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Neuroinflammation and Microglia: Considerations and approaches for neurotoxicity assessment

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Abstract

Background—The impact of an inflammatory response, as well as interactions between the immune and nervous systems, are rapidly assuming major roles in neurodegenerative disease and injury. However, it is now appreciated that the exact nature of such responses can differ with each type of insult and interaction. More recently, neuroinflammation and the associated cellular response of microglia are being considered for their contribution to neurotoxicity of environmental agents; yet, to date, the inclusion of inflammatory endpoints into neurotoxicity assessment have relied primarily on relatively limited measures or driven by *in vitro* models of neurotoxicity.

Objective—To present background information on relevant biological considerations of neuroinflammation and the microglia response demonstrating the complex integrative nature of these biological processes and raising concern with regards to translation of effects demonstrated *in vitro* to the *in vivo* situation. Specific points are addressed that would influence the design and interpretation of neuroinflammation with regards to neurotoxicology assessment.

Conclusion—There is a complex and dynamic response in the brain to regulate inflammatory processes and maintain a normal homeostatic level. The classification of such responses as beneficial or detrimental is an oversimplification. Neuroinflammation should be considered as a balanced network of processes where subtle modifications can shift the cells toward disparate outcomes. The tendency to over-interpret data obtained in an isolated culture system should be discouraged. Rather, the use of cross-disciplinary approaches to evaluate multiple endpoints should be incorporated into the assessment of inflammatory contributions to the neurotoxicity of environmental exposures.

Keywords

microglia; neuroinflammation; mercury; neurotoxicology; immunotoxicology; neurodegenerative disease; aging

1. Introduction

Inflammation is a normal response of the organism to infection, injury, and trauma. Within the brain, this response can be elicited from the resident cells or it can be induced with infiltration of immune cells from the periphery. A complex series of immune-like reactions is initiated to neutralize invading pathogens, repair injured tissues, and promote wound healing with the end goal of restoring tissue homeostasis. While neuroinflammation has been considered a mediator

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of secondary damage, the local immune response also has beneficial effects on the traumatized tissue. Such effects include the clearance of cellular debris, secretion of neurotrophic factors, cytokines, and activation of proteases for matrix remodeling. Therefore, while inflammation in the brain can have negative effects on recovery following injury, other actions appear to be beneficial and essential. In this framework, inflammation can be viewed as a complicated series of local immune responses that serve to deal with a threat to the neuronal microenvironment. Such a threat can occur with disease, physical trauma, ischemia/hypoxia, or with cellular damage due to multiple initiating stimuli, including exposure to neurotoxicants. For additional reading on the issue of heterogeneity of microglia responses the reader is directed to the following references [1,2,3,4,5].

The central nervous system is immune privileged with regards to self-regulation of its immunological components [6,7]. As in other organ systems, there are multiple contributions to, as well as outcomes from, an immune-mediated response in the CNS. For example, we now know that molecules of the systemic innate immune system are able to stimulate the immune cells of the brain, as well as, directly impact neuronal populations. While the exact level of influence this has on the intact brain has not been clearly determined, the fact that the CNS is not impervious to cellular or soluble pathogens and immunogens from the circulation presents a different framework than originally held. Within this framework, one could envision that systemic modulating factors, such as stress, immune-status, and genetic background may influence the normal neuroimmune interactions and shift the normal response to injury. The reader is directed to the following references for further reading on the influence of systemic inflammation of neuroinflammation [8,9,10,11].

Research efforts in neuroinflammation and neuroimmunology have significantly expanded over the last decade. Quite a bit of this work has been directed toward understanding the mechanisms involved in immune signaling and the contribution of inflammation to neuronal and white matter degradation with autoimmune diseases, such as multiple sclerosis, human immunodeficiency virus, or brain infections. More recently, consideration has been directed toward the possible role of such responses in the neurotoxicity of pharmacological and environmental agents. In regards to neurotoxicology of environmental agents, this is a newly evolving field and quite often the studies are based on a limited number of endpoints, neuropathology inclusive of a microglia response, or the generation of oxidative stress by-products and pro-inflammatory cytokines, and quite often generated in an isolated *in vitro* system. In this opinion, it is our goal to provide a general background on unique features of the neuroinflammatory and neuroimmune responses in the brain. This is set in a framework of the potential contribution of infiltrating cells, the development and aging of resident immune cells, and the endogenous regulatory processes. These distinct features of the inflammatory response and microglia will impact the design and interpretation of experimental models to assess effects within a neurotoxicology framework.

2. Microglia as the primary immune cell of the brain

Microglia serve as the resident mononuclear phagocytes of the brain and are highly heterogeneous within the healthy CNS. They comprise only 10% of the total cell population of the brain; yet, have multiple morphological and possible functional profiles as influenced by their environment [12,13]. The presence of activated glial cells localized to regions of brain injury was initially considered as a sign of pathology and, as such, considered for use as a sensitive marker to identify injury sites predestined for imminent tissue destruction [5]. However, we now know that microglia are in intimate contact with neurons, for which they serve important maintenance functions, are capable of responding to subtle changes in the microenvironment, and dynamically survey the CNS [14,15,16,17]. The benefit of the actions of microglia is evident with an exacerbation of neuropathology induced in mouse models that

are deficient in monocytes/macrophages. For example, in the *op/op* mouse, there is an absence of functional protein for colony stimulating factor that results in a depletion of monocytes, including microglia. When these animals were subjected to hippocampal damage by the organometal, trimethyltin, a shift in the dose response curve was seen with an exacerbation of neuronal death in the absence of microglia [18]. A similar exacerbation of damage has been demonstrated for ischemic injury using ganciclovir treatment to ablate proliferating microglia [19].

