Chapter 4

Glial Reactivity in Response to Neurotoxins: Relevance and Methods

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Abstract

Microglia and astrocytes become activated in response to diverse toxic exposures, regardless of the cellular or molecular targets affected; biomarkers of these responses, therefore, can be used to detect and localize damage to any area of the CNS. A variety of cellular and molecular markers of reactive microglia and astrocytes have been implemented to reveal all types of neural injuries, including those caused by chemical insults of the CNS. Recent advances in approaches to evaluate the cell-specific transcriptome in the CNS allow for an expansion of the existing repertoire of glial activation biomarkers. Here, we show how the approach we used to validate assays of glial fibrillary acidic protein (GFAP) as a biomarker of astrogliosis can be extended to a cell signaling-based assay via phosphorylation of signal transducer and activator of transcription 3 (STAT3). We also introduce new methods to assess cell type-specific gene expression, glial-specific pharmacological inhibition, and genetic manipulation that can be used to evaluate glial reactivity, with the overall goal of defining the microglial and astroglial activation phenotype that results from exposures to broad classes of neurotoxicants.

Key words Astrocytes, Microglia, Hypertrophy, Gene expression, Protein expression, ELISA, Neuroinflammation

1 Introduction

The history of neuroscience in large measure has been dominated by a focus on the structure and function of neurons with relatively little attention devoted to the other nervous system cell type: glia. More recently, however, microglia, oligodendroglia, and astrocytes, the principal glial subtypes, have been subject to renewed attention at the molecular to cellular levels. Microglia and astrocytes, in particular, have received attention for their role in neuroinflammation and their potential contribution to disease (for examples, see More et al. [1], Cai et al. [2], Phillips et al. [3], Crotti and Glass [4], and Hooten et al. [5]). Our focus here is on the propensity of microglia and astrocytes to become "activated" in response to neurotoxic insults regardless of the brain area

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affected or the particular neurotoxicant or neurotoxic mixture involved. It is this "reactive" property of microglia and astrocytes that make them a useful source for broadly applicable biomarkers of neurotoxicity. We review, herein, some of the approaches we have taken to validate existing biomarkers of glial activation and present contemporary methods for discovering and characterizing novel glial biomarkers of neurotoxicity.

1.1 Glial Reactivity and CNS Damage

As the "microsensors" of the CNS, the activation of glia serves as a biological response/sensor, driven by the brain's reaction to diverse sets of insults [6, 7]. Since the glial activation response is triggered regardless of affected brain area or neuron type, it can serve as a sensitive and specific measure of neurotoxicity that is not specific to the compound or mixture of compounds that induced it. Microglia and astrocytes are the main "reactive" glial cells within the CNS, with each responding to various stimuli, both centrally and peripherally generated. With their similarity to macrophages, microglia are typically viewed as the "resident immune cells" of the CNS, secreting proinflammatory mediators like cytokines and chemokines and demonstrating phagocytic functions once activated. Astrocytes are also immune-like cells capable of secreting cytokines and chemokines and creating "glial scars" to isolate areas of significant damage but additionally provide metabolic support to neurons and their synapses and are crucial to the formation of the blood-brain barrier. While oligodendrocytes are also major constituents of the glial population of the CNS and can be affected by toxic exposures, their role in neurotoxicity has been less well studied; most focus has been on developmental effects on oligodendroglia, such as those caused by lead, alcohol, and anesthetics [8–10].

Glia can be activated by a variety of stimuli, including infection, disease (e.g., Alzheimer's and Parkinson's), and cell damage (e.g., toxicity- or traumatic injury-induced) (for review, see Burda and Sofroniew [7]). Generally, glial activation results in a combination of morphological changes to the cell, as well as the presentation of a different molecular and biochemical pattern. The morphological changes to microglia and astrocytes are the most straightforward indication of glial activation; however, this response cannot be generalized across both cell types. Microglia transition from a ramified state to a more amoeboid state, losing their processes and, consequently, resulting in a larger cell body when activated. While reactive microglia have historically been classified into categories (M1 and M2), their response is more graded, and their morphology can represent a continuum between ramified, "resting" cells to activated, amoeboid phagocytic cells. Astrocytes also experience hypertrophy (reactive gliosis, astrogliosis) but also tend to increase the number and/or size of their processes, as opposed to losing them. Additionally, reactive astrocytes, under severe

injury conditions such as trauma, can undergo proliferation, cell migration, and conglomeration to form "scars." This response can result from signaling molecules that are released by neighboring cells or carried to the brain by circulation.

