reactive Astrocytes

Besides *Gfap*, what other genes are upregulated by reactive astrocytes? Recent studies have used gene profiling to investigate the transcriptional programs triggered by specific signaling cascades that result in either acute or chronic changes in reactive astrocyte transcriptome, morphology, and most importantly, function. This "reactive profile" can persist from several hours to days or even decades. Zamanian et al. (2012) purified and genetically profiled reactive astrocytes from mice treated either with a systemic injection of LPS (an agonist of TLR4 receptors on microglia and other immune cells) to induce neuroinflammation or by middle cerebral artery occlusion to induce cerebral ischemia. Neuroinflammation and ischemia induced two different types of reactive astrocytes, termed "A1" and "A2," respectively. This terminology parallels the "M1" and "M2" macrophage nomenclature, which has also been applied to microglia in the CNS. Microglia, the resident immune cells within the CNS, are extremely heterogeneous. Their diverse functional phenotypes range from pro-inflammatory "M1-like" phenotypes characterized by upregulation of inflammatory mediators such as *Tnf* (Tumor Necrosis Factor), *Il1b* (Interleukin 1 beta), and reactive oxygen species (see Block et al., 2007) to immunosuppressive "M2-like" phenotypes characterized by upregulation of *Chil3* (Chitinase-like 3), *Fzd1* (Frizzled class receptor 1), and *Arg1* (Arginase 1, see Boche et al., 2013). It should be noted that this nomenclature is under current refinement because macrophages and microglia can display more than two polarization states (Martinez and Gordon, 2014; Heppner et al., 2015), and it is not yet clear whether microglia themselves can ever adopt an M2-like state as opposed to invading macrophages. Similarly, reactive astrocytes might well have more than two states of polarization (see Figure 1), which will be an important area for future research.

These gene transcriptome analyses of reactive astrocytes show that A1 neuroinflammatory reactive astrocytes upregulate many genes (e.g., complement cascade genes) that have been previously shown to be destructive to synapses, suggesting that A1s might have "harmful" functions. By contrast, ischemia-induced A2 reactive astrocytes upregulated many neurotrophic factors, which promote survival and growth of neurons, as well as thrombospondins, which promote synapse repair. This upregulation suggests that A2s might have "helpful" or reparative functions. Consistent with this, previous studies have shown that reactive astrocytes induced by ischemia promote CNS recovery and repair (Gao et al., 2005; Zador et al., 2009; Hayakawa et al., 2014). For instance, Sofroniew and colleagues selectively ablated scar-forming, proliferative reactive astrocytes by using a *Stat3* driver (STAT3 is a specific scar-forming reactive astrocyte marker). This removal of scar-forming reactive astrocytes after a stab wound led to a sustained 25-fold increase in infiltration of monocytes, macrophages, neutrophils, and lymphocytes; failure of BBB repair; and substantial neurodegeneration (Bush et al., 1999). Similar experiments involving *Stat3*-mediated ablation of proliferative scar-forming astrocytes in the context of spinal-cord injury led to a worsened injury outcome, including extensive axon dieback (Anderson

et al., 2016). These "helpful" reactive astrocytes might well correspond to the A2 reactive astrocytes induced by ischemia in Zamanian et al. (2012).

The finding that different types of reactive astrocytes are induced by different types of injury—and that ischemic injury produces a so-called trophic "A2" reactive astrocyte, whereas an inflammatory insult produces a more toxic "A1" reactive astrocyte (Zamanian et al., 2012)—raises many questions. How many reactive astrocyte types are there? What are the cell-cell interactions that induce reactive astrocytes? And what are the relevant extracellular and intracellular signaling pathways that induce reactive astrocytes? Studies strongly suggest that *Stat3*-mediated (possibly A2) reactive astrocytes proliferate and support neuronal regeneration in models of acute trauma (Anderson et al., 2016), whereas A1 neuroinflammatory reactive astrocytes might be induced by NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling (Lian et al., 2015; Liddelow et al., 2017; see below). In much the same way that microglia can have multiple simultaneous reactive profiles, do the A1 and A2 phenotypes represent the extremes of a continuous spectrum of reactive profiles (see Figure 1)? The heterogeneity of reactive astrocytes at the cellular level needs to be more thoroughly investigated. Modern developments in single-cell sequencing techniques are likely to provide a wealth of information about the reactive state and about timing after chronic infection, disease, or acute injury. It also remains unclear how this heterogeneity may or may not be pre-determined. Is it is established during development, poising individual astrocytes to react in a specific manner? How does this heterogeneous priming and response contribute to the vulnerability of neurons susceptible to astrocyte-derived toxins? A more comprehensive understanding of the pathways involved in inducing astrocyte reactivity will provide a great deal of this information.