Microglia share innate immunological functions with other mononuclear phagocytes, such as monocytes, macrophages, and dendritic cells [20]. They share phenotypic characteristics and lineage-related properties with bone-marrow-derived monocytes and macrophages. Such properties include the ability to secrete cytokines common to immune accessory cells and, while significantly less than that seen in peripheral organs, microglia serve as antigen-presenting cells [21,22,23,24,25].

Just as there are multiple neuronal phenotypes within the brain, multiple morphological, and likely functional, phenotypes exist for microglia. It has even been suggested that microglial responses are tailored in regional and insult-specific manners [26]. Unfortunately, with the exception of a fully activated microglia showing an amoeboid morphology consistent with phagocytic activity, to date there is little functional information to accompany these morphological differences. This however, is beginning to change with the increased awareness of microglia heterogeneity and the inability to directly translate effects seen in culture systems to the *in vivo* state.

While microglia are not solely responsible for the inflammatory or immune mediated responses in the brain, they are poised to be able to rapidly respond to environmental changes. The presence of activated microglia within injured brain regions and in post-mortem tissue from patients with various neurodegenerative diseases, has led to the initial assumption that all reactive microglia would contribute to an adverse and degenerative process. However, this makes the assumption that somehow, a brain resident glial cell can be transformed into an aggressive effector cell that can attack healthy neurons either physically, as by phagocytosis, or via secreted factors. However, in the mature brain, microglia seem to alter their morphology dependent upon the specific task at hand. Such resident immune responders may be beneficial in the healing phases of CNS injury by actively monitoring and controlling the extracellular environment, walling off areas of the CNS from non-CNS tissue, and removing dead, damaged, or dysfunctional cells. Microglia can upregulate a variety of surface receptors and produce multiple secreted factors including pro- and anti-inflammatory cytokines, nitric oxide, reactive oxygen species (ROS), glutamate, and growth factors. However, microglia also express the glutamate transporter, GLT-1 and, thus, may provide a level of protection through the elimination of extracellular glutamate [27]. In addition, microglia can facilitate the apoptosis and phagocytosis of infiltrating T cells through various signaling pathways leading to a subsequent down regulation of microglial immune activation [28]. This plethora of responses is representative of the dichotomy of microglia reactivity in promoting neuronal survival or degeneration.

Structurally, microglia display a dynamic and active phenotype with ongoing retraction and extension of processes into the brain parenchyma [17]. This supports the idea of a surveillance function for microglia in the healthy brain, but it also raises the question of whether or not microglia have an active role to maintain optimal neuronal function. These cells are also known to aid in brain remodeling through their assistant role in synapse stripping and matrix reorganization.

Very distinct interactions have been demonstrated between neurons and glia. If neuronal activity is blocked with tetrodotoxin, microglia upregulate their expression of major histocompatibility complex (MHC) class II [29], suggesting an interdependency between normal functioning neurons and microglia. In support of this, healthy neurons express several molecules that actively downregulate microglia and macrophage activation states. In mice lacking CD200 or CD200 receptor, microglia show an altered morphology and increased expression of MHC and CD11b [30,31]. In these mice, inflammatory reactions are exacerbated following mechanical injury, exposure to the toll-like receptor ligand (TLR), lipopolysaccharide (LPS), and induction of experimental autoimmune encephalomyelitis.

2.1. Development and Aging of Microglia

During brain development, the resident population of microglia changes as a function of maturation. Prior to the formation of the blood-brain-barrier, blood-borne monocytes are able to migrate into the brain, maturing into resident microglia. In the human, microglia are present as early as 13 weeks of gestation, however, they display different morphological phenotypes [32]. In the cortex, they display a ramified morphology while, in the germinal matrix; they display a more amoeboid morphology. Developmentally, this is a period of active brain remodeling and the microglia are believed to participate in the clearance of apoptotic cells [33]. A more aggressive role of microglia is seen in inducing neuronal death during normal development in the cerebellum [34]. With birth, and during the first few postpartum weeks, the number of amoeboid microglia decrease and an increase is seen in ramified cells with long, thin, branched processes [35]. The classical bone-marrow-derived microglial cells have a small soma with arborized or ramified processes and reside in the brain parenchyma. Under normal conditions, these cells do not display macrophage-like functions and serve as resident immune cells with a very slow turnover rate [36]. In addition to resident microglia, perivascular macrophages line blood vessel walls but do not penetrate into the brain parenchyma, and macrophages are found in brain meninges and choroid-plexus. These cells display a different morphology than microglia and have a slightly higher turnover rate.