At the molecular level, each cell presents a unique response upon activation that is largely dependent upon the type and severity of the stimuli. The responses in microglia and astrocytes are regulated via both autocrine and paracrine signaling mechanisms. As immune-like cells, microglia act similarly to macrophages largely mounting an inflammatory response (neuroinflammation) upon detection of pathogen-associated or damage-associated molecular patterns. The signaling instigated through ligation of microglial pattern recognition receptors results in the altered expression of pro- and anti-inflammatory signaling molecules, which can stimulate surrounding microglia or other neural cells. The hallmark of astrogliosis is the increased expression of glial fibrillary acidic protein (GFAP), which reflects the hypertrophy and associated accumulation of glial intermediate filaments in reactive astrocytes. Like microglia, astrocytes also secrete inflammatory molecules, as well as respond to external signals received from microglia and other cells.

It is important to note that while neurotoxicity often results in neuronal cell damage, glial reactivity is not exclusive to neurodegeneration. This is crucial to the efficacy of glial reactivity as a means to detect neurotoxicity, because many toxic compounds may have more subtle effects on neurons or result in neuroinflammation as opposed to degeneration. While the study of disease states like traumatic brain injury, Alzheimer's disease, Parkinson's disease, multiple schlerosis, etc. has made it difficult to separate neural damage from neuroinflammation [11–14], it is possible for neuroinflammation (in the absence of damage) to create adverse symptomology. A striking example of this is observed in Gulf War Illness, where veterans of the 1991 Persian Gulf War have presented with a persistent, likely toxicity-driven, multi-symptom illness [15, 16] that seems to be the result of underlying chronic neuroinflammation [17–19]. While these individuals present with various cognitive impairments [16, 20–22], the only structural brain pathology that has been noted constitutes some small changes in white matter volume [23, 24], further highlighting the importance of non-neuronal indices of neurotoxicity.

1.2 Methods for Detecting and Measuring Glial Reactivity

The utility of glial reactivity as a broadly applicable index of neurotoxicity is that it overcomes the unpredictable nature of a given toxic compound's target by taking advantage of a cellular response that occurs without regard for the location of the damage or for the agent(s) causing the damage. The glial response to neurotoxicity, by definition, is an indirect response of glial cell types to the damaged target, one that reflects cellular interconnectivity and

Table 1
"Toolbox" neurotoxicants and their targets

Toxicant	Regional target	Cellular target
Trimethyltin	Limbic structures	Neurons
Triethyltin	Limbic structures	Neurons
Kainic acid	Limbic structures	Neurons
Domoic acid	Limbic structures	Neurons
Ibotenic acid	Limbic structures	Neurons
MPTP	Neostriatum	Dopaminergic neurons
Amphetamine	Neostriatum	Dopaminergic neurons
Methamphetamine	Neostriatum	Dopaminergic neurons
MDA	Neostriatum	Dopaminergic neurons
MDMA	Neostriatum	Dopaminergic neurons
6-Hydroxydopamine	Neostriatum	Dopaminergic neurons
Cadmium	Striatum	Neurons, glia
Methylmercury	Cortex, hippocampus	Neurons
Methylazoxymethanol	Cortex, hippocampus	Neurons
Bilirubin	Cerebellum	Purkinje neurons
Colchicine	Hippocampus	Dentate neurons
3-Acetyl pyridine	Inferior olive	Neurons
Iminodipropionitrile	Cortex, brain stem, olfactory bulb	Neurons
MK-801 (Dizocilpine)	Cortex	Neurons
Ketamine	Cortex	Neurons
5,7-Dihydroxytryptamine	Hippocampus	Serotonergic neurons
Fenthion	Eye	Retinal neurons
2,6-Dichlorobenzonitrile	Olfactory bulb	Sensory neurons

communication of "damage signals" to activate microglia and astroglia. The requirement of cell-to-cell signaling to elicit glial activation emphasizes the importance of having a model that keeps this signaling intact. Thus, it is a requirement to evaluate these responses in an intact animal model of toxic exposure. The utility of a given method for the measurement of glial reactivity is dependent upon its ability to be used in the assessment of various toxic agents. Thus, the methods developed for this task should prove to be relatively consistent across a battery of toxic compounds targeting different cells in different brain areas (see Table 1).