New Tools for Studying Astrocytes and Reactive Astrocytes

It has been very difficult to distinguish contributions of astrocytes from those of microglia because they usually become reactive in concert, and both are involved in neuroinflammation. These delicate interactions are difficult to study in in vivo systems because many of the key proteins and genes are present or expressed by multiple cell types. As a result, it is generally better use culture systems to complete such mechanistic investigations. The first in vitro systems to investigate astrocytes were developed in the early 1980s (McCarthy and de Vellis, 1980), and although they allowed for the study of isolated astrocytes for the first time, they had several shortcomings. First, purification took several weeks, and there was considerable contamination of other CNS cell types, including microglia and progenitor cells. Second, these cultures are maintained in serum-containing media, which because of the presence of BBB is usually excluded from the CNS (except in instances of trauma or vascular distress following stroke). Serum exposure appears to alter the transcriptome and morphology of these cells irreversibly (Foo et al., 2011). As useful as these culture systems have been for the investigation of important astrocyte physiological functions (Christopherson et al., 2005; Eroglu et al., 2009; Allen et al., 2012), they are not representative of physiological astrocytes in vivo.

More recent immunopanning methods rapidly purify astrocytes from the postnatal rodent brain in under a day (Foo et al., 2011). These immunopanned astrocytes are maintained in serum-free media with the trophic support of Heparin-Binding EGF-like growth factor (HBEGF). These newer in vitro cultures of astrocytes retain their resting in vivo gene profiles and, unlike previous purification and culture methods, are not highly proliferative in culture. There is also evidence that astrocytes grown on a three-dimensional polymer matrix might be even more appropriate: they show less upregulation of *Gfap* than do cells grown in a two-dimensional monolayer, and their many branching processes make them morphologically complex (Puschmann et al., 2014). However, even these morphologically complex astrocytes are prepared according to the original serum-containing methods of the 1980s, and as such they are probably transcriptomically different from astrocytes present in the normal healthy CNS. Human astrocytes have also been purified and grown in vitro, either as pure monocultures (Sharif and Prevot, 2012; for a comprehensive review see Krencik and Ullian, 2013) or as three-dimensional organoids (Paşca et al., 2015). Combined, these multiple purification and growth paradigms suggest that perfect culture systems for modeling a "normal" in vivo astrocyte are likely to require a combination of multiple features, including specific trophic support, the correct substrate, and possibly other unknown factors. Whole transcriptomic analyses of three-dimensional cultures of immunopanned, serum-free cultures will most likely provide additional insights into astrocyte functions in the normal, healthy brain.

Unfortunately, culture systems for microglia have largely lagged behind the successes of astrocyte culture systems, although recent advances have provided newer methods for studying these cells in vitro (Butovsky et al., 2014; Bohlen et al., 2017). This alone has been a major impediment to further investigations into how these important immune cells might interact with astrocytes in both a physiological and reactive setting—whether this activation is due to infection, disease, trauma, or immune response.

Another complication in finding an appropriate methodology for studying astrocytes has been in the lack of an in vitro model of astrocyte reactivity that recapitulates real reactivity seen in disease and after trauma. Although many of the methods listed above show upregulation of a whole suite of reactive-astrocyte genes, none mimic the exact profile seen with acute purification of astrocytes in animal models of injury or disease. These models will be of ever-increasing importance because they will provide a powerful way of cataloging the specific mechanisms of reactive-astrocyte induction, as well as allow one to probe interactions of reactive astrocytes with other CNS cells. Additionally, they will provide a useful model for screening drugs for the ability to treat neurodegenerative disease.