The progressive, age-related change in the expression of MHC II antigens by microglia led to the assumption that microglial activation occurs with aging. The pattern of expression was reported to be similar to what occurs in experimental animal models of injury. However, in the aged brain, neuronal injury and death, which stimulate microglia activation, are not apparent [37]. Further investigation showed morphological changes of microglia cytoplasmic structure in the aged human brain reflective of dystrophy and senescence [13,38]. Thus, while the cells change as a function of age, the morphological phenotype is suggestive of a decreased ability to mount a normal host-resistance response rather than a transformation to an activated cell. With an increasing focus on aged animals, such a shift is beginning to be reported [39]. It has been suggested that age-related neurodegeneration might not only be due to a loss of neuroprotective properties, but also the actual loss of microglia [40]. A recent hypothesis put forth to explain the phenotypic changes in aging microglia suggest that microglial senescence is triggered by an intracellular oxidative stress response due to enhanced intracellular accumulation of iron [41]. Under this hypothesis, an oxidative stress response, often attributed to microglia based primarily upon *in vitro* studies, would lead to senescence rather than activation.

2.2. Microglia Phagocytosis

An efficient, active process for the phagocytosis and clearance of aberrant or excess proteins is necessary for maintaining homeostatic balance of the protein burden in the brain and preventing development of proteinopathies and neurodegeneration. While the actual mechanism is not known, the inability of microglia to efficiently clear β -amyloid ($A\beta$)₄₂ is a major contributor to Alzheimer's disease (AD) and amyloid deposition is seen in other

neurodegenerative diseases such as, Parkinson's, Batten, and prion diseases. This may be due to a senescence process in microglia, an overload of the cell, or to an altered cell signaling. While inflammation is not a hallmark of AD, recent data suggests that both A β deposition and neurofibrillary tangles activate a potentially pathological innate immune response [42,43]. Clearance of debris is facilitated by the expression of highly conserved pattern recognition receptors (PRRs). On glial cells, these include the complement receptors [44,45] TLRs, and scavenger receptors [46,47,48]. In the CNS, astrocytes, microglia, and neurons can participate in the complement cascade which, when activated, can lead to a local inflammatory response as triggered by aberrant proteins and peptides leading to phagocytosis of excess material. For example, complement component 1q (C1q) co-localizes with amyloid deposits and triggers the complement cascade [49]. While the phagocytic activation serves in a beneficial manner to eliminate aggregated and toxic proteins from the brain, it has been proposed that an extended activation or dysregulation of this system can lead to an adverse environment and subsequent toxicity to surrounding cells. Data obtained from cultured microglia suggest that activation of the complement system can lead to increased phagocytosis of A β [50]. In transgenic mouse models of AD, less neuropathology is demonstrated in the absence of C1q [51]; however, inhibition of complement activation diminishes microglial activity, increases amyloid load, and enhances neuronal degeneration [52].

2.3. Redox Signaling

Reactive oxygen species (ROS) are multi-potent, diffusible molecules capable of carrying out signal transduction processes in response to extracellular stimuli. A role of ROS in gliosis has been proposed from studies showing an inhibition by antioxidant treatment. Recently, NADPH oxidase was identified as the ROS-producing molecule in astrocytes, where cytokine stimulation leads to a rapid activation followed by the expression of pro-inflammatory products, such as inducible nitric oxide synthase (iNOS; [53]). The generation of ROS leading to the induction of iNOS enhances nitric oxide (NO) production from glial and endothelial cells. NO is a vasorelaxant, a neurotransmitter, and, in excess concentrations, NO forms peroxynitrite, and through nitrosylation of cell signaling messengers, serves in immune regulation [54]. ROS signaling has been considered as a unifying mechanism underlying multiple forms of neuroinflammation and microglia responses. However, many of these studies have been conducted *in vitro* and raise the question of how to translate to *in vivo*. Given the high activation level that occurs in peripheral macrophages, it is possible that what may be seen *in vivo* is predominantly reflective of infiltrating macrophages rather than a contribution from the resident microglia. For example, not all injuries to the brain result in the induction of NOS but rather may rely on signaling via the TNF receptor for neuronal death [55,56]. These reports demonstrate that a tumor TNF receptor mediated neuronal death can occur both with a neurotoxicant injury-model and in a model of brain ischemia. This would suggest that the production of TNF from microglia could contribute to this process; however, the exact dynamics are complicated and may be initiated by the injured neuron rather than the microglia.

3. Pro-inflammatory cytokines

The onset of inflammation is often characterized by the release of pro-inflammatory cytokines, such as (TNF), interleukin (IL)-1, as well as, adhesion molecules. Many of these secreted factors influence the system in both an autocrine and paracrine fashion. In addition to the activation of local glia and, often, the recruitment of blood-borne leukocytes, local synthesis of inflammatory-related cytokines such as IL-1 and TNF α elicits a wide range of effects including, cell adhesion, migration, survival, differentiation, replication, secretory function, and cell death. In the same cell, a cytokine can exert contrary effects, inducing cell death, survival, or proliferation depending upon the functional context in which it acts. For IL-1 and

TNF α , these effects are mediated primarily by IL-1 type I receptor (IL-1R1) and TNFR1 (p55) or 2 (p75), respectively.