As described previously, microglia and astrocytes exhibit hallmarks of reactivity, and these changes serve as the basis of the methods used to detect these cellular activation responses. The morphological changes exhibited by activated microglia and astrocytes are best evaluated by neurohistology utilizing cell-specific markers. However, due to the morphological continuum of reactive gliosis and the more qualitative evaluation involved with histology, the evaluation of salient protein biomarkers associated with cell morphological changes or the signaling cascades involved in the "activation" response by Western blotting and enzyme-linked immunosorbent assays (ELISAs) techniques is more desirable.

As noted previously, a large component of microglial and astroglial reactivity is the expression and secretion of inflammatory mediators. While cytokines and chemokines are typically measured, particularly clinically, via protein-based analysis, the measurement of these proteins in the brain has proven difficult and inconsistent without explanation. Thus, evaluation of gene expression can serve as a means to detect glial reactivity when protein-based measurements fail. Large-scale gene expression analysis, such as RNA sequencing, can be applied in conjunction with novel genetically modified mouse strains to capture a more extensive response profile to a neurotoxic insult.

2 Materials

2.1 Animal Tissue Preparation

In general, all experiments use either C57BL/6J mice purchased from the Jackson Laboratory or Long Evans rats purchased from Charles River Laboratories, depending on the neurotoxic model used. For instance, trimethyltin instigates a much more robust response in rats than in mice [25], whereas MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is very effective at damaging dopaminergic nerve terminals in mice, but not so in rats [26]. Conscious decapitation is the preferred method of euthanasia when evaluating brain targets as the use of anesthetics can alter the expression of various genes and proteins [27, 28]. The brain is rapidly and carefully removed from the skull and moved to a thermoelectric cold plate for dissection of discrete brain areas, typically the cortex, hippocampus, and striatum (Fig. 1). First, fine curved tweezers are used to pinch and separate the left and right hemispheres along the midline of the brain (along the corpus callosum) to allow folding back of the cortex. The cortex is then gently teased away from the underlying tissue exposing the striatum and hippocampus (Fig. 1a). The hippocampus is toward the posterior of the brain, having an elongated and curved shape, and can be rolled out from the underlying brain tissue (Fig. 1b). The striatum is toward the anterior of the brain and is removed by "cupping out" the visibly striated tissue (Fig. 1c, d). At this point, the entire cortex

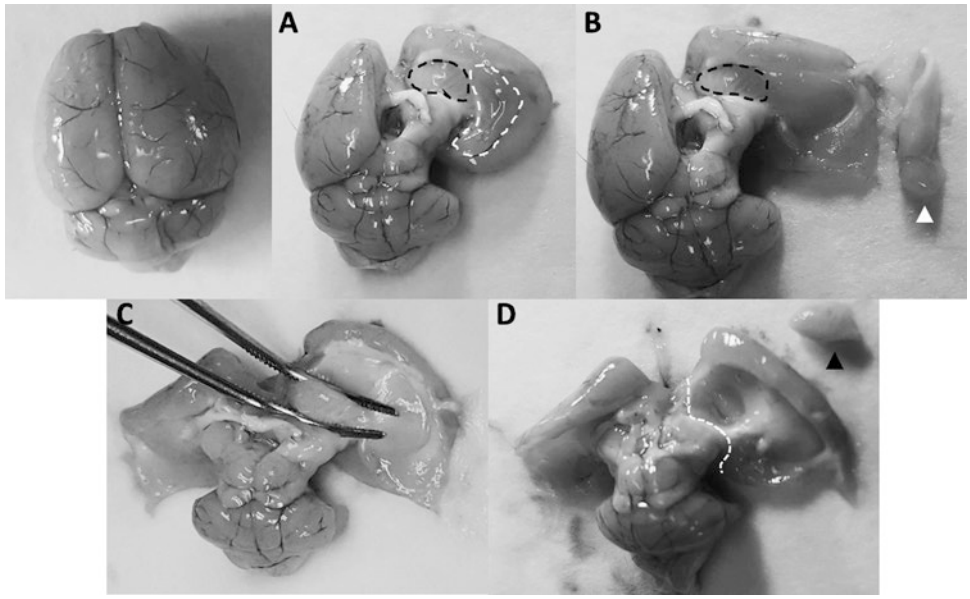

Fig. 1 Dissection of discreet brain regions. Representative images are shown for mouse brain. (a) After breaking the white matter connections of corpus callosum along the midline, the cortex can be folded back to reveal the striatum (within black dashes) and the hippocampus (within white dashes). (b) The hippocampus (arrowhead) can be rolled away from the cortex for isolation, leaving the striatum behind. (c) The striatum is then scooped out using fine curved forceps. (d) After removal of the striatum (arrowhead), the cortex can be removed from the rest of the brain along the dashed line