Recently, we developed a new model system that enables pure A1 reactive astrocytes to be studied in a culture dish (Liddelow et al., 2017). This was possible thanks to our ability to rapidly purify astrocytes from the uninjured postnatal brain, grow them in serum-free cultures, and finally supplement these cultures with a reactive astrocyte-inducing microglial-derived cytokine cocktail. Microglial activation, either by acute CNS injury or by systemic LPS injection, induces A1 reactive astrocytes both in vitro and in vivo. We found that microglia induce these A1s

by releasing three cytokines: Interleukin 1 alpha (IL1α), Tumor Necrosis Factor alpha (TNFα), and the Complement Component Subunit 1q (C1q), which together are sufficient in vitro to induce A1 reactive astrocytes whose gene profiles closely mirror that of A1 reactive astrocytes in vivo (Liddelow et al., 2017). The resulting cultures of pure A1 reactive astrocytes provide a powerful tool with which to investigate their functions. Using this model, we found that A1s have a striking loss of most main astrocyte functions; they have a decreased ability to induce synapse formation and function, a loss of ability to phagocytose synapses, and a loss of ability to promote neuronal survival and growth. In an improvement to GFAP staining as a marker for reactivity, single-cell data showed that the complement component C3 was specifically upregulated in A1 reactive astrocytes (and not in resting or A2 reactive astrocytes). This marker now provides a way to distinguish between different activation states of reactive astrocytes in both rodent and human tissue. Surprisingly, the A1 reactive astrocytes also exhibited a new function in which they secrete a yet-to-be-identified neurotoxin that induced apoptosis of neurons and oligodendrocytes but no other CNS cell types. Importantly, we found that A1 reactive astrocytes were rapidly induced after CNS injury and were responsible for the death of axotomized CNS neurons. When A1 formation was prevented genetically or pharmacologically, the death of the axotomized CNS neurons was entirely prevented. Interestingly, the activated microglia used for inducing A1s were insufficient to induce death of neurons or oligodendrocytes.

Thus, improved methods of separating and highly purifying microglia and astrocytes will allow their relative contributions to be dissected and enable improved dissection of their interactions. This will be important because it is unclear whether microglia lack the capacity to induce death of neurons. On the one hand, although some studies show microglial toxicity (Boje and Arora, 1992; Chao et al., 1992; Burguillos et al., 2011), these are in vitro studies that do not recapitulate in vivo microglia phenotypes (see above; Bohlen et al., 2017). On the other hand, animal-model experiments that inactivate or deplete microglia are not associated with beneficial outcomes for the brain, suggesting that microglia are not responsible for neurotoxicity and are instead protective. It is possible that the function of these normally protective microglia could change in chronic neurodegeneration, but to date these studies have been hamstrung by a lack of effective models with which to study glia in disease. New methods of purifying and culturing microglia (Bohlen et al., 2017) and astrocytes (Foo et al., 2011; Liddelow et al., 2017) should enable future studies to address many other questions, including whether astrocytes signal back to the microglia to control their activation state or signal to infiltrating peripheral immune cells.

Astrocytes and Disease

Reactive astrocytes, such as reactive microglia, accompany every acute injury and chronic neurological disease. Given that it is now clear that reactive astrocytes exist in at least two different states of activation, A1 and A2, a critical question is now to determine whether they are present in both mouse models and human disease, and another is what their specific contributions to disease pathophysiology are. Although it is easy to understand how scar-forming reactive astrocytes that encapsulate injury or seal a damaged BBB are beneficial, other forms of astrocyte reactivity appear to be harmful. Reactive astrocytes, while providing trophic support to regenerating axons (Anderson et al., 2016), can also inhibit axon regeneration (see Silver and Miller, 2004). Reactive astrocytes also upregulate some genes responsible for the induction of synapse formation. Such genes include those encoding thrombospondins, which can help repair the brain (Liauw et al., 2008; Zamanian et al., 2012; Liddelow et al., 2017), but nevertheless these changes might also result in unwanted synapses that lead to epilepsy or neuropathic pain (Boroujerdi et al., 2008). Astrocytes cultured from the Sod1G93A mutant mouse release a factor that can kill motor neurons (Di Giorgio et al., 2007; Nagai et al., 2007; Lobsiger et al., 2007). Similarly, a neurotoxic factor that is released from A1 reactive astrocytes is toxic to alpha motor neurons, which degenerate in human patients with ALS. Interestingly this factor is not toxic to other motor neuron subtypes spared in the disease (Liddelow et al., 2017). Indeed, A1 reactive astrocytes are present in brain regions involved in neurodegeneration in a variety of human diseases, including Alzheimer's disease, multiple sclerosis, ALS, Parkinson's disease, and Huntington's disease (Liddelow et al., 2017). Given that A1s release many classical complement cascade components that can enhance synaptic degeneration (Stevens et al., 2007; Hong et al., 2016; Sekar et al., 2016) as well as a neurotoxin that induces the death of neurons and oligodendrocytes, this brings renewed focus to the potential importance of reactive astrocytes in chronic neurodegenerative disease. Although it has long been speculated that neuroinflammation is secondary to neurodegeneration, the existence of A1s in human neurodegenerative disease suggests that neuroinflammation might be helping or even driving neurodegeneration. It will be important to determine the identity of this toxin so that new therapeutics can inhibit its production or antagonize its effects.