While a pathophysiological role for IL-1 as part of the inflammatory process in the brain has been established, exactly how the signaling process may contribute to neuronal death and protection is still unclear. However, data support its role as a key mediator of inflammation and neuronal death in acute brain injuries, such as stroke and trauma [57]. Microglia functions related to an innate immune response are associated with TNF signaling and its regulation of both inflammation and apoptosis. TNF is a multipotent, inflammatory cytokine that can induce apoptosis via activation of receptors containing a homologous cytoplasmic sequence identifying an intracellular death domain. This includes TNFR1 and CD95 (APO-1/Fas) with their corresponding death ligands, TNF and the structurally related type II transmembrane protein, FasL. The release of TNF α and FasL shedding by microglia are implicated in neurotoxicity [58,59]. Membrane receptor mechanisms of apoptosis are implicated in neuronal death involving downstream intracellular death-signaling complexes, such as AP-1, NF- κ B, and caspases. Further, TNFR1 activation may provide a molecular mechanism for the rapid apoptosis of injured or sick neurons through a caspase 3-mediated pathway [60]. The tissue distribution of the receptor and the differentiation state of the target cell influence the cellular response to cytokines. Although neither TNF α or IL-1 β has been demonstrated to cause neuronal death in healthy brain tissue or normal neurons [61] and normal cellular architecture is maintained in mice deficient in pro-inflammatory cytokines or receptors, this mechanism supports the hypothesis that localized pro-inflammatory cytokine activation could initiate neuronal death. Increases in TNF α and IL-1 β have been observed prior to neuronal death [62,63,64] and recent studies suggest that, indeed, the activation of immune factors such as TNF can participate in causation of neuronal death [55,56].

4. Downregulation of Inflammation

In order to counter pro-inflammatory signaling pathways, cells utilize multiple mechanisms including concurrent production of anti-inflammatory factors such as IL-10, IL-4, soluble TNF receptors, IL-1 receptor antagonist, and transforming growth factor (TGF) β . Other anti-inflammatory cellular processes include the activation of protein kinase A to hinder the expression of iNOS [65]. Further, upon exposure to pro-inflammatory cytokines, cells rapidly upregulate their normally low levels of a family of proteins called suppressors of cytokine signaling (SOCS [66]). These signaling molecules can negatively regulate the response of immune cells, either by inhibiting the activity of JAK or by competing with signaling molecules for binding to the phosphorylated receptor. Data suggests that the up-regulation of SOCS represents a step towards suppressing glial inflammation via negative regulation of the JAK-STAT pathway [67]. Inhibition also occurs with cytokine-induced iNOS expression. Both rapid transcription of SOCS 1 and 3, as well as the phosphorylation of SHP2, down-regulate the JAK-STAT pathway. This leads to the suppression of inflammatory responses [67]. Specific anti-inflammatory cytokines in the CNS such as, IL-10, IL-13, and IL-4 down-regulate the inflammatory process by stimulating biosynthesis of pro-inflammatory cytokine inhibitors, such as soluble receptors [68]. They also intercept signals that are generated by pro-inflammatory cytokine, receptor-ligand complexes.

5. Influence of Systemic Signals

Although the blood-brain-barrier serves as an effective barrier for the majority of the tissue, the circumventricular organs may present a vulnerable location for signaling from the systemic circulation. A second mechanism for communication between the systemic and central immune systems is somewhat indirect. In this case, cytokines circulating within the bloodstream can signal via binding to receptors on neurovascular endothelial cells. Pro-inflammatory cytokines

released upon immune challenge can activate vagal afferent signaling and subsequent direct or indirect activation of vagal efferents. This has been proposed as a demonstration that the sensory vagal afferents, together with the regulatory vagal efferents, form an inflammatory reflex that continually monitors and modulates the inflammatory status in the periphery [69]. Additional observations suggest that the cholinergic, anti-inflammatory pathway is activated during the inflammatory response. For example, inflammatory events in the thoracic abdominal cavity are signaled to the brain through the vagal-nerve sensory afferents.

Recently, peripheral lymphocytes (T and B cells) that infiltrate the brain have been shown to participate in the local response to an acute or chronic CNS injury, in addition to resident microglia and infiltrating macrophages. T-cells primed to react to specific myelin antigens promote neuronal survival in animal models of nerve crush and other physical injuries [70] and have been shown to protect hippocampal neurons against chemical exposure to the organometal, trimethyltin [71]. Overall, these studies demonstrate that CNS responses previously considered to be the result of CNS tissue specific cells may involve contributions from multiple cell types whose actions are significantly influenced by the systemic state of the organism.