should be easily separated from the rest of the brain. Care should be taken to remove the white matter from the surface of the dissected brain regions. For the isolation of RNA, the tissues (in microcentrifuge tubes) are frozen on dry ice and moved to a –80 °C freezer. For protein evaluation, the tissue is weighed and then sonicated in ten volumes of hot (90–95 °C) 1% sodium dodecyl sulfate (SDS) buffer (in 1× phosphate buffered saline). For example, 100 μg of tissue weight is homogenized in 1000 μL of 1% SDS. The homogenization in SDS buffer is crucial for the GFAP ELISA (Sect. 3.1) to adequately release GFAP protein into the supernatant while still generating a sample that is suitable for ELISA.

2.2 Microwave Tissue Fixation

When evaluating the expression of phosphoproteins, like pSTAT-3^{Tyr705}, it is best to sacrifice the animal by focused microwave irradiation (Muromachi Microwave Fixation Applicator, model TMW-4012C, Tokyo, Japan) to preserve the phosphorylation state of the protein [29]. Using the appropriate water-jacketed adaptor, microwave fixation is achieved using 3.5 kW of applied power for 0.9 seconds for mice (approx. weight 30 g) and 1.5–1.7 seconds for rats (approx. weight 350–400 g).

2.3 ELISA Assay Preparation

Protein homogenates are diluted in 0.5% Triton X-100 in 1× phosphate buffered saline (PBS). The antibodies used in this assay are rabbit polyclonal α-GFAP (1:300; DAKO), mouse monoclonal α-GFAP (1:1500; Sigma-Aldrich), and alkaline phosphatase-conjugated α-mouse IgG (1:1500; Jackson ImmunoResearch Laboratories, Inc.), all diluted in 1× PBS. The colorimetric reaction with alkaline phosphatase is made using the alkaline phosphatase substrate kit (Bio-Rad), per manufacturer's instruction. Wash buffers are 1× PBS or 0.5% Triton X-100 in 1× PBS (PBS-T), and BLOTTO blocking buffer is 5% dry milk in 1× PBS. All steps are performed at room temperature, except for initial coating of the plate with polyclonal antibody (37 °C).

2.4 Translating Ribosome Affinity Purification (TRAP)

The isolation of actively translating mRNA bound to the cell-specific, eGFP-tagged ribosomes present in the bacTRAP transgenic mouse lines is relatively straightforward and consists of a combined procedure of standard immunoprecipitation and RNA purification [30–33]. A detailed protocol for the "TRAP-ing" procedure, as well as mouse genotyping, is publically available online at www.bactrap.org.

3 Methods

3.1 Quantitative GFAP ELISA

GFAP is ubiquitously recognized as the hallmark of astrocytes, present in both resting and reactive cells, making it a common astrocytic marker for histology. While certainly useful for morphological evaluation of astrocytes and their hypertrophy upon activation, using histology as a means of assessing neurotoxicity when the cellular targets within the brain are unpredictable would prove cumbersome, requiring multiple sections throughout the entirety of the brain to assess the response. Furthermore, a qualitative assessment of gliosis or toxicity, in general, is less desirable than a quantitative measure that could hint at the level of toxicity or damage that results from a particular dosage of exposure. As astrogliosis results in the accumulation of astrocytic filaments and, thus, the GFAP which is associated with these filaments, the increased expression of GFAP can also be used to *quantitate* astrogliosis.

Using the "toolbox" of known neurotoxicants described in Table 1, a quantitative ELISA for GFAP was developed [34, 35] and validated for its efficacy at measuring levels of astrogliosis associated with neurotoxicity [36]. As outlined by O'Callaghan and Sriram [36], the GFAP assay demonstrated that all types of neurotoxic compounds, regardless of target brain region or cell type, resulted in the increased expression of GFAP even at doses not associated with detectable histological or behavioral changes (Fig. 2a). Furthermore, this response is specific to conditions of neural damage, as the enhanced expression of GFAP is not seen under strictly neuroinflammatory conditions (Fig. 2b).