How can the relative roles of A1s and A2s in disease be determined from mouse models? One way forward will be to identify the key intracellular signaling pathways that polarize the induction of astrocytes into either helpful or harmful cells. Disabling these inducing pathways within astrocytes will prevent the formation of either A1 or A2 reactive astrocytes. Harmful effects of reactive astrocytes have already been reported in various mouse models of disease.

The NFκB pathway controls cytokine production and cell survival and is strongly associated with neuroinflammation, as well as neuroinflammatory reactivity in astrocytes and microglia (Mattson and Meffert, 2006; Kaltschmidt and Kaltschmidt, 2009; Hsiao et al., 2013). It is also widely activated during neurodegenerative disease (Migheli et al., 1997; Gilmore, 2006). Many pro-inflammatory agents, such as cytokines, bacterial or viral antigens, amyloid, stress, free radicals, and many other factors activate the NFκB pathway (Gilmore 2006; Kaltschmidt and Kaltschmidt, 2009). The ubiquitous nature of activation of the NFκB pathway in disease has meant the connection between activation and astrocyte reactivity has so far been ambiguous; it has not yet been investigated whether it is essential to initiate astrocyte reactivity. It is possible that the requirement for NFκB pathway activation is only of transient importance to astrocyte reactivity, given that inhibition of NFκB signaling selectively in astrocytes only temporarily altered *Gfap* expression levels at the onset of motor dysfunction in a murine model of ALS, the Sod1G93A

mutation (Crosio et al., 2011). It is unknown to what extent these cells were actually reactive, however; *Gfap* is one of the least upregulated reactive transcripts after injury (Zamanian et al., 2012). A comprehensive analysis of astrocyte gene alterations in this model might lead to very different conclusions. Similarly, NFκB-GFP reporter mice crossed with Sod1G93A ALS mice show more robust activation in microglia cells than in astrocytes, but again astrocyte reactivity was only assessed via GFAP immunoreactivity (Frakes et al., 2014). Toxicity assays in co-cultured cells detected motor-neuron cell death that was attributed to microglia-released factors in this model. It is unclear, however, what percentage (if any) of contaminating astrocytes might be present in these culture systems because it is now apparent that traditionally activated neuroinflammatory microglia have strong NFκB pathway activation that is required for the activation of neuroinflammatory reactive astrocytes (Liddelow et al., 2017). Similarly, studies of NFκB activation of astrocytes in rodent models of Alzheimer's disease (Carrero et al., 2012; Lian et al., 2015), as well as in mouse models of Huntington's disease (Hsiao et al., 2013), provide evidence that NFκB activation in astrocytes might play an important role in chronic inflammation and progression of these diseases. NFκB pathway activation in astrocytes is present in the spinal cord of human patients with ALS (Migheli et al., 1997). Thus, NFκB-activated astrocytes might represent harmful astrocytes that help promote neurodegeneration in a variety of mouse models of disease. Given that A1 reactive astrocytes also exhibit NFκB activation (Lian et al., 2015), taken together these studies suggest that A1 reactive astrocytes are probably present in many models of mouse disease, where they might exert a variety of harmful effects that contribute to disease pathophysiology (see below).

In contrast, recent studies taken together suggest that the JAK-STAT3 pathway is probably mediating the activation of A2 (ischemic) scar-forming reactive astrocytes. This pathway regulates multiple cell functions, including cell proliferation, differentiation, and growth, and some inflammatory functions (Ceyzériat et al., 2016). The JAK-STAT3 pathway in astrocytes is important during early brain development, when it helps to control the onset of astrogliogenesis and the later maturation of astrocytes (He et al., 2005; Kanski et al., 2014). Many studies have implicated JAK-STAT3 in scar-forming astrocyte reactivity after acute injury (Herrmann et al., 2008; Anderson et al., 2016; Ceyzériat et al., 2016). The *Drosophila* homolog of STAT3, STAT92E, is also responsible for mediating "reactivity" in astrocyte-like cells of the fly (Doherty et al., 2014).