6. Acute versus Chronic Inflammation

The complex interdependency of neuronal injury and glial activation makes it difficult to distinguish between an initiating, prerequisite activation versus a compensatory, functional response of the glia. While this is a difficulty with acute insults or infection, it becomes next to impossible to ask such questions in a progressive, chronic condition. The best data to date comes from various models of acute injury. However, the majority of these models are limited with regards to a well-defined and characterized dose and temporal response. Resident microglia offer neuroprotection following acute brain injury [72]. With an acute physical lesion such as a nerve transection, an increase is seen in immunoreactive microglia 3–5 days after the lesion. This response declines rapidly within the first 1–2 weeks but the number of microglia cells can remain elevated a month after lesion [73,74]. Currently, the available models of acute injury are compromised by the presence of infiltrating macrophages, which may not represent the early stages of a chronic disease or neurotoxicity produced from exposure to an environmental agent. The critical need for an appropriate model to examine the response of resident microglia in the absence of infiltrating cells has prompted significant efforts from various investigators. However, any interpretation of data with regards to levels of inflammatory factors or morphological changes in microglia requires the consideration of non-resident cells in the process.

The interest in neuroinflammation in chronic degenerative disease is based upon human data suggesting a decreased risk for AD with extended use of non-steroidal anti-inflammatory drugs (NSAIDs). In many animal models of chronic human disease, reports refer to an over expression of the pro-inflammatory cytokines, $\text{TNF}\alpha$ and $\text{IL-1}\beta$; however, the basal levels are extremely low, such that, even “over expressed”, the cytokine levels still remain very low. Many of these changes could reflect a shift in the microglia population as a function of aging [13,41]. It is of interest that much of this data is based upon determining mRNA levels for each cytokine. While the mRNA levels for $\text{TNF}\alpha$ are elevated 2 to 3-fold in many models when measured, the expression of TNF protein is rarely detected. In addition, there often is very little, if any, data on the message or protein levels of the receptors or receptor antagonist. It is possible that, in these proposed models of chronic inflammation, the elevated mRNA levels for TNF in the absence of a comparable elevation in protein or receptors reflect a “primed” system similar to what has been seen with peripheral macrophages expressing high levels of untranslated TNF mRNA. Additionally, the data could reflect a change in action of anti-inflammatory molecules such as $\text{TGF}\beta 1$ and IL-10 to regulate TNF translation. This would be

consistent with reports that a systemic inflammatory challenge in an animal model of chronic neurodegenerative disease resulted in exaggerated brain inflammation, exaggerated sickness behavior, and an increase in acute neurodegeneration [75]. Overall, the data suggests that the pro-inflammatory response to neurodegeneration is maintained under tight regulatory control.

As mentioned earlier, when microglia are exposed to the same stimulus over an extended period of time, the cells adjust to this new environment in their overall efforts to maintain homeostatic balance. For example, in the AD brain, microglia are in contact with plaques yet, they are not stimulated to phagocytize and effect clearance. Thus, the chronic exposure appears to lead to the down-regulation of this normal microglial activity. A similar response occurs for repeated exposures of microglia to LPS [76]. When a progressive model of Parkinson's disease was examined with reference to the adverse impact of microglial activation and inflammation on damage to dopaminergic neurons, Wright and colleagues [77] clearly demonstrated *in vivo* that, while the immunosuppressant drug, tacrolimus (FK506), reduced the initial stage of neuronal death in the substantia nigra, it did not alter the morphological response of microglia. While the tendency is to view a neurodegenerative disease process as a chronic, slow progression of cell death, it may instead be a series of acute, individual events. If that is the case, further examination into short-term injury responses, such as might occur in models of delayed neuronal death, might actually be more relevant to a chronic human condition than originally thought. Of interest is the recent work of Meyer-Luehmann and colleagues [78] demonstrating, by longitudinal *in vivo* multiphoton microscopy, that plaques in the brain form quickly over a period of 24 hrs. Within 1–2 days of a new plaque's appearance, microglia are activated and recruited to the site. These issues raise questions as to what features need to be incorporated in the generation of a model of chronic neuroinflammation. They also raise significant questions with regards to how to interpret any changes induced as a result of exposure to a toxic compound.

7. *In vitro* culture systems to study inflammatory responses

Studies in cell culture have demonstrated both neurotoxic and neuroprotective effects of microglia or microglia conditioned media on cultured neurons. *In vitro*, microglia have been shown to be capable of releasing several potentially cytotoxic substances, including reactive oxygen intermediates, NO, proteases, arachidonic-acid derivatives, excitatory amino acids, quinolinic acid, and cytokines. Much of the *in vitro* work examining the "neurotoxic" properties of microglia have used isolated microglia cultures with the assumption that the induction of microglia activation and production of such factors would be representative endpoints of toxicity. In various *in vitro* assay systems of cultured microglia, stimulation with LPS and IFN γ induce the cells to develop a neurotoxic phenotype [79,80]. However, if the same compounds are injected into the brain, peripheral macrophages are recruited into the CNS and the infarct is self-resolving. This resolution is paralleled by an activation of microglia throughout the CNS, yet no neuronal death is observed. Importantly, upon isolation of the resident microglia from the infiltrating macrophages, co-culturing with neurons demonstrate that infiltrating macrophages can induce neuronal death, while such toxicity is not observed with co-cultures of neurons and resident microglia [80]. Some of the differences between *in vitro* and *in vivo* observations of microglia may be attributable to the inability to culture these cells in a quiescent, resting state and the fact that much of the major published data comes from cultures grown in serum, allowing for serum-dependent anomalies.