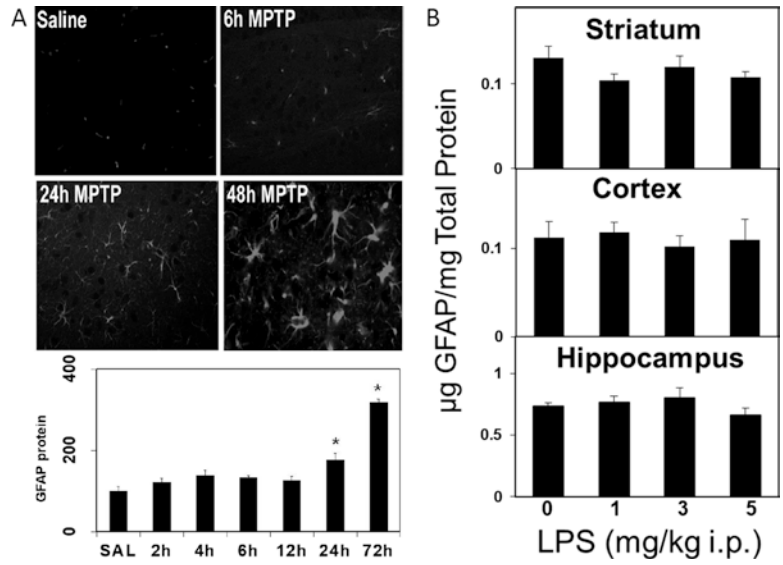

Fig. 2 Time course of GFAP expression post-MPTP and post-LPS. (a) Mice were exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (12.5 mg/kg, s.c.), and the induction of GFAP was measured by immunohistochemistry and ELISA. (b) Mice were exposed to LPS at various concentrations and GFAP measured in multiple brain regions by ELISA. Bars represent mean ± S.E.M., $*p \leq 0.05$. (Modified from O'Callaghan and Sriram [36] and O'Callaghan et al. [37])

In this assay, the plate is incubated with polyclonal GFAP antibody for a minimum of 1 h, washed four times with PBS, and then blocked with BLOTTO for 1.5 h. Samples and appropriate protein standards (100 µL/well) are added to the plate, incubated for 1 h, and then washed four times with PBS-T. The protein standards constitute a set of serial dilutions ranging from 8 to 0.2 ng of GFAP for mice and 24–0.6 ng of GFAP for rats, accounting for differences in brain anatomical size across the two species. This standard can be created with either purified GFAP protein or a total protein sample with known GFAP concentration. The monoclonal GFAP and alkaline phosphate-conjugated antibodies are added simultaneously to the plate, incubated for 1 h, and then washed four times with PBS-T. Finally, the plate is incubated for 20 min with P-nitrophenyl and stopped with 0.4N NaOH prior to reading at 405 nm.

3.2 Western Blot Analysis of pSTAT3^{Tyr705}

While astrogliosis and the measurement of GFAP have been established as a quantitative measure to determine the level of neurotoxicity, astrogliosis is also associated with neuroinflammation. This correlation allows for the use of neuroinflammatory markers associated with astrogliosis as indicators of neurotoxicity, in addition to the previously described GFAP assay. As such, it has been shown that activation of the JAK2-STAT3 pathway, indicated by phos-

phorylation of STAT3 at Tyr705 (pSTAT3^{Tyr705}), correlates with astrogliosis [37, 38]. More specifically, neurotoxic insult results in an increased expression of inflammatory cytokines and chemokines in the brain that is detectable prior to astrogliosis, which then activates the JAK2-STAT3 pathway in reactive astrocytes (Fig. 3). Interestingly, while associated with neuroinflammation, the activation of STAT3 by neurotoxic damage is not suppressible with anti-inflammatory treatment, yet is responsive to neuroprotective drugs [37]. This response in astrocytes is distinguishable from purely neuroinflammatory activation of STAT3, through an inflammagen like lipopolysaccharide, which is suppressible with anti-inflammatory treatment and likely originates from microglia [37]. Thus, STAT3 activation can also be used as an indicator of astrogliosis.