Therapeutic Potential

How might drugs that target these pathways mediate the onset or progression of diseases that involve astrocyte reactivity? Because A1 formation can be prevented by delivery of neutralizing antibodies to TNFα and IL1α and because FDA-approved drugs targeting TNFα and IL1α already exist, this approach deserves further investigation for treatment of a variety of acute CNS injuries and chronic neurodegenerative diseases. A new drug that inhibits C1q is currently in clinical trial. Mice that lack TNFα, IL1α, and C1q live for prolonged periods without obvious abnormalities. Similarly, identification of the A1 secreted neurotoxin could lead to the development of drugs that inhibit this toxin or its actions.

Similarly, drugs that control Stat3 and NFκB could hold potential for controlling the activation state of reactive astrocytes. Upregulation of NFκB signaling is associated with several neurodegenerative diseases, such as Parkinson's disease (Hunot et al., 1997), Huntington's disease (Hsiao et al., 2013), and Alzheimer's disease (Kaltschmidt et al., 1997; Mori et al., 2010). In addition to NFκB activation, C3 (an A1 astrocyte marker) is activated by amyloid in both mouse models and human brain from patients with Alzheimer's disease (Lian et al., 2015). Because we now understand the negative interactions of A1 reactive astrocytes, it is tantalizing to attempt to treat neurodegeneration by blocking NFκB signaling. However, it should be noted that although NFκB is activated under pathological conditions, the pathway is also involved in synapse formation and plasticity (Meffert et al., 2003; Koo et al., 2010; Boersma et al., 2011). It is unknown, therefore, whether inhibition of NFκB would have negative effects on the CNS. It is possible that although neuron-killing A1 astrocytes would be stopped, regenerative efforts or synaptogenic capacity would still be impeded. Unfortunately, very few genetic overexpression models aimed at determining the extent to which NFκB might be detrimental to the CNS in pathological conditions have been generated (Gerondakis et al., 2006).

Treatments aimed at increasing the proportion of STAT3-mediated trophic scar-forming astrocytes could potentially be detrimental to the CNS. STAT3 is not normally activated in healthy brains under basal conditions; instead, the pathway involves highly activated human-disease-like brain glioma (Gu et al., 2008). STAT3 also targets genes that promote the cell cycle and inhibit apoptosis (these are probably key for the proliferative nature of scar-forming reactive astrocytes), two key mechanisms integral to tumorigenesis. Tumor recognition by the immune system is also blocked when STAT3 is overactive as a result of the inhibition of activity of microglia and macrophages (Hussain et al., 2007; Brantley and Benveniste, 2008). Would drugs that increase STAT3 signaling cause formation of astrocytomas? Would treatment to produce more "trophic" reactive astrocytes have an alternative effect and ultimately impair CNS function?

Drugs that inhibit NFκB activation in astrocytes or promote STAT3 activation might hold therapeutic potential, but they should be approached with care until further dissemination of their roles in the healthy CNS. Equally, the result of increases and decreases in the balance of A1 and A2 astrocyte number in the context of disease and trauma will need to be ascertained before safe treatment paradigms will be possible. If we have learned anything from decades of neuron-focused neurodegenerative research, it is that such diseases of the brain are complex. As a result, although treatments focused on astrocytes (and glia in general) are providing real promise for human patients, care needs to be taken in applying our current understanding until we fully comprehend the delicate balance between emerging multiple activation states.

Directions for Future Research

One highly mysterious area is the nature and functional significance of the interactions between immune cells and astrocytes. There is mounting evidence that astrocyte activation relies on the multitude of signals pouring from the activation of other resident CNS cells (namely microglia) and infiltrating peripheral immune