The microglia gene expression profile has been described for primary murine [81] and rat cells [82] and a murine cell line [83]. Additional gene expression profiles have been generated from cultured microglia stimulated with IFN γ [84], TGF- β [85], fractalkine [86], colony-stimulating factor [81], or Gram-positive bacteria [47]. Attempts to directly compare the gene expression profile in culture with what occurs *in vivo* have been extremely limited. Recent work by Lund

et al., [87] demonstrated similarities, but also some important differences, in the gene and protein expression profiles of a microglial response *in vivo* versus *in vitro*. Using LPS, they demonstrated a relative concordance in the inflammatory-related genes; however, as one might expect, the counter-regulatory response was significantly less evident. The authors concluded that this represented a deficiency in homeostatic control of the innate immune response and the presence of regulatory factors in the nervous system that were not present *in vitro*. This is consistent with the hypothesis that the simplistic view of inflammation as an important mediator of neuronal damage is not necessarily correct, but rather that the outcome from an inflammatory event is decided by how the response is controlled.

The secondary addition of microglia cells or supernatants from microglia cultures to isolated neurons has been another common approach. Several reports suggest that astrocytic and neuronal factors are key in determining the toxic potential of microglia [88,89]. However, isolated neurons in dissociated cultures are particularly susceptible to negative effects of microglia as a result of the absence of endogenous neuroglial and neurovascular interactions. Quite often, *in vitro* studies employ activation of a single receptor to examine microglial responses, or to prime the cells for a subsequent exposure. For example, one common approach uses the bacterial component, LPS as an activator. Exposure to LPS activates TLRs, causing microglia to acquire a phenotype that is both inflammatory and cytotoxic. This has led to an inaccurate acceptance of this specific induction as typical of all activated microglia. *In vivo*, microglia encounter danger signals from both exogenous (infectious agents) and endogenous factors (excessive and aberrant cellular products and neurotransmitters). Thus, reliance on a one-dimensional stimulus may lead to findings that will not translate to the cascade of responses that occur in the normal *in vivo* situation.

It is becoming readily apparent that the neuroinflammatory component of various degenerative conditions is of equal, and often greater, importance than the dysfunction of the constitutive neuron population. As the majority of these responses are elicited by the supportive glial cells, in particular microglia and astrocytes, the incorporation and evaluation of these cell types in *ex vivo* assay systems is of particular interest in the pharmaceutical and toxicological fields. For example, a proposed critical feature for microglia in culture is the ability to migrate to the site of injury; however, this has been somewhat difficult to demonstrate *in vivo*. However, in brain slice cultures, an initial retraction of microglia ramifications and the extension of new pseudopodia, presumably for migration, have been demonstrated, as well as, the dependency between cell types for this process to occur [90,91].

7.1. Organotypic slice cultures

Organotypic slice cultures (OSCs) are heralded as being the quintessential model system for neuroinflammatory studies. The three dimensional architecture of the slice model recapitulates the majority of cellular contacts between these neurons, the supportive glia, and vasculature, at least for some period of time *in vitro*. OSCs are thick sections (250–400µm) of early postnatal brain tissue grown in either roller tube or interface cultures. In the roller tube method, the culture is maintained attached to a coverslip rotating within the incubator, thinning down to a monolayer of cells in a few weeks. The more commonly used “interface” culture relies upon a semi-permeable membrane to facilitate the maintenance of a gas: media interface at the surface of the slice, which, from 400µm, thins down to ~100µm by 14 days *in vitro* (DIV). The trauma of culturing has a profound effect on the survivability of neurons, as well as the synaptic organization, at least over the short term. As early as four hours after culturing, swollen fibers and degenerating neurons are observable. This robust neuronal cell death continues up to at least 6DIV followed by a low-level, spontaneous neuronal degeneration that persists up to at least 28DIV [92].

A profound “reorganization of glia” occurs in organotypic cultures [93], with astrocytes forming a scar-like layer over the slice [94] that can significantly hinder the delivery of any exogenous factor after 24 hrs in culture. This scar covers the top and bottom of the slice by 3DIV and the entire slice by 7DIV, persisting until at least 20DIV [95,96]. Investigators have attempted to bypass the barrier by direct injection into the slice; however, this seems to offer a migratory tract for surface microglia. The proliferation and typified migration of astrocytes to the outer rim of the intact OSCs may be mediated, in part, by release of IL-1 from microglia. Perhaps more interestingly, slice astrocytes appear to maintain an immature phenotype, regardless of culture duration [97].

Microglia respond immediately to the trauma of preparing the organotypic culture by initiating an orchestrated series of changes in morphology, function, and proliferation. Microglia respond to the cut edge within hours, converting to an amoeboid morphology as early as 2 hours *in vitro* [91] and their response to subsequent insults can occur in less than 10 min, as shown following glucose deprivation [98]. The majority of microglia become amoeboid by 1-2DIV, remaining in an activated state over the first 6DIV; facilitating the phagocytosis of debris and damaged nuclei. Between 7 and 14DIV, many microglia in the center of the slice regain their ramified phenotype [90,95]. However, there remain sporadic, amoeboid microglia with phagocytic activity evenly distributed throughout the slice [94]. Their migration to the cut edge geographically blunts their responsiveness to localized neuronal insults and limits their ability to survey the immediate neuronal microenvironment or communicate with other cell types via short-lived soluble mediators such as ATP. Importantly, though, the functions of organotypic microglia seem to be integral in regulating and quelling the degenerative response of resident immune cells that results from the trauma of culturing. In organotypic cultures, microglial processes are seen separating neuronal processes, presynaptic endings and cell bodies, presumably performing synaptic stripping in order to facilitate the synaptic deafferentiation necessary for synaptic reorganization and repair [99].