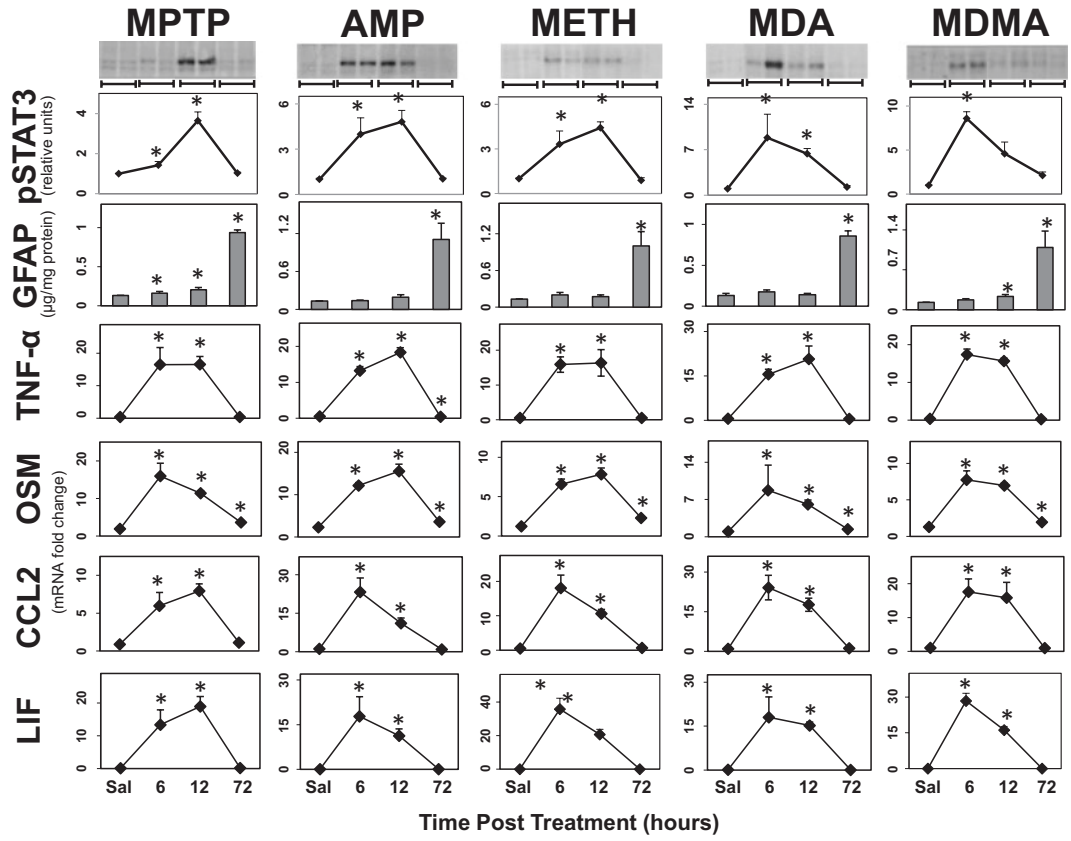

Fig. 3 Time course of STAT3 activation following neurotoxicant exposure. Mice were exposed to MPTP (12.5 mg/kg, s.c.), amphetamine (AMP) (10 mg/kg, s.c., three injections at 2 h intervals), methamphetamine (METH) (20 mg/kg, s.c., three injections at 2 h intervals), 3,4-methylenedioxyamphetamine (MDA) (10 mg/kg, s.c., three injections at 2 h intervals), or 3,4-methylenedioxy methamphetamine (MDMA) (20 mg/kg, s.c., three injections at 2 h intervals). pSTAT3^{Tyr705} and GFAP proteins were measured by immunoblot and ELISA, respectively. Inflammatory cytokine/chemokine mRNA (TNF-a, OSM, CCL2, and LIF) was measured by qRT-PCR. Points represent mean ± S.E.M. and * indicates $p \leq 0.05$. (Modified from O'Callaghan et al. [37])

While detection of pSTAT3^{Tyr705} is achievable through standard Western blotting procedures, the key to reliably detecting phosphorylated proteins is protecting against their dephosphorylation postmortem [29]. Microwave fixation of the brain tissue is a simple, straightforward method of achieving stable phosphorylation without affecting the downstream tissue processing. The optimal Western blotting conditions for detecting pSTAT3^{Tyr705} are to use a 10% SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) separating gel to resolve samples of 20 μg of total protein, typically with a concentration of 1 μg/μL in Laemmli/β--mercaptoethanol buffer, with overnight protein transfer to nitrocellulose membrane.

3.3 ALDH1L1 bacTRAP Transgenic Mice and RNAseq-Based, Astrocyte-Specific Gene Expression Analysis

There are many methods that have been developed for the isolation of specific cell populations in order to evaluate their unique expression profiles, whether they be naïve or challenged responses (for review see Chew et al. [39]). The most common means to achieve this sort of evaluation is either through tissue microdissection, cell culture of isolated primary or cultivated cells, or by fluorescence-assisted cell sorting (FACS) from homogenized whole tissue alone or in combination with other methods. However, these methods have their inherent issues. As stated earlier, much of the brain's response to any insult, including neurotoxicity, is dependent upon the connectivity between cells and across different brain areas. While neuron, astrocyte, or microglia cell cultures can certainly provide you with a cell-specific response to a neurotoxin, it fails to replicate the brain's complexity. Even when using techniques like co-culture, it is unclear whether these in vitro conditions produce the same connections as found in vivo. In contrast, FACS has the advantage of beginning with an in vivo model where the cells retain their spatial relationship with each other during the exposure. However, the actual method of dissociation and cell sorting often can impart cellular and molecular changes [40] that may layer on top of the exposure-induced response.