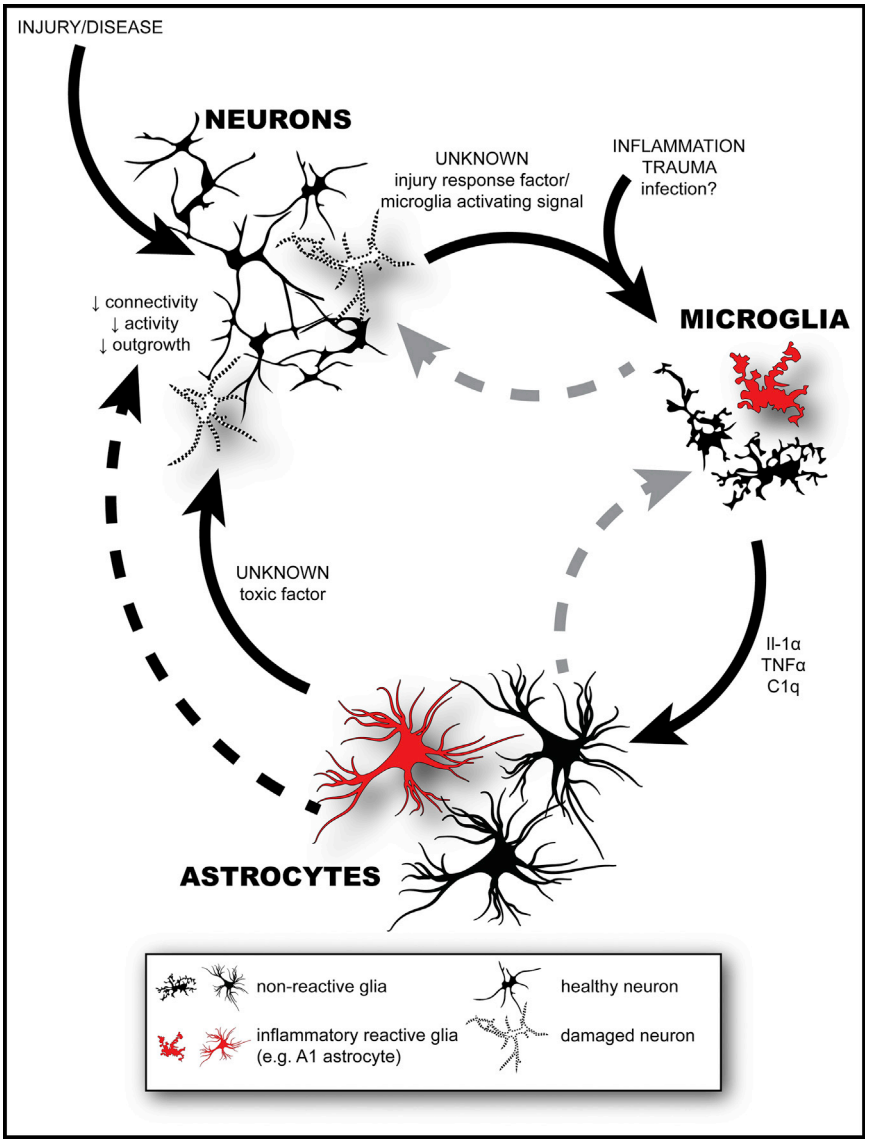

Figure 2. A Model of Astrocyte-Neurotoxicity Specificity

A model in which only damaged neurons are targeted by an A1 (neuroinflammatory) reactive-astrocyte-derived toxin. After injury, disease, or inflammatory insult, damaged neurons could release a factor (or factors) that could activate microglia; the neurons might also present a "neurotoxin receptor" on the cell surface. This release of microglia-activating signals would in turn cause microglia to release astrocyte-activating signals (Il1α, TNFα, and C1q), which then would cause astrocytes to release a neurotoxin. In this multistep process, one would see broad and robust activation of both astrocytes and microglia (which could also be activated by the initial injury itself), but targeted death of only diseased or damaged neurons (dependent on "neurotoxin receptor" expression), and no toxicity to innocent bystanders. This way, the neural network would be largely maintained and could be rewired to protect overall function.

cells. What is less well understood, however, is how these reactive astrocytes integrate into the community of CNS cells and how they might interact with them in a positive or negative way. Do they alter their homeostatic and trophic functions? Do they proliferate to impede further immune-cell infiltration into the CNS? What molecules that have remodeling or toxic effects on the surrounding brain tissue do they secrete? Recent work has illuminated the importance of microglia-astrocyte crosstalk, namely microglia-derived signals that induce a specific form of reactive astrocyte. Interleukin 1 alpha, TNFα, and C1q induce A1 neuroinflammatory reactive astrocytes, present after LPS stimulation (and presumably bacterial infection, Zamanian et al., 2012; Liddelow et al., 2017). In contrast, TGFβ signaling to astrocytes, presumably from TGFβ originating in microglia or other immune cells, reduces subacute neuroinflammation after stroke in mice (Cekanaviciute et al., 2014; Cekanaviciute and Buckwalter, 2016). In turn, similar to activated microglia, reactive astrocytes highly express many complement components, cytokines, and chemokines that probably signal to microglia or various other immune cell types to attract them into the CNS or regulate their immune functions.