7.1.1. Alterations to inflammatory cytokines and cell surface markers—Pro-inflammatory cytokines (IL-1 β , IL-6, and TNF α) are expressed at increased levels in OSCs and cannot be induced further with a controlled mechanical injury, whereas stimulation of the pro-inflammatory cascade of microglia directly via chronic application of LPS transiently increases IL-1 β and IL-6 [100,101]. Organotypic culture preparation also causes temporary increases in secreted IL-6 and TNF α . These return to baseline levels after 4DIV [90]; however, microglia express IL-1 β up to 10DIV, regardless of whether they have a ramified or amoeboid morphology [94]. The co-stimulatory molecule B7-2, normally displayed on antigen presenting cells for T cell stimulation, is quickly upregulated from at least 0-7DIV. The heterogeneity of the microglia response is demonstrated in OSCs by the observation that microglia that are nearby degenerating neurons, not those in areas with intact neuronal architecture, can be induced to express MHC II in response to IFN γ [102].

The aberrant environment of the OSC detracts from their use to examine neuroinflammation. Efforts to downregulate this process and maintain a non-inflamed slice have not been overly successful. While it is difficult to address questions with regard to an up-regulation of inflammation and a glia response or in identifying neuronal/glia interactions, OSC may be useful to examine an inherent difference in genetically modified mice that would result in a down-regulation of the response. Whether or not one could adequately evaluate similar changes as a function of *in vivo* drug or environment toxicant exposure remains a question. With long-term cultures, the inflammatory response may have abated, but the priming or pre-conditioning of the glia may result in a shift in cellular responsiveness to damage such that it would be an inappropriate representation of the *in vivo* situation. In support of this, a recent paper demonstrated increased susceptibility of neuronal populations in slice cultures following pre-exposure to an inflammatory environment [103]. One item requiring consideration when

interpreting soluble signals in organotypic tissues is the lack of vascular system influences. The capillary structure begins to break down immediately after culture and slices lose RECA-1 positive and eNOS positive vascular structures after two weeks, although some inducible components may persist over the short term.

8. Application of Inflammation and Microglia Responses in Evaluation of Chemical-Induced Neurotoxicity

While there is a significant amount of interest in the microglial response and contribution to neuronal dysfunction, there is surprisingly little work actually conducted within the area of environmental neurotoxicology. Much of this has relied on the use of *in vitro* systems for which the relevance of the findings has let to be translated to the *in vivo* situation. Some of the initial work has focused on heavy metals such as, mercury and inorganic lead that are well known to have neurotoxic properties and induce an alteration in the systemic immune system. Some of the initial work on mercury demonstrated a transient increase in microglia in the neuropil following acute exposure of adult rats [104]. Mercury deposits were demonstrated in microglia and, with additional studies in non-human primates [105], and the accumulation in astrocytes and microglia was suggested to identify a primary location for the demethylation of mercury. Further work to determine if the mercury-induced activation of microglia was involved in neuronal apoptosis showed no relationship. With exposure to low levels of mercury, a neuroprotective effect was observed as a result of the microglial reactivity and the induction and release of IL-6 from astrocytes [106,107]. More recent work [108] utilizing a continuous (10 – 50 days) application of the heavy metals, lead and mercury, to aggregating brain-cell cultures demonstrated an increase in amyloid precursor protein with lead and the formation of insoluble A β by mercury. In each case, a microglia response was observed that would be consistent with normal phagocytic activity of these two classic stimuli. A significant amount of research has examined the role of microglia in neurotoxicity produced by the organometal, trimethyltin [109,110,111]. Interestingly, in these studies, the induction of TNF α by microglia may directly contribute to the neuropathology [56,112,113]. Additional *in vivo* work continues to demonstrate heterogeneity of the microglia response, even within targeted brain structures and individual classes of chemical or drug-induced injuries, continuing to demonstrate heterogeneity of microglia responses [56,114].

Recent interest in environmental exposure and inflammation in brain tissue has arisen from *in vivo* and *in vitro* studies showing that nearly all of the proposed etiologies for Parkinson's disease, including bacteria, viruses, pesticides, drug contaminants, and head trauma, are known to produce an inflammatory response. However, a mechanism by which microglia would specifically target healthy dopamine neurons remain un-identified [115]. Interestingly, recent work demonstrated that exposure to polychlorinated biphenyls induces a systemic inflammatory response in mice with an associated reduction in striatal dopamine related proteins, potentially related to IL-6 expression [116]. Thus, there is a body of literature for assessment of neurotoxicity of environmental agents that, to date supports the diversity and heterogeneity of the microglia and neuroimmune or neuroinflammatory response.