A method developed by the Heintz and Greengard laboratories at The Rockefeller University for detecting cell-specific gene expression changes [30–33] circumvents the issues encountered with cell culture and FACS by providing a purely in vivo method (Fig. 4). Bacterial artificial chromosome-translating ribosome affinity purification (bacTRAP) technology isolates the actively translating mRNA from specific cell types. Transgenic mice are generated using a BAC that expresses eGFP-tagged ribosomal RNA (L10a) expressed under the control of a cell-specific marker gene. While one may question the use of a BAC clone as opposed to a traditional transgenic construct, the bacTRAP transgenics employ more than just the promoter region of the cell-specific gene that is typically used to control traditional transgenics, using

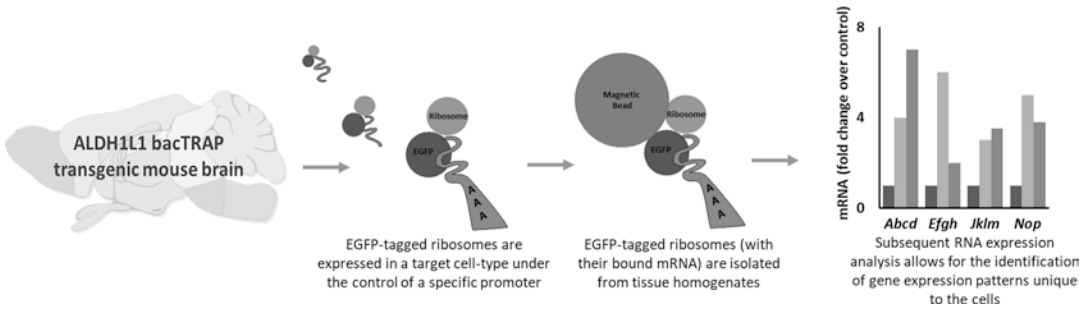

Fig. 4 bacTRAP methodology. By utilizing targeted, cell-specific expression of eGFP-tagged ribosomes, the bacTRAP transgenic mice allow for the isolation of cell-specific mRNA that are actively being transcribed by and, thus, bound to ribosomes. Following isolation of the cell-specific mRNA, gene expression patterns can be evaluated by a number of methods

a large portion of the gene upstream of the 5′ start site that includes not only the promoter but also the transcriptional control elements constituting the need to use a BAC to contain the large amount of DNA. Due to their size, BAC-based transgenics also tend to have a lower number of inserts of the transgene, a condition ideal when expressing rRNA components as the goal is to isolate translating mRNAs without affecting ribosomal numbers in the cell and, by extension, transcriptional rates. By using the eGFP tag to affinity purify ribosomes from brain tissue, the translating mRNA attached to the ribosomes can be isolated and utilized to evaluate the gene expression response of the target cell type. This can then be combined with any method of gene expression analysis, including real-time PCR, microarray, and RNA sequencing, to develop a response profile to the exposures administered in vivo.

The utility of the bacTRAP technology is reliant upon the availability of high fidelity, cell type-specific markers. While a number of bacTRAP transgenic lines exist for various cell types throughout the brain, the Aldh1L1 bacTRAP mouse, relying on the control of the astrocyte-enriched, aldehyde dehydrogenase one family member L1 gene [32], is specific for isolating astrocyte expression profiles [41–45]. Historically, it has been difficult to identify a microglial marker that captures the majority of the brain's microglial population and does not overlap with macrophages. Often, the markers are specific to the different activation stages of the cells, such as CD11b or Iba1 which increase in activated microglia. However, a bacTRAP transgenic mouse was recently created for microglia using CD11b [46].