It has long been a mystery why the mammalian CNS fails to regenerate severed axons after injury (Brosius Lutz and Barres, 2014). Reactive gliosis is a prominent response, and it is suggested that reactive astrocytes form a barrier to axons (Silver and Miller, 2004). However, recent work has established that after spinal-cord injury at least some of the reactive astrocytes formed help promote regrowth of the severed axons (Bush et al., 1999; Anderson et al., 2016). Still, A1 reactive astrocytes that fail to promote neuron survival and growth and are actively inhibitory to it rapidly form within the optic nerve after crush injury (Liddelow et al., 2017). They also lose phagocytic ability and thus would be unable to clear myelin debris, which is strongly inhibitory to axon growth. This raises the important question for future work to investigate as to whether ablation or prevention of the A1 response helps to promote successful axon regeneration. A provocative question raised by recent studies revealing the existence of A1 reactive astrocytes after injury or in disease is why the CNS would ever produce a neurotoxic, "harmful" astrocyte. One possibility is that these toxic reactive astrocytes are only targeting neurons injured beyond repair: those neurons with excessive damage or dysfunctional firing that could be of major detriment to the neural network as a whole. In this model (see Figure 2), diseased and injured neurons would do one of two things: first, they could release a factor (or factors) that could activate microglia; or second, they could present a "neurotoxin receptor" on the neuronal surface. This release of microglia-activating signals would cause microglia to release A1 astrocyte-activating signals (Il1α, TNFα, and C1q), and A1 astrocytes in turn would release a neurotoxin. In this multistep process, one

would see broad and robust activation of both astrocytes and microglia (which could also be activated by the initial injury itself) but only targeted death of diseased or damaged neurons (dependent on "neurotoxin receptor" expression), and no innocent bystander toxicity. This way, the neural network would be largely maintained and could be rewired to protect overall function. For instance, synapses released from the dead neurons could rewire onto neighboring uninjured neurons.

Another possibility is that neurotoxic A1 reactive astrocytes are not neurotoxic by design but instead have evolved to help to fight off infections. Although A1 astrocytes are not directly toxic to bacteria (Liddelow et al., 2017), they do secrete a vast array of complement cascade components that probably enhance the clearance of bacteria by the immune system—such components include both resident microglia and infiltrating peripheral myeloid cells. Similarly, they might halt viral infection or kill virally infected neurons in order to prevent the spread of viruses, or even modify CNS immune responses by mitigating the responses of specific types of infiltrating immune cells. In any case, it seems unlikely that the A1 response has evolved for use only in the CNS. An important question for future work is whether cells in non-CNS tissues undergo A1-like transformations in response to inflammatory processes and, if so, whether the A1 toxin is involved in mediating cell death, for instance the death of cardiac cells in cardiomyopathy or insulin secreting islet cells in diabetes.

Finally, although it is clear that A1s are induced by activated microglia, it remains unclear what the cellular and molecular basis of A2 induction is. Are microglia, perhaps in a different activation state than M1, able to induce A2s? Alternatively, are other cell types alternatively or additionally responsible? It is possible that ischemia itself will act directly on astrocytes to induce their polarization to the A2 state. A better understanding of the signaling mechanisms that induce different types of reactive astrocytes will be an important step toward the development of drugs that allow us to control and harness reactive astrocytes to promote repair in disease.

Concluding Remarks

The study of astrocyte-immune interactions has provided significant advances and new insights in recent years. As the link between microglia, infiltrating peripheral immune cells, and astrocytes during normal physiology and during pathological conditions is becoming apparent, the altered communication that results and the expanding importance of crosstalk between immune cells will require increased research focus. We find ourselves at a time when the methods of studying astrocytes both in culture systems and in animal models are finally available, providing a powerful approach to addressing questions about astrocyte functions during disease and after trauma. With more appropriate markers for reactivity, and the ability to rapidly purify cells from both rodents and humans, it will be possible to investigate and visualize different types of reactive astrocytes. Ultimately, this will provide a more comprehensive understanding of what astrocytes do in disease and how we might ameliorate disease by targeting astrocytes.

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