9. Expert Opinion

The presence of activated microglia during almost any neurological insult and the reliance on, and possible over-interpretation of, data obtained from *in vitro* systems has lead to the assumption that activated microglia and associated inflammatory responses are harmful to the brain. Recent work, however, has clearly demonstrated that the cellular activities are, for the most part, beneficial. It is when the strict regulatory control normally imposed on them is altered that these cells may begin to show detrimental effects. One could speculate that such functions could be related to senescence and the inability to perform normal activities or the production

of pro-inflammatory cytokines exceeding the down-regulatory capacity of the system with either an acute or chronic induction. Classification as beneficial or detrimental oversimplifies the interactions between diverse cell types of the brain and the signaling cascades they initiate. Various families of cytokines, growth factors, and chemokines influence the apoptotic or survival pathways of neurons and the inflammatory state of the CNS. Understanding signaling pathways activated by such factors continues to increase; however, it is still unclear which specific pathways regulate inflammatory processes in more chronic neurodegeneration and neuroimmunological diseases. What is becoming clear is that there is a complex and dynamic response of the brain to regulate inflammatory processes and maintain a normal homeostatic level. Any role in dysregulation is complex and due to the overlapping, synergizing, and antagonizing effects of various factors. This then requires consideration of the process as a balanced network where subtle modifications can shift the cells toward disparate outcomes, such as death, proliferation, migration, the induction of inflammation, or the inhibition of immune responses.

In addition, it is possible that the resident microglia of the brain parenchyma are more receptive to the regulatory controls of the environment and that, many of the damaging effects observed, stem from infiltrating peripheral macrophages recruited to the site of damage. This may explain the dichotomy of microglial responses observed: the rather diffuse response of process bearing microglia throughout the brain but the more activated morphology seen at focal injury sites. In addition, the possibility exists that activated microglia are not committed to a particular behavioral phenotype but, rather, may be able to vacillate between destructive and supportive phenotypes. In this case, characterizing the temporal pattern of such changes becomes important when interpreting data on the nature of the microglial response and how it might play a role in neurotoxicity or neuroprotection.

A significant amount of our current knowledge on microglia comes from *in vitro* studies. While such studies can provide information on the cellular mechanisms of a specific response, they do not reflect the heterogeneity of microglia or the complexity of their responses *in vivo*. Responses observed *in vitro* will be one-dimensional, given that the cells are deprived of their physiological environment and often stimulated on only a single receptor, such as with LPS. Thus, their use within the context of toxicology may be limited to the ability to determine if the exposure to an environmental agent can directly induce a microglial response. This, however, does not conclude that a similar response will occur *in vivo* or with environmentally relevant exposures and byproducts. The net outcome of the multiple microglia-derived factors remains un-resolved. The continued *in vitro* and *in vivo* work clearly demonstrates that the network of effects and the interactions between the various cell types of the brain, as well as a contribution from non-CNS- resident cells, requires that any evaluation of the process employ multiple endpoints. The final outcome and effect of the process is dependent upon the nature of the insult and the underlying biological state of the organism, including age, genetic background, stress level, disease, drug, and environmental exposure history. The physiological and pathological nature of interactions between the immune and nervous systems is different from the localized response of resident immune cells in the brain (104). Each should be considered individually, as well as, in concert, when evaluating clinical and experimental data with regards to determining the detrimental or beneficial consequence of the response.

The source and history of CNS-resident macrophage populations becomes of importance in determining the impact of exposure-related changes. For example, if the source of amoeboid macrophages is from a blood-borne population, then the systemic effects of exposure to drugs or environmental agents becomes of issue if a challenge or injury occurs that recruits blood-borne cells. Conversely, if the source of macrophages is from a self-renewing or a long-lived source located within the CNS, this then reflects the length of time that the cell has had to adapt to any environmental differences (i.e. matrix components, cellular contact, etc.) or homeostatic

signals (i.e. extracellular ligands) from the environment. As has been assumed for development of the nervous system with regards to neurons and neural connections, the developmental ontogeny of microglia and the limited turnover of these cells suggest that development may also reflect a vulnerable period for disruption that may manifest as dysfunction over a long period of time. Additionally, the demonstration of microglia senescence suggests a second vulnerable period occurring in the aged population.

Our understanding of the complexity of the monocyte populations throughout the body, the heterogeneity of microglia cells, the possible contribution of cells recruited from the periphery or those activated within the brain parenchyma, and the multiple factors involved in the regulation of neuroinflammation, sets a daunting task in the design and interpretation of studies evaluating the impact of environmental exposure. While it is tempting to morphologically examine the response of glia or to measure levels of pro-inflammatory cytokines, each in isolation, it is clearly evident from the current state of the field that this will offer little understanding of the system. Within the field of neurotoxicology, there are a few laboratories trying to use an integrated approach to understand how neuroinflammation and alterations in this process may contribute to neurotoxicity. Of interest is the evolving approach of not only determining the impact on degeneration but also on neuronal survival. The research efforts within neurotoxicology cited above represent work conducted with this broader perspective in mind and, as such, offer a framework for future studies.

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