As an in vivo animal model, the Aldh1L1 bacTRAP mouse can be treated with any potential neurotoxicant and evaluated for astrocyte-specific gene expression postmortem, preserving the integrity of the astrocyte responses. Isolating the gene expression profile in this manner means that all intra- and extracellular communication

remained intact prior to isolation, allowing for the most accurate representation of the astrocyte's specific response. The streamlined tissue processing and RNA isolation provided by the bacTRAP model avoids any potential alterations in the response by more lengthy tissue/cell processing required by alternative methods. As noted by Sloan and Barres [40], there were minor differences between the transcriptome profiles generated for "resting" microglia using their combination immunopanning and FACS procedure compared to the bacTRAP databases; however, these changes may be significantly greater when evaluating the rapid and transient responses to exposure. By removing the requirement to extensively process the brain tissue and/or cells prior to transcriptomic analysis, it is important to note that by isolating only the actively translating mRNA bound to the ribosomes, the RNA yield following the immunoprecipitation and purification procedures of the TRAP protocol is low compared to traditional RNA isolation. As high-throughput gene expression analysis like microarray and RNA sequencing generally requires at least 1 μg of RNA for analysis, it is necessary to pool samples from smaller brain areas, like the striatum and hippocampus, or use nearly the entire cortical region for a mouse.

Beyond capturing the cell-specific gene expression profiles to neurotoxic exposures, the bacTRAP technology also allows for the comparison of cell-specific responses to the profile of the total brain, mixed cell population. As RNA yields are higher without the restriction of isolating ribosome-bound mRNA, this can be achieved with larger brain areas, like the cortex, by setting aside a small piece of cortical tissue for "traditional" RNA isolation prior to the TRAP procedure. With smaller brain areas that require pooling for analysis, preparing separate pools by hemisphere can achieve similar results as long as the exposure effects are not expected to be bilateral or homogenous across the brain. This comparison can reveal a great deal regarding the cell-specific responses to a neurotoxic exposure, not only highlighting the astrocyte-enriched gene expression profile but also contextualizing the contribution of the astrocyte in the level of a particular gene's expression. By exposing the Aldh1L1 bacTRAP mice to the "toolbox" of known neurotoxicants and evaluating by high-throughput gene expression analysis, the large datasets generated by microarray or RNA sequencing can be cross-compared to narrow down common neurotoxicant-responsive genes that can be used as markers of agent-induced astrogliosis.

3.4 Evaluating Microglial Reactivity

As noted earlier, the study of microglia has largely been impeded by the absence of a ubiquitous microglia-specific marker, impacting the success of FACS and other technologies like bacTRAP transgenics to isolate cell-specific responses. As such, much of the

study of microglial reactivity relies on morphological evaluation by histology using several microglial markers, including Iba1, CD11b, and Isolectin B4. The other methods of studying microglia reactivity involve using pharmacological inhibitors or genetically modified mice to evaluate the brain's response to a neurotoxic insult in the absence of fully functioning microglia. While several pharmacological agents have been identified to inhibit microglia [47], one of the most commonly used inhibitors is the tetracycline antibiotic, minocycline. Minocycline was found to significantly reduce neuroinflammation, leading to the hypothesis that the drug is a selective microglial inhibitor [48, 49]. Additionally, the recently developed pharmaceutical colony-stimulating factor 1 receptor (CSF1R) inhibitor, pexidartinib, has been identified to render microglia quiescent, severely reducing the microglial population [50, 51]. While much of the work using this drug has focused on protective effects related to neurodegenerative diseases and injury [52–56], the potential for pexidartinib to be used as a tool to evaluate neurotoxicity still requires investigation.

In addition to pharmacological inhibition, genetically modified mice can also be used to evaluate the microglial response to a neurotoxic exposure. While several knockout/conditional knockout mouse lines exist that can be manipulated to study microglia, the most commonly used line is the fractalkine (Cx3cr1) knockout mouse. While these mice do not display the "absence" of microglia which may be achieved with using a pharmacological inhibitor like pexidartinib, these mice exhibit a suppressed or altered microglia immune response [57–61]. As with the inhibitors, this provides a less direct method of evaluating the microglial contribution to a neurotoxic response, because the knockout response profile must be compared to the wild type in order to evaluate the role of the microglia in neurotoxicity.

4 Conclusions

In this chapter, we have highlighted the usefulness of glial reactivity as an indicator or biomarker of neurotoxicity and presented several reliable methods for evaluating the response of astrocytes and microglia to neurotoxic exposures. The significance of measuring glial reactivity to assess neurotoxicity lies in the ability of this process to highlight areas of toxicant-induced damage in the absence of understanding the chemical's mechanism of action or cellular/regional target. By shifting the focus away from the neurons themselves, the evaluation of whether a novel (or existing) chemical, drug, or other agent has detrimental effects on the brain can be reliably measured.

5 Notes

While it is highly recommended to use microwave fixation-based euthanasia for the evaluation of phosphorylated proteins, pSTAT-3^{Tyr705} in particular seems to be relatively stable and can be reliably screened in banked, fresh frozen tissue.

Acknowledgments